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REVIEW PAPER

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Chemiluminescence-driven Dye Excitation for Dark Photodynamic Therapy

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ABSTRACT

Photodynamic therapy is a treatment that uses a combination of light-absorbing photosensitizers and dissolved oxygen to kill cancer. One specific limitation of photodynamic therapy is that the visible light used for photosensitizer excitation has a short tissue penetration depth of several millimeters. This limits the application of photodynamic therapy to surface cancers in the absence of a technique to illuminate deeper tissue. Efforts to extend tissue depth to which photodynamic therapy can be applied have been attempted with use of up-conversion and persistent-luminescent nanoparticles that absorb near infrared light and emit visible light for photosensitizer excitation, yet an initial excitation with an external light source is still required. More recently, systems employing chemiluminescence as an excitation energy source designed to bypass the use of external light have been developed and investigated as potential agents that could overcome the problem of achieving photodynamic therapy in deep tissue. We wish to provide an overview of several systems that have been recently reported that employ both radiative and non-radiative chemiluminescent energy transfer for photosensitizer excitation that have been developed in the hope of achieving "dark" photodynamic therapy. This article reviews several of these important new developments in the design of photodynamic therapeutic systems that utilize chemiluminescence.

Keywords. singlet oxygen, chemiluminescence, bioluminescence, photodynamic therapy

Introduction

Photodynamic therapy (PDT) is a cancer treatment that uses photo-generated reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide and hydroxyl radical to damage targeted cells. For generation of ROS, a PDT treatment method employs photosensitizers (PS) that are excited by external illumination provided by visible light at power levels that do not damage healthy tissue. The primary ROS generated is $^1O_2$ which reacts with cell molecules ultimately resulting in tissue damage and cell death. The mechanism of $^1O_2$ formation in these system is energy transfer from excited PS to ground state oxy-

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gen. Other ROS such as superoxide and hydroxyl radical are also generated to a much lesser extent and can be also toxic to cells. Singlet oxygen is short lived (< 1 μs) in biological tissue thus for photodynamic therapy it is desirable to generate $^1O_2$ within a cell. Delivery of PS to diseased tissue is accomplished systemically by injection and PS tends to accumulate on or internalize into targeted cells and in healthy tissue to some extent. After PS delivery to targeted cells, PS is remotely activated by an external light source producing toxic ROS in the presence of oxygen. This methodology imparts spatial and temporal selectivity in treatment as the PS localized on targeted cells is activated by selectively illuminating the region of diseased tissue resulting in necrosis and/or apotosis.[1] Photosensitizers cannot generate ROS in the absence of light or other excitation energy source and ideally PS is nontoxic to heathy cells in the dark. Research in PDT is ongoing as this approach has yet to reach its full clinical potential although several PS are approved for use.[1] Current limitations in PDT include tissue irradiation with visible wavelengths that have a short tissue penetration depth. This particular limitation may be addressed in the design of PDT systems that can excite an acceptor PS by donors via chemiluminescence.

A simplified mechanism for the generation of singlet oxygen is presented in Figure 1. Photosensitizer (PS) excitation from the ground state (^{1}PS) to the first excited state singlet (^{1}PS*) can be accomplished by excitation with external visible light or by chemiluminescence. Intersystem crossing (ISC) to the first excited state triplet (^{3}PS*) followed by collisional energy transfer to ground state oxygen ($^3O_2$) generates cytotoxic singlet oxygen ($^1O_2$) which can induce cell apoptosis and/or necrosis. This review article will address several systems that employ a chemiluminescent donor (chemilumigen) to excite an acceptor PS either by remote illumination or by chemiluminescence resonance energy transfer (CRET). An excellent review article covering mechanisms, structural characteristics of chemilumigens, and applications of chemiluminescence and bioluminescence in PDT has been published in 2016.[2] This review includes several recent developments in the field where a chemilumigen is covalently attached to PS and can excite PS by through-bond energy transfer.

An overview of chemiluminescence-driven PDT systems

One of the first systems developed for overcoming of the need for external light sources in PDT employed the well-known chemilumigen donor luminol. Firer and coworkers investigated the possibility of using luminol as a molecular donor of intracellular chemoluminescence for the destruction of leukemic cells.[3] In this study, murine hybridoma cells were cultured with a transferrin-hematoporphyrin conjugate PS and luminol, hydrogen peroxide and ferrous sulfate and kept in the dark. The results of the study confirmed that the compound luminol induces intracellular chemiluminescence and was able to activate the hematoporphyrin conjugate resulting in 95% cytotoxicity.[3] Although luminol chemiluminescence was sufficient to generate high cytotoxicity by excitation of PS, the concentration of the conjugate PS required to attain LD_{max} was 6 times higher than that needed with an external light source.[3] It is also worth noting that in this study luminol itself induced about 15% cytotoxicity. This study demonstrated the potential for utilizing luminol chemiluminescence as an excitation source in PDT.

Wang and coworkers also proposed a PDT system in which the photosensitizer was activated by a chemiluminescent chemical donor rather than by an external light source.[4] The chemiluminescent molecule, as in the previously described system, was luminol in the presence of hydrogen peroxide and horseradish peroxidase as oxidants. Cationic oligo (p-phenylene vinylene) (OPV) was used as the acceptor PS. Electrostatic binding of the cationic OVP with the negatively charged oxidized luminol dianion placed the donor and acceptor in close proximity for chemiluminescent resonance energy transfer (CRET). Excitation of OVP was achieved as luminol chemiluminescence has a maximum at 425 nm and OVP has an

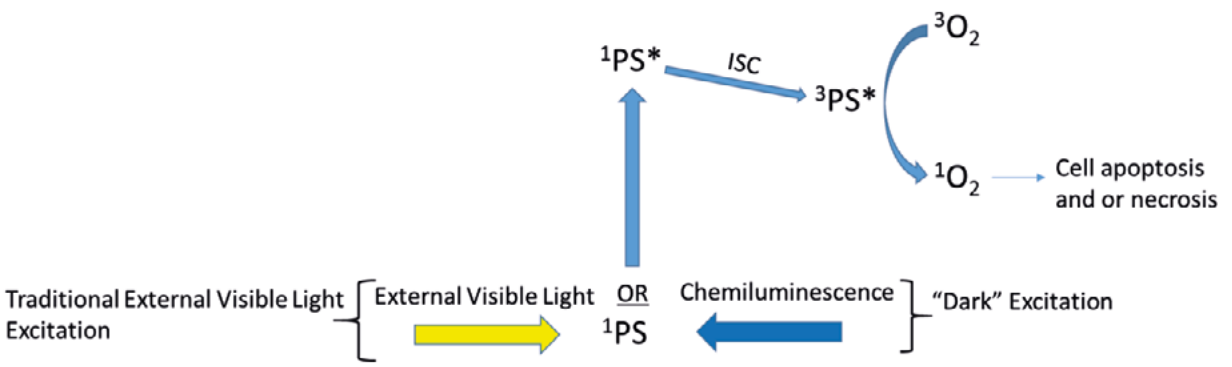

Figure 1. Photosensitizer (PS) excitation from the ground state (^{1}PS) to the 1st excited state singlet (^{1}PS*) by excitation with external visible light or, alternatively, by chemiluminescence. Intersystem crossing (ISC) to the first excited state triplet (^{3}PS*) followed by energy transfer to ground state oxygen ($^3O_2$) generates cytotoxic singlet oxygen ($^1O_2$)

overlapping absorption between 350-550 nm.[4] Oxygen molecules in the surroundings were sensitized through excited OPV to produce reactive oxygen species (ROS) which presumably included $^1O_2$.

Renilla luciferase-immobilized quantum dot-655 (QD-RLuc8) have been employed for bioluminescence resonance energy transfer (BRET) mediated PDT and was tested as an intracellular excitation source.[5] Studies showed that QD-RLuc8 exhibited chemiluminescence at 655 nm after coelenterazine addition for excitation of Foscan® loaded micelles for PDT. This system displayed two separate modes of energy transfer. The first was BRET to the quantum dots. Secondly, the BRET excited quantum dots transferred energy to the PS Foscan® via fluorescence resonance energy transfer (FRET) to yield ROS. Mice tested were transfected with human lung adenocarcinoma epithelial A549 cells and to assess the effectiveness of this system, tumor growth rates were measured in the presence and absence of the QD-RLuc8- coelenterazine-Foscan® system. A significant increase in tumor volume in untreated mice (4.5–6×) compared with mice treated with QD-RLuc8- coelenterazine- Foscan® system indicated delayed tumor growth.[5] In addition, the QD-RLuc8-coelenterazine- Foscan® was also apparently responsible for decreasing vascularization of the tumors. This study showed that the efficiency of bioluminescence-mediated PDT is sufficient for eliciting a photodynamic effect *in vivo* thus the QD-RLuc8- coelenterazine- Foscan® system has the potential to act as a chemiluminescene-driven PDT.[5]

A similar study to evaluate bioluminescence driven PDT were performed by Kim et al. The study was conducted to determine whether bioluminescence is sufficient as an PS excitation source in PDT.[6] To investigate the effect of bioluminescent PDT on tumor growth in mice *in vivo*, the Renilla luciferase-immobilized quantum dot-655 coelenterazine system was activated intracellularly for excitation of the PS chlorin e6. An interesting result was a comparison between the amount of chlorin e6 excitations per minute from a continuous wave laser and from Renilla luciferase-immobilized quantum dot-655 coelenterazine system. Studies determined that chlorin e6 had an excitation rate of 4×10^7 times per minute from a 660 nm 2.2 mW laser and 3×10^8 times per minute from the Renilla luciferase-immobilized quantum dot-655 coelenterazine system This is a very important result showing that bioluminescence resonance energy transfer can generate stronger photochemical activation in the cell membrane than a laser in some cases.[2,6]

The chemiluminescent system developed by Zhang et al. employed functionalized nanoparticles consisting of semiconducting polymer dots (poly[2-methoxy-5-((2-ethylhexyl)oxy)-p-phenylenevinylene) with folic acid and horseradish peroxidase covalently attached on pendant Janus dendrimers.[7] The PS (meta-tetra(hydroxyphenyl)-chlorin) was covalently attached to the core semiconducting polymer dots. Addition of luminol and hydrogen peroxide in the vicinity of these nanoparticle dots was determined to excite PS in two distinct pathways. The first directly by CRET to PS and the secondly CRET to the functionalized nanoparticle dots which in turn excited PS by FRET. This system was tested *in vitro* by incubating the functionalized nanoparticles with MCF-7 breast cancer cells, C6 glioma cells, and NIH 3T3 fibroblast cells. The nanoparticle dots were found to be biocompatible and cell viabilities for fibroblast cells were 72%, glioma cells 32% and breast cancer cells 17% at 10 μg mL.[7] These results demonstrated that the functionalized nanoparticle dot-luminol system was sufficient for chemiluminescent excitation of bound PS for potential use in PDT.

Very recently, Lee et al. have reported the synthesis of "chemi-dynamic nanoparticles" as "dark" PDT systems based on peroxalate chemiluminescence.[8] Peroxalate oxidation by hydrogen peroxide forms an unstable dioxetanedione which decomposes into carbon dioxide and energy which can in turn be used for PS excitation. A hydroxybenzyl alcohol-incorporating copolyoxalate with peroxalate ester linkages and the PS protoporphyrin was evaluated for potential PDT using a cell culture models.[8] The hydroxybenzyl alcohol-incorporating copolyoxalate and protoporphyrin individualy showed minimal apoptotsis in cell models.[8] Interestingly, pre-treatment of cells with hydrogen peroxide-generating cinnamaldehyde followed by addition of chemiluminescent "chemi-dynamic nanoparticles" produced significant dose dependent apoptosis.[8] This study has successfully shown that peroxalate chemiluminescence is sufficient excite PS for cell death in the absence of light.

Chemilumigen donor PS acceptor covalent conjugates

There have been several reported systems that covalently attach a chemilumigen (luminol) to dyes for chemical generation of fluorescence by CRET.[9] Algi et al. studied the photophysical properties and energy transfer efficiency of 2,3-dihydrophthalazine-1,4-dione (a luminol derivative) covalently linked to a boron-dipyrromethene (BODIPY) dye.[9] This conjugate could generate chemiluminescence upon treatment with alkaline hydrogen peroxide in the presence of Fe(III) ions resulting in CRET from 2,3-dihydrophthalazine-1,4-dione to the BODIPY fluorophore. Interestingly, these conjugated donor/acceptor pairs can emit visible light via CRET. These systems can be modified specifically for chemiluminescent driven PDT and, to date, one such system has been developed as depicted in Figure 2.

In this case, in attempts to achieve $^1O_2$ generation, iodine was added to the xanthene core to promote intersystem crossing and the authors observed a significant decrease in fluorescence.[10] Activation of the donor/accep-

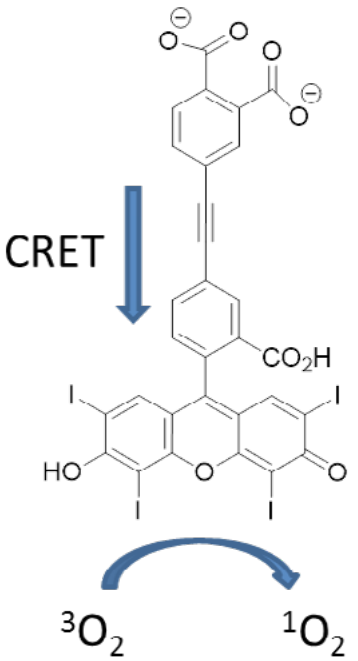

Figure 2. A system developed by Akkaya et al. for excitation of pendant tetraiodoxanthene and subsequent generation of $^1O_2$ via through-bond CRET

tor conjugate with hydrogen peroxide is advantageous since this may also occur readily in cancer cells.[11] Generation of $^1O_2$ was inferred by trapping with 1,3-diphenylisobenzofuran. As far as we know, this is the first system developed utilizing CRET by covalent attachment of a chemilumigen to a PS and further studies are expected to be reported in the near future.

Conclusion

Photodynamic therapy is growing in popularity and usefulness as a clinical treatment for cancer. One main drawback of PDT is that the chemiluminescence driven systems aim to overcome is limited PS excitation light penetration depth. The systems discussed herein are promising as attempts to overcome this limitation although further research and optimization is needed. Given the importance of PDT, we expect that the development of systems that are capable of chemiluminescence driven "dark" excitation of PS will continue to advance.

References

1. Rai P, Mallidi S, Zheng X, et al. Development and applications of photo-triggered theranostic agents. Adv Drug Deliv Rev. 2010;62:1094-124.
2. Magalhães CM, da Silva JCGE, daSilva LP. Chemiluminescence and bioluminescence as an excitation source in the photodynamic therapy of cancer: A critical review. ChemPhysChem. 2016;17:2286-94.
3. Laptev R, Nisnevitch M, Siboni G, Malik Z, Firer MA. Intracellular chemiluminescence activates targeted photodynamic destruction of leukemic cells. Br J Cancer. 2006;95:189-96.
4. Yuan H, Chong H, Wang B, et al. Chemical molecule-induced light-activated system for anticancer and antifungal activities. J Am Chem Soc. 2012;134:13184-7.
5. Hsu CY, Chen CW, Yu HP, Lin YF, Lai PS. Bioluminescence resonance energy transfer using luciferase-immobilized quantum dots for self-illuminated photodynamic therapy. Biomaterials. 2013;34:1204-12.
6. Kim YR, Kim S, Choi JW, et al. Bioluminescence-activated deep-tissue photodynamic therapy of cancer. Theranostics 2015;5:805-17.
7. Zhang Y, Pang L, Ma C, et al. Small molecule-initiated light-activated semiconducting polymer dots: an integrated nanoplatform for targeted photodynamic therapy and imaging of cancer cells. Anal Chem. 2014;86:3092-9.
8. Singh SV, Berwin JK, Hoyeon P, Gilson K, Dongwon L. Novel chemi-dynamic nanoparticles as a light-free photodynamic therapeutic system for cancer treatment. Macromol Res. 2017. https://doi.org/10.1007/s13233-017-5078-91–7.
9. Degirmenci A, Algi F. Synthesis, chemiluminescence and energy transfer efficiency of 2,3-dihydrophthalazine-1,4-dione and BODIPY dyad. Dyes and Pigments. 2017;140:92-9.
10. Nisa Y, Uyar TB, Seven O, Akkaya EU. Singlet oxygen generation with chemical excitation of an erythrosine–luminol conjugate. ACS Omega. 2017;2:1367-71.
11. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by Human tumor cells. Cancer Res. 1991;51:794-8.

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REVIEW PAPER

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Investigation of pharmaceuticals by nuclear magnetic resonance imaging and spectroscopy

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ABSTRACT

Currently, new and easier ways of analyzing pharmaceutical drug forms and drug delivery mechanisms are being sought. Magnetic resonance imaging (MRI) is a non-invasive imaging technique that images drug forms such as tablets, liquids and topicals and drug form behavior in living organisms on both the tissue and cellular scale. The advantages of MRI include non-invasiveness, variable sample capacity and ease of transfer of phantom results to *in vitro* and *in vivo* studies. This review concerns the usefulness of clinical MRI that cannot be understated as this technique provides non-invasive and non-destructive insight into the properties of drug delivery systems. The research discussed here concerns the use of magnetic resonance, spectroscopy and chromatography to investigate selected pharmaceuticals and covers work of selecting drugs and antibodies for modification by synthesis for evaluation by MRI. Modifications have been aimed at improving therapeutic efficacy, delivery, and MRI. Modification conditions such as (pH, concentration, temperature, and the influence of other components present in the solutions) will be discussed to understand drug delivery system improvements and the reliability and repeatability of the results obtained. We hope to explore and expand the scope of pharmaceutical imaging with MRI for application in clinical medicine.

Keywords. drug delivery systems, drug forms, magnetic resonance imaging, pharmaceuticals

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Introduction

Currently one of the most accurate non-invasive imaging methods is magnetic resonance imaging (MRI). This method allows one to make sections in any plane of both living organisms and non-anatomical structures. The signals we receive in MR depend on the object being tested and its properties. We have the ability to obtain data with morphological, functional and metabolic information. To non-invasively monitor drugs inside the human body is a challenge. However, MRI has not been yet used in its full capacity in the field of pharmacy. The application of MRI in the sphere of pharmacy began in 1995 and is constantly developing. The main applications of MRI *in vitro* are monitoring of water and other solvents, controlled release of dosage forms, hydration and diffusion. The use of MRI in pharmacy can provide a platform to transfer knowledge from an *in vitro* study to an *in vivo* study in drug delivery and controlled release of dosage forms. This transfer of knowledge already takes place in research and there are several example studies on neurological drugs, anticancer drugs and vitamins. The first application of MRI in pharmacy to study pharmaceutical tablets was published by Nebgen et al. where the authors showed the distribution of porosity in tablets which is an important subject for the generation of solid drugs.[1] In another work published by Hyde *et al*., the first quantitative MRI investigation based on a study of water migration from phosphate buffered solution into monolithic implants made of poly(glycolic acid-co-DL-lactic acid) produced by an extrusion process was conducted.[2] MRI is interesting for the magnetic properties of the nuclei of individual atoms. Each nuclei has its magnetic moment, which along with the applied magnetic field align with the lines of the field. The resulting weak net magnetization precess when disturbed from equilibrium. The frequency of precession (ω) is equal to the applied magnetic field (B) multiplied by the magnetogyric ratio (γ). The magnetogyric ratio, γ, is a property that varies for different nuclei, being largest for the hydrogen nucleus $\gamma=42{,}58\ s^{-1}T^{-1}$. Radiowaves of angular frequency (ω) show a resonant interaction with the nuclei. A pulse of radio waves at this frequency can therefore be used to disturb the nuclei from equilibrium and set them into precession. Unfortunately, the MR signal is intrinsically weak, but increases in strength with increasing γ and B. MRI is therefore generally only applied to samples containing ^{1}H nuclei in high concentrations. One MRI technique is magnetic resonance spectroscopy (MRS). MRS is a diagnostic tool used to characterize tissues in terms of their chemical composition.[3-5] MRS is used to determine the chemical properties of a given area, focusing on the metabolites of the cells. The method is based on the effect of the chemical shift of the atom (nuclei of different cells precess at different frequencies).[6-7] Most commonly performed experiment is single-voxel spectroscopy (SVS), where the signal is received from the selected location. Measurements are made using PRESS (Pointed-Resolved Spectroscopy) or STEAM (Stimuled Echo Aquisition Mode) sequences. Based on the recorded signal from a given voxel, a Fourier transform is calculated and then spectra are generated on which individual peaks correspond to individual metabolites.[8] A chemical shift graph of signal frequency in parts-per-million (ppm) is generated from the signal amplitude. The area under the peak corresponds to the concentration of the metabolite. This provides the possibility of quantification of signal by using internal standards. Measurements are made using the PRESS (Pointed-Resolved Spectroscopy) or STEAM (Stimuled Echo Aquisition Mode) sequences. Based on the recorded signal from a given voxel, Fourier transform is calculated, and then spectra are generated on which individual peaks correspond to individual metabolites. In a technique called Magnetic Resonance Spectroscopic Imaging (MRSI), we can obtain color maps where the concentration level of a particular metabolite is encoded by color. Identification and quantification of the metabolite such as N-acetyl l-aspartic acid (2.02 ppm), creatine (3.02 ppm), choline (3.22 ppm) and lactate (1.33 ppm) in phantom were performed using SAGE post processing software. In order to evaluate the performance of an MR system, an MR phantom has been developed to accurately analyze errors of MR systems.[9-11] This makes it possible to visualize the distribution of metabolites throughout the brain. This is problematic, however, because the data received may include voxel bleeding, that is, voxel noise from the surrounding voxels.[12-14]

MRI in pharmacy

The pharmaceutical sector has MRI related examples where formulations have been studied by observing tablet hydration and its effect during dissolution. MRI has been used to study internal mechanisms underlying *in vitro* drug release behavior in dosage forms, to monitor events within pharmaceutical processes, and *in vivo* to investigate the behavior of drug delivery systems in the body.[15] Examples of pre-clinical and *in vitro* MRI in new drug design studies include forms such as nanoparticles.[16-20] and nanogels.[21] Drug delivery systems use MRI to map drug transport and physiological response. Drug development [22-23] and drug release[24-28] have also been studied by MRI. As shown in the PubMed Data Base, the number of total publications regarding the applications of MRI in pharmacy is constantly increasing.

The authors provide innovative and creative examples of the use of MRI research in pharmacy. Also, the number of publications on contrast agents has increased due to intensive searches for improvement of diagnostic methods. With MRI, we are able to provide non-invasive ways to visualize events during controlled-release dosage. Using MRI, we also have a tool that is helpful in

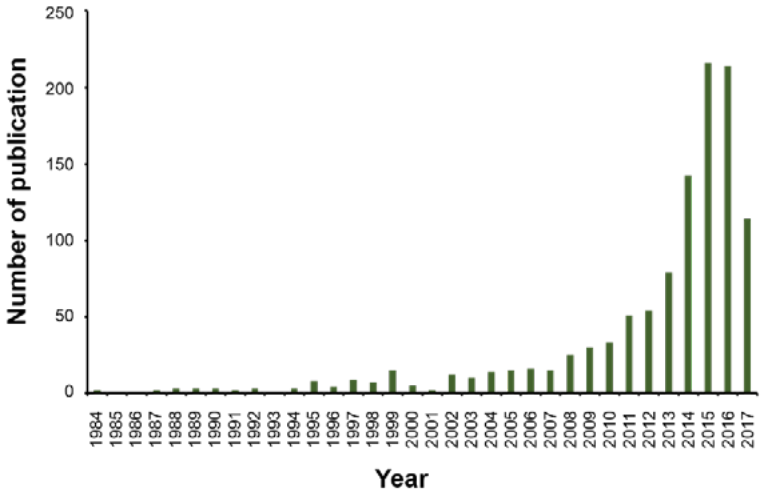

Figure 1. Publications on MRI Applications in Pharmacy

understanding the processes that occur in drug metabolism. This may have a significant impact on the development of a new generation of pharmaceuticals[29]. Porosity and compaction density are important parameters in the manufacture of tablets by compression. Nebgen *et al.* have shown how MRI can provide a noninvasive method for mapping the density distribution within a compacted tablet at a spatial resolution of (95 μm). However, factors such as paramagnetic materials, water–air, and solid–air interfaces can cause MRI artifacts.[30-32] MRI can identify tissue macromolecules such as nucleic acids, lipids, collagen and proteoglycans using parameters such as chemical shift, relaxation rates, and magnetic spin couplings. The enormous potential of MR to translation of the complex physical and mathematical concepts into biological material is recently an emerging area of empirical and theoretical interests. The MR techniques to determine proton relaxation times spin - lattice T_1 and spin - spin T_2 are numerous. These include a method fully relaxed Inversion recovery (IR)[33], Fast Inversion Recovery (FIR)[34], Modified Fast Inversion Recovery (MFIR)[35], Progressive Saturation (PS)[36] , Saturation Recovery (SR)[37], Variable Nutation (VN)[38], Look Locker (LL)[39], choice of flip angles, delay intervals, and amount of signal averaging. These methods in a greater or lesser extent take advantage to provide a T_1 and T_2 measurements.[40-41] T_1 and T_2 in MRI are functions of spin density and also instrumental parameters such as the pulse sequence timing and slice selective sensitivity profile.[42] In liquids at higher temperatures T_1 and T_2 are almost equal. However, in solids and at low temperatures, there little molecular motion, T_1 may be many seconds while T_2 is only microseconds. The most commonly used methods in MRI for generating T_1 maps are based on the basic pulse sequences used for T_1 measurements in Nuclear Magnetic Resonance (NMR) spectroscopy: PS and IR. These radio-frequency pulse sequences can be combined with several imaging techniques and are used frequently in MRI.[43] The relaxation process is characterized by two exponential time constants T_1 and T_2. The transient time-domain signal

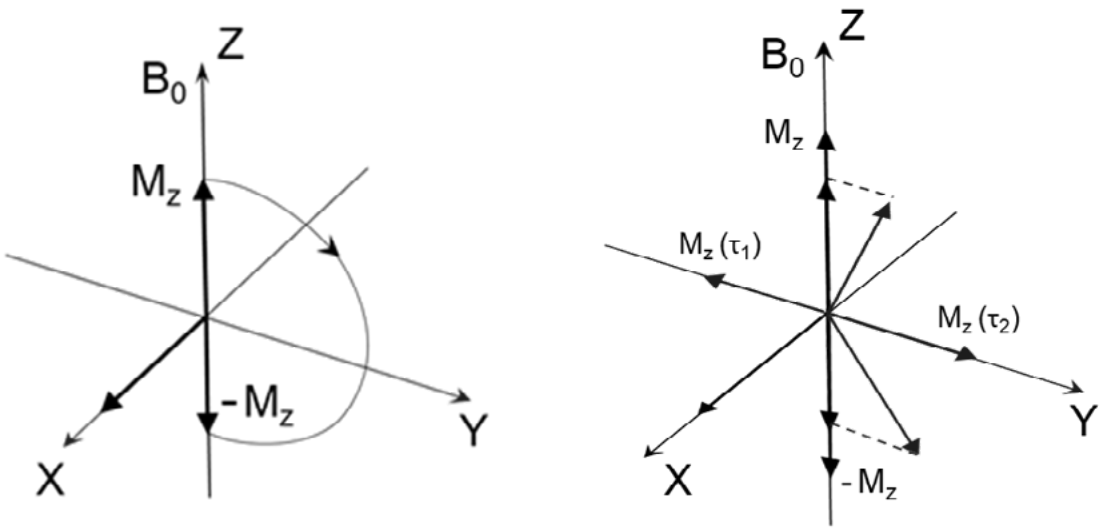

Figure 2. The MRI phenomenon

is digitized and stored in a computer. In MRI, the resultant magnetization aligned with the static magnetic field, which is called longitudinal magnetization, is tipped into the transverse plane, where it can be detected as an electric signal. This so-called transverse magnetization decays exponentially with a time constant T_2. The longitudinal magnetization relaxes back to its equilibrium orientation parallel to the static magnetic field exponentially with a time constant T_1. The mechanisms by which contrast agents enhance relaxation involve the magnetic moments. Relaxation does not occur spontaneously, it must be stimulated. Longitudinal magnetization relaxes toward equilibrium as excited spins undergo transitions to lower energy states.[44] These transitions must be stimulated by a changing magnetic field. A magnetic field oscillating in strength at the Larmor frequency supplies a quantum of energy exactly equal to the energy difference between the two states, thereby stimulating relaxation. The magnetic moments associated with particles such as nuclei, electrons, and atoms supply changing magnetic fields to stimulate relaxation (Figure 2). These magnetic field fluctuations are vital for relaxation.[45]

The first published calculated T_1 image was generated in 1978 using sequence PS showed in Figure 3. Briefly, after the first 90 degree pulse, the magnetization is perturbed within the selected slice into the transverse plane. The transverse magnetization processes during the time interval TE and relaxes exponentially with a time constant as the 180 degree pulse refocuses any dephasing due to field in homogeneities. Longitudinal relaxation occurs during the interval TR until the next sequence repetition. If the next 90 degree pulse is applied the longitudinal magnetization has been allowed to recover completely during the intermediate period.[46]

Most MR studies indicated that the often used pulse sequence to measure T_1 relaxation time is the IR measurements.[40] The pulse diagram for IR is shown in Figure 4. Briefly, 180 degree pulse inverts the magnetization vector MZ. After this the magnetization lies along the negative z axis and MZ = - MZ. The T_1 relaxation makes the magnetization increase during time interval from - M0

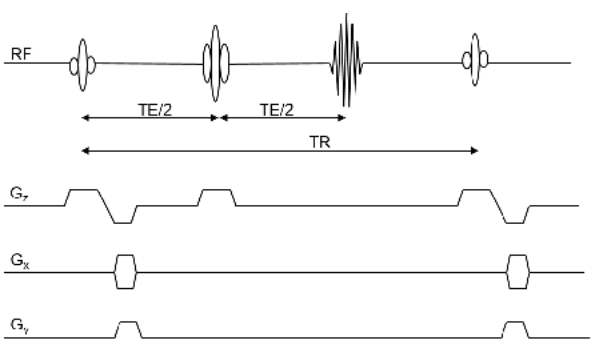

Figure 3. Partial or progressive saturation in a 2D Fourier Transform spin-echo pulse sequence

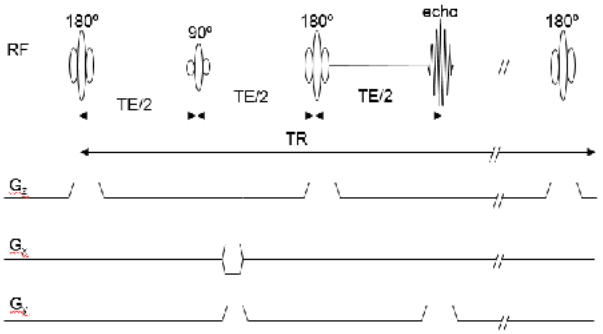

Figure 4. Inversion Recovery

throughout zero until it is back to original value MZ = M0. If at some time following the 180 degree pulse, the 90 degree pulse is applied MZ is rotated around the X axis and will then lie somewhere along the Y axis. A T_1 of a 90 degree pulse reads the relaxed magnetization.[47]

T_1 maps can also be generated by using a variable-tip-angle pulse during the MRI experiment. In this method, a pulse of tip angle zero-0 is used to perturb the magnetization, which is then left to partially relax back to its thermal equilibrium value during the short TR. As the excitation pulse is generally other than 90 degree only a fraction of the thermal equilibrium magnetization is tipped into the transverse plane. This transverse magnetization is then a function of both the pulse tip angle 0 and the amount of longitudinal relaxation that has occurred during the time interval TR.[48] In this case, the application of a 180 refocusing pulse to form a spin echo cannot be used, because such a pulse also would invert the magnetization that has remained along the longitudinal axis. Instead, an echo is formed through the use of gradients. This radio-frequency pulse sequence can then be incorporated into any imaging regime Figure 5. The transverse relaxation during TE is now described by the effective transverse relaxation time constant.[49]

Figure 5 presents a pulse sequence where T_1 derives from the ratio of the STE of the SE and is formed from the first two pulses. Equation 1 shows the ratio of STE and SE where TM is the time between the second and third pulses.

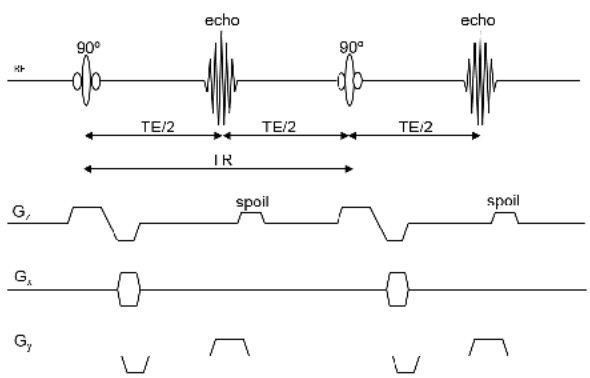

Figure 5. Variable tip angle, 2D Fourier transform gradient recalled echo pulse sequence

$$STE/SE = \exp(-TM/T_1)$$

This assumes perfect 90° pulses. Also to eliminate diffusion effects, a plot of $1/T_{1eff}$ against TE2 yields a straight line whose intercept at TE = 0 is $1/T_1$-T_{1eff} is the measured T_1 at each TE.[50] A SE sequence in which the magnetization stored on the z axis during TM is sampled with a series of pulses with increasing flip angles designed to give equal SI in the absence of T_1 relaxation. T_1 is calculated from $SI=S_0 \exp(TM/T_1)$. Two pulses produce a SE, and a series of low-angle pulses then produce a series of STEs. The T_1 is derived from the SE and STE intensities, which would all be the same if there were no T_1 relaxation. SE is very sensitive to flip-angle errors and could be improve by the SNR by changing the flip angle of the second pulse to 180 degree. It was forms a composite echo with a SE and a STE 90 degree out of phase and calculated T_1 from the phase of the signal. Phantom STE data are similar to IR data, but human STE data were not compared to IR phantom data (Figure 6-7). [51]

MRI and MRS measurements show that engineered liposomes can detect the mildly acidic pH of the tumor microenvironment with 0.2 pH unit precision and they release their content into C6 glioma tumors selectively, *in vivo*.[52] MRSI of the pH sensitive probe (+/-)2-(imidazol-1-yl)succinic acid disodium salt and a pH map of a tumor *in vivo* was investigated. Citrate, choline-containing compounds, creatine and polyamines have also been described by MRSI. The protons of Citrate resonate around 2.6 ppm, but the precise chemical shift and the scalar coupling of these protons depend on pH and cation concentration in cellular membranes.[53] MRSI was used in neurooncology but also in multiple sclerosis, stroke and epilepsy. However, a major challenge in conventional MRSI is the longer acquisition time required for adequate signal to be collected.[54]

Studies to characterize the reliability of MRSI thermometry, including contributions from inter-scan and interexamination variability have been performed. In addition, measurments of the variation between subjects and assessment of the extent of brain temperature variation.[55] MRSI also provids spatially localized maps of metabolite concentrations.[56] MRSI provides a unique modality to non-invasively study tissue metabolism *in vivo*; it acquires metabolic information reflecting tissue function and provides a sensitive assessment of chemical alterations.[57] Table 1-3 presents the applications of MRI to study the forms of drugs and properties.

The study provided by Zeitler and co-worker showed as a calibration set such that the MRI signal from the pore space of the tablet could provide a measure of tablet density. Different sets of production tablets were analyzed using the MRI method and the density distribution within the dosage form was determined.[76] Since T_1 of pure water can be 3–5 s and the image acquisition time is proportional to TR, this can lead to poor temporal resolution. In addition, the shortest achievable TE is restricted by the finite time required to turn the gradients on and off, limited by the hardware, as well as the time to record the signal. The use of low-field MRI (typically 0.5 Tesla) for pharmaceutical research, and specifically for tablet dissolution, has been limited partly due to the availability of instrumentation and also the perceived lack of sensitivity.[77] Nickel-doped agarose/sucrose gels can be used as reference materials for MRI diffusion measurements and show excellent short-term stability with respect to ADC. A phantom made of these materials can be invaluable in optimizing DW-MRI protocols, developing novel pulse sequences for DW-MRI, or comparing ADC values among field strengths, vendors, and imaging centers.[78] Magnetic Block Ionomer Clusters with hydrophilic ionic cores and nonionic coronas have been prepared that have ultrahigh transverse NMR relaxivities together with capacities for incorporating high concentrations of polar antibiotic payloads. Magnetite-polymer nanoparticles were assembled by adsorbing the polyacrylate block of an aminofunctional poly(ethylene oxide-b-acrylate) (H2N-PEO-b-PAA) copolymer onto magnetite nanoparticles.[79] Magnetic nanoparticles possess unique magnetic properties and the ability to function at the cellular and molecular level of biological interactions making them an attractive platform as contrast agents for MRI and as carriers for drug delivery. Recent advances in nanotechnology have

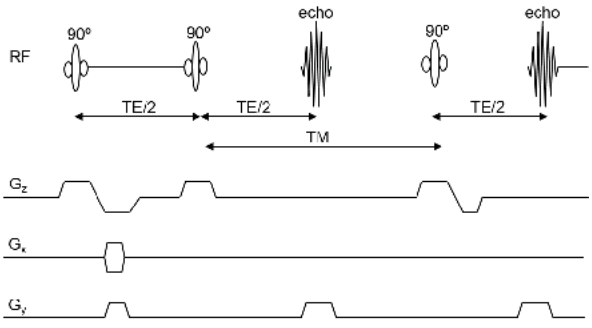

Figure 6. STE sequence

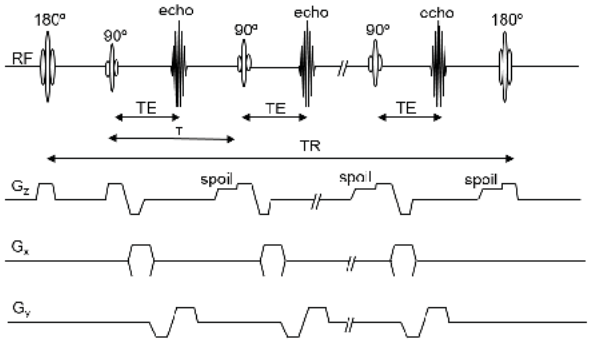

Figure 7. Multiple gradient echo

Table 1. MRI in Pharmacy

Authors	Ref	Sample	Experiment
Nebgen G et al.	[31]	circular tablet	qualitative study on the hydration of HPMC tablets
Soussi B. et al.	[58]	implant	extruded monolithic zero-order release kinetics implant; characterization of the liquid in polymer by T_2 maps
Hyde T. and Gladden L.	[59]	circular tablet	qualitative imaging study of the erosion of the drug containing compartment of a dry coated controlled release tablet
Peppas N. et al.	[60]	extrudate	measurement of water distribution in lipid/MCC extrudates processed at different speed and different water content in the formulation
Fyfe C. and Blazek-Welsh A.	[32]	spherical pellet	drug release from different formulations of API loaded lipid matrix pellets; diffusion measurements using PGSE sequence
Harding S. et al.	[61]	lipophilic matrix heophylline beads	quantitative imaging experiment of the liquid concentration, T_2 distribution and self-diffusion coefficient within poly(glycolic lactic acid) controlled release drug delivery system
Malveau C. et al.	[62]	capsule plug	dissolution study of coated pulsatile release capsules; release is triggered by swelling hydrogel plug
Johns M. and Gladden L.	[63]	circular tablet	quantitative measurement of the polymer concentration during the hydration of tablets
Baumgartner S. et al.	[64]	circular tablet	swelling and water diffusion was studied in samples of high amylose starch tablets
Richardson JC et al.	[15]	circular tablet	porosity imaging of tablets penetrated by gadolinium-doped silicon oil
Djemai A and Sinka I.	[65]	circular tablet	water penetration into the tablets is studied experimentally
Karakosta E. and McDonald P.	[66]	spherical pellet	pore structure evolution in pellets during dissolution; pellets were made of lactose and MCC
Marchessault R. et al.	[67]	circular tablet	porosity measurement of tablets made of three different excipients (MCC, lactose and anhydrous calcium phosphate) compressed at different pressures
Mäder K. et al.	[68]	capsule plug	dissolution study of capsules formulated from HPMC and L-dopa using flow through cell in a horizontal magnet

Table 2. MRI measurements of drugs

	MRI drugs
Maximum sample size	5 mm to 30 cm
Measurement possibilities	chemical specificity to nuclei of interest (intrinsic signal)
	nuclear spin relaxation times
	molecular mobility
Chemical sensitivity	high
Advantages	chemical specificity
	in situ dissolution studies are possible
	quantitative technique
	ability to study flow and diffusion processes
	wide range of imaging sequences is available to specifically emphasise certain properties of the sample
Limitations	only some solids can be imaged directly
	the experiments are usually destructive as they require the interaction of a liquid phase with the sample
	operation of strong magnetic fields requires special safety precautions
	restricted sample size in magnetic more
	paramagnetic materials (such as most metals) have to be eliminated from the sample setup

Table 3. T_1 and T_2 times measurements *in vitro*

MRI of samples; T_1 and T_2 times measurements *in vitro*		
Author / [Ref]	**Sample**	**Relaxation times**
Haas A. / [69]	phantom containing: six tubes filled with water, doped with different concentrations of Gd(DTPA)	T_1 values varied from 140 ms up to 2400 ms
Aboagye E. *et al*. / [60]	phantom containing: (BT) background tissue compartment; (P1) and (P2) pathological tissues compartments; (OF) fat compartment; (A) air compartment	T_1 = 1093 ms, T_2 = 91 ms for P1; T_1 = 993 ms, T_2 = 88 ms for P2; T_1 = 657 ms, T_2 = 84 ms for BT
Keenan K. *et al*. / [70]	phantom: $NiCl_2$ solutions with varying concentration (0.3 mM to 69.68 mM), and $MnCl_2$ solutions with varying concentration (0.013 mM to 1.704 mM)	T_1 value of 2033-22 ms and T_2 value of 1669-20 ms for $NiCl_2$; T_1 value of 2376-83 ms and T_2 value of 939-8 ms for $MnCl_2$
Hiraki Y. *et al*. / [71]	phantom: $GdCl_3$ concentration was varied from 0–140 μmol/kg and the agarose concentration was varied from 0–1.6% in a fixed carrageenan concentration of 3%	T_1 value of 202–1904 ms and T_2 value of 38–423 ms
Kato *et al*. / [72]	CAGN phantom containing: carrageenan, $GdCl_3$, agarose, NaCl, NaN_3 and distilled water	T_1 values of 202–1904 ms and T_2 values of 38–423 ms when the concentrations of $GdCl_3$ and agarose are varied from 0–140 μmol/kg, and 0%–1.6%
Jensen M., Caruthers S., Jara H. / [73]	phantom composed of several fluid-filled containers; phantom contains materials spanning the T_1 and T_2 relaxation times of the biologic range; (dilutions of gadolinium, distilled water, dilutions of ultrasound gel, corn oil, isopropyl alcohol, dilutions of glycerol, dilution of copper sulfate)	accurate T_1 measurements within the biologic range; T_2 measurements are accurate for T_2 values of less than about 500 ms, thus covering all known gel-like biologic tissues
Wickline S. *et al*. / [74]	phantom with highly potent paramagnetic liquid perfluorocarbon nanoparticle contrast agent (minimum concentration needed for diagnostic contrast)	input parameters: T_1 value of 1120 ms and T_2 value of 100 ms
Adriaensen H. *et al*. / [75]	four paramagnetic nickel sulfate ($NiSO_4$) aqueous solutions with different relaxation times, and fruit (apple, tomato)	T_2 values of 26-1248 ms for $NiSO_4$ solutions; T_2 values of 53-506 ms for apple and T_2 values of 114-835 ms for tomato

improved the ability to specifically tailor the features and properties of MNPs for these biomedical applications.[80] The objective of this study was to prepare and characterize magnetic nanoparticles embedded in polylactide-co-glycolide matrixes (PLGA-MNPs) as a dual drug delivery and imaging system capable of encapsulating both hydrophilic and hydrophobic drugs. Magnetic resonance imaging was carried out both *in vitro* and *in vivo* to assess the efficacy of PLGA-MNPs as contrast agents. PLGA-MNPs showed a better contrast effect than commercial contrast agents due to higher T_2 relaxivity with a blood circulation half-life~47 min in the rat model.[81] The inverse relationship between T_1 and nanoparticle concentration accounts for the nonlinear increase in contrast, resulting in a modest leveling of the contrast effect at high concentrations when TE is kept to a minimum (~7 nM). The close agreement between the model and the phantom data supports extrapolations to lower concentrations of nanoparticles. If a CNR >= 5 is defined as the minimum diagnostically meaningful contrast, the model shows that only picomolar concentrations of nanoparticles need be present within a typically-sized imaging voxel to produce diagnostic contrast enhancement for molecular imaging.[74]

Conclusion

The number of papers regarding the applications of MRI in pharmacy shows a huge progress in MRI hardware and software applied to biomedical research.

References

1. Nebgen G, Gross D, Lehmann V, Muller F. ^{1}H NMR microscopy of tablets. J Pharm Sci. 1995;84:283-91.
2. Hyde T, Gladden L, Payne R. A nuclear-magnetic-resonance imaging study of the effect of incorporating a macromolecular drug in poly(glycolicacid-co-DL-lactic acid). J Control Release. 1995;36:261-75.
3. Lee P, Adany P, Choi IY. Imaging based magnetic resonance spectroscopy (MRS) localization for quantitative neurochemical analysis and cerebral metabolism studies. Anal Biochem. 2017;529:40-7.
4. Cudalbu C, Cooper AJL. Editorial for the special issue on introduction to in vivo Magnetic Resonance Spectroscopy (MRS): A method to non-invasively study metabolism. Anal Biochem. 2017;529:1-3.
5. Capati A, Ijare OB, Bezabeh T. Diagnostic Applications of Nuclear Magnetic Resonance–Based Urinary Metabolomics. Magn Res Insights. 2017;10:1.

6. Gillinder L, Yi GS, Cowin G, et al. Quantification of intramyocardial metabolites by proton magnetic resonance spectroscopy. Front Cardiovasc Med. 2015;2:24.
7. Mandal P, Shukla D, Govind V, Boulard Y, Ersland L. Glutathione Conformations and Its Implications for *in vivo* Magnetic Resonance Spectroscopy. J Alzh Dis. 2017;10.3233/JAD-170350.
8. Zhang Y, An L, Shen J. Fast Computation of Full Density Matrix of Multispin Systems for Spatially Localized In Vivo Magnetic Resonance Spectroscopy. Med Phys J. 2017;10.1002/mp.12375.
9. Sheikh ASF, Mohamed M. Magnetic resonance spectroscopy and magnetic resonance spectroscopic imaging in Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy: A literature review. J Pak Med Assoc. 2017;67:912-16.
10. Younisa S, Hougaarda A, Vestergaardb M, Larssonb H, Ashinaa M. Migraine and magnetic resonance spectroscopy: a systematic review. Curr Opin Neur J. 2017;30:246.
11. Guo L, Wang D, Bo G, Zhang H, Tao W, Shi Y. Early identification of hypoxic-ischemic encephalopathy by combination of magnetic resonance (MR) imaging and proton MR spectroscopy. Exp Therap Med J. 2016;12:2835-42.
12. Bertholdo D, Watcharakorn A, Castillo M. Brain proton magnetic resonance spectroscopy: introduction and overview. Neuroimag Clin N Am. 2013;23:359-80.
13. Soares DP, Law M. Magnetic resonance spectroscopy of the brain: review of metabolites and clinical applications. Clin Radiol. 2009;64:12-21.
14. Pagani E, Bizzi A, Di Salle F, De Stefano N, Filippi M. Basic concepts of advanced MRI techniques. Neurol Sci. 2008;29,3:290-5.
15. Richardson JC, Bowtell R, Mäder K, Melia C. Pharmaceutical applications of magnetic resonance imaging (MRI). Adv Drug Deliv Rev. 2005;57:1191.
16. Figueiredo P, Lintinen K, Kiriazis A, et al. In vitro evaluation of biodegradable lignin-based nanoparticles for drug delivery and enhanced antiproliferation effect in cancer cells. Biomaterials. 2017;121:97-108.
17. Sua X, Chana C, Shia J, et al. A graphene quantum dot@ Fe3O4@SiO2 based nanoprobe for drug delivery sensing and dual-modal fluorescence and MRI imaging in cancer cells. Biosens Bioelectron. 2017;92:489-95.
18. Feng Q, Zhang Y, Zhang W, et al. Programmed near-infrared light-responsive drug delivery system forcombined magnetic tumor-targeting magnetic resonance imaging and chemo-phototherapy. Acta Biomat. 2017;49:402-13.
19. Banoa S, Afzal M, Waraich M, Alamgire K, Nazir S. Paclitaxel loaded magnetic nanocomposites with folate modified chitosan/carboxymethyl surface; a vehicle for imaging and targeted drug delivery. Intern J Pharm. 2016;513:554-63.
20. Chen Y, Ai K, Liu J, Sun G, Yin Q, Lu L. Multifunctional envelope-type mesoporous silica nanoparticles for pH--responsive drug delivery and magnetic resonance imaging. Biomaterials. 2015;60:111-20.
21. Ma Y, Ge Y, Li L. Advancement of multifunctional hybrid nanogel systems: Construction and application in drug co-delivery and imaging technique. Mat Sci Eng. 2017;71:1281-92.
22. Raggi P, Baldassarre D, Day S, de Groot E, Fayad ZA. Non--invasive imaging of atherosclerosis regression with magnetic resonance to guide drug development. Atherosclerosis. 2016;251:476-82.
23. Lin B, Su H, Jin R, Li D, Wu C, Jiang X, Xia C, Gong Q, Song B, Ai H. Multifunctional dextran micelles as drug delivery carriers and magnetic resonance imaging probes. Sci Bull. 2015;60:1272.
24. Punčochová K, Ewing AV, Gajdošová M, et al. Identifying the mechanisms of drug release from amorphous solid dispersions using MRI and ATR-FTIR spectroscopic imaging. Intern J Pharm. 2015;483:256.
25. Um SY, Par JH, Chung MW, Choic KH, Lee HJ. ^{1}H-Nuclear magnetic resonance-based metabolic profiling ofnonsteroidal anti-inflammatory drug-induced adverse effects in rats. J Pharm Biomed Anal. 2016;129:492-501.
26. Pacheco-Torres J, Mukherjee N, Walko M, et al. Image guided drug release from pH-sensitive Ion channel-functionalized stealth liposomes into an in vivo glioblastoma model. Nanomed: Nanotech Biol Med. 2015;11:1345-54.
27. Li L, Zhang R, Guo Y, et al. Functional magnetic porous silica for T1–T2 dual-modal magnetic resonance imaging and pH-responsive drug delivery of basic drugs. Nanotech. 2016;27:485702.
28. Gao N, Bozeman E, Qian W, et al. Tumor Penetrating Theranostic Nanoparticles for Enhancement of Targeted and Image-guided Drug Delivery into Peritoneal umors following Intraperitoneal Delivery. Theranostics. 2017;7:1689-94.
29. Melia CD, Rajabi-Siahboomi A, Bowtell R. Magnetic resonance imaging of controlled release pharmaceutical dosage forms. Pharm Sci & Techn Today. 1998;1:32-5.
30. Bowtell R, Sharp J, Peters A, et al. NMR microscopy of hydrating hydrophilic matrix pharmaceutical tablets. Magn Reson Imag. 1994;12:361-4.
31. Tung NT, Tran CS, Nguyen TL, Hoang T, Trinh TD, Nguyen TN. Formulation and biopharmaceutical evaluation of bitter taste masking microparticles containing azithromycin loaded in dispersible tablets. Eur J Pharm Biopharm 2017; doi: 10.1016/j.ejpb.2017.03.017
32. Fyfe CA, Blazek A. Investigation of hydrogel formation from hydroxypropylmethylcellulose (HPMC) by NMR spectroscopy and NMR imaging techniques. Macromolecules. 1997;30:6230.
33. Wang X, Roeloffs V, Kłosowski J, et al. Model-based T1 mapping with sparsity constraints using single-shot inversion-recovery radial FLASH. Magn Reson Med. 2017; DOI: 10.1002/mrm.26726.
34. Mazumdar A, Siegel MJ, Narra V, Luchtman-Jones L. Whole-Body Fast Inversion Recovery MR Imaging of Small Cell Neoplasms in Pediatric Patients: A Pilot Study. Am J Roentgen. 2002;179:1261-126.

35. Gupta RK. A modified fast inversion-recovery technique for spin-lattice relaxation measurements. J Magn Reson. 1980;38:447-452.
36. Helms G, Hagberg GE. *In vivo* quantification of the bound pool T_1 in human white matter using the binary spin–bath model of progressive magnetization transfer saturation. Phys Med & Biol. 2009;54:23-25.
37. Chow K, Flewitt JA, Green JD, Pagano JJ, Friedrich MG, Thompson RB. Saturation recovery single-shot acquisition (SASHA) for myocardial T_1 mapping. Magn Reson Med. 2013;71:2082-95.
38. Deoni SCL, Peters TM, Rutt BK. Determination of optimal angles for variable nutation proton magnetic spin-lattice, T_1, and spin-spin, T_2, relaxation times measurement. Magn Reson Med. 2003;51:194-9.
39. Messroghli DR, Nordmeyer S, Dietrich T, et al. Assessment of Diffuse Myocardial Fibrosis in Rats Using Small-Animal Look-Locker Inversion Recovery T_1 Mapping. Cardiovascr Imag. 2011;4:636-40.
40. Träber F, Block W, Lamerichs R, Gieseke J, Schild HH. ^{1}H metabolite relaxation times at 3.0 tesla: Measurements of T_1 and T_2 values in normal brain and determination of regional differences in transverse relaxation. J Magn Reson Imag. 2004;19:537-45.
41. Mlynárik V, Gruber S, Moser E. Proton T_1 and T_2 relaxation times of human brain metabolites at 3 Tesla. NMR in Biomed. 2001;14:325-31.
42. Bouhrara M, Bonny JM. B_1 mapping with selective pulses. Magn Reson Med. 2012;68:1472-80.
43. Dai W, Garcia D, de Bazelaire C, Alsop DC. Continuous flow-driven inversion for arterial spin labeling using pulsed radio frequency and gradient fields. Magn Reson Med 2008;60:1488-97.
44. Gelman N, Ewing JR, Gorell JM, Spickler EM, Salomon EG. Interregional variation of longitudinal relaxation rates in human brain at 3.0 T: Relation to estimated iron and water contents. Magn Reson Med. 2001;45:71-9.
45. Kiessling F, Pichler BJ. Small Animal Imaging: Basics and Practical Guide. Springer Science & Business Media. 2010;150-5.
46. Shah B, Anderson SW, Scalera J, Jara H, Soto JA. Quantitative MR Imaging: Physical Principles and Sequence Design in Abdominal Imaging. RadioGraphics. 2010;31:867-80.
47. Huang J, Zhang M, Lu J, Cai C, Chen L, Cai S. A fast chemical exchange saturation transfer imaging scheme based on single-shot spatiotemporal encoding. Magn Reson Med. 2017;77:1786-96.
48. Bydder GM, Pennock JM, Steiner RE, Khenia S, Payne JA, Young IR. The short TI inversion recovery sequence--an approach to MR imaging of the abdomen. Magn Reson Imaging. 1985;3:251-4.
49. Kurland RJ. Strategies and tactics in NMR imaging relaxation time measurements. I. Minimizing relaxation time errors due to image noise--the ideal case. Magn Reson Med. 1985; 2:136-58.
50. Bydder GM, Pennock JM, Steiner RE, Khenia S, Payne JA, Young IR. The short TI inversion recovery sequence-an approach to MR imaging of the abdomen. Magn Reson Imaging. 1985;3:251-4.
51. Hardy CJ, Edelstein WA, Vatis D, Harms R, Adams WJ. Calculated T1 images derived from a partial saturation-inversion recovery pulse sequence with adiabatic fast passage. Magn Reson Imaging. 1985;3:107-16.
52. Pacheco-Torres J, Mukherjee N, Walko M, et al. Image guided drug release from pH-sensitive Ion channel-functionalized stealth liposomes into an *in vivo* glioblastoma model. Nanomedicine. 2015;11:1345-54.
53. Tayari N, Heerschap A, Scheenen TWJ, Kobus T. *In vivo* MR spectroscopic imaging of the prostate, from application to interpretation. Anal Biochem. 2017;529:158–70.
54. Al-Iedani O, Lechner-Scott J, Ribbons K, Ramadan S. Fast magnetic resonance spectroscopic imaging techniques in human brain- applications in multiple sclerosis. J Biomed Sci. 2017;24:17-20.
55. Thrippleton MJ, Parikh J, Harris BA, et al. Reliability of MRSI brain temperature mapping at 1.5 and 3 T. NMR in Biomedicine. 2013;27:183-90.
56. Kasten J, Lazeyras F, Van De Ville D. Data-driven MRSI spectral localization via low-rank component analysis. IEEE Trans Med Imaging. 2013;32:1853-63.
57. Fu Y, Serrai H. Fast magnetic resonance spectroscopic imaging (MRSI) using wavelet encoding and parallel imaging: *in vitro* results. J Magn Reson. 2011;211:45-51.
58. Madhu B, Hjartstam J, Soussi B. Studies of the internal flow process in polymers by H NMR microscopy at 500 MHz. J ControllRelease. 1998;56:95-04.
59. Hyde T, Gladden L. Simultaneous measurement of water and polymer concentration profiles during swelling of poly-(ethylene oxide) using magnetic resonance imaging. Polymer. 1998;4:811-9.
60. Narasimhan B, Snaar J, Bowtell R, Morgan S, Melia C, Peppas N. Magnetic Resonance Imaging Analysis of Molecular Mobility during Dissolution of Poly(vinyl alcohol) in Water. Macromolecules. 1999;32:704–10.
61. Harding S, Baumann H, Gren T, Seo A. NMR microscopy of the uptake, distribution and mobility of dissolution media in small, sub-millimetre drug delivery systems. J Control Release. 2000;66:81–99.
62. Malveau C, Baille W, Zhu X, Marchessault R. NMR imaging of high-amylose starch tablets. 2. Effect of tablet size. Biomacromolecules. 2002;3:1249–54.
63. Johns M, Gladden L. Magnetic resonance studies of dissolving particulate solids. Magn Reson Imag. 2003; 21: 395–6.
64. Baumgartner S, Lahajnar G, Sepe A, Kristl J. Quantitative evaluation of polymer concentration profile during swelling of hydrophilic matrix tablets using ^{1}H NMR and MRI methods. Eur J Pharm Biopharm. 2005;59:299–06.
65. Djemai A, Sinka I. NMR imaging of density distributions in tablets. Intern J Pharm. 2006;319;55–62.

66. Karakosta E, McDonald P. An MRI analysis of the dissolution of a soluble drug incorporated within an insoluble polymer tablet. Appl Magn Reson. 2007;32:75–91.
67. Therien-Aubin H, Zhu X, Ravenelle F, Marchessault R. Membrane formation and drug loading effects in high amylose starch tablets studied by NMR imaging. Biomacromolecules. 2008;9:1248–54.
68. Strübing S, Abboud T, Contri R, Metz H, Mäder K. New insights on poly(vinyl acetate)-based coated floating tablets: Characterisation of hydration and CO_2 generation by benchtop MRI and its relation to drug release and floating strength. Eur. J. Pharm. Biopharm. 2008;69:708–17.
69. Haas A. Snapshot FLASH MRI. Applications to T_1, T_2 and Chemical-Shift Imaging. Magn Reson Med. 1990;13,77-89.
70. Keenan K, Stupic K, Boss M, et al. Multi-site, multi-vendor comparison of T_1 measurement using ISMRM/NIST system phantom. ISMRM 24th Annual Meeting. 2016;55:3290.
71. Yoshimura K, Kato H, Kuroda M, et al. Development of a Tissue-Equivalent MRI Phantom Using Carrageenan Gel. Magn Reson Med. 2003;50:1011–17.
72. Kato H, Kuroda M, Yoshimura K, et al. Composition of MRI phantom equivalent to human tissues. Am Assoc Phys Med. 2005; DOI: 10.1118/1.2047807.
73. Jensen M, Caruthers S, Jara H. Quantitative Magnetic Resonance Imaging With The Mixed Turbo Spin-echo Pulse Sequence: A Validation Study. The Internet J Radiol. 2000;2(1).
74. Morawski AM, Winter PM, Crowder KC, et al. Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI. Magn Reson Med. 2004;51:480-6.
75. Adriaensen H, Musse M, Quellec S, Vignaud A, Cambert M, Mariette F. MSE-MRI sequence optimisation for measurement of bi- and tri-exponential T_2 relaxation in a phantom and fruit. Magn Reson Imag. 2013;31,1677–89.
76. Zeitler JA, Gladden LF. *In-vitro* tomography and non-destructive imaging at depth of pharmaceutical solid dosage forms. Eur J of Pharm Biopharm. 2009;71:2-22.
77. Nott KP. Magnetic resonance imaging of tablet dissolution. Eur J Pharm Biopharm 2010;74:78-83.
78. Lavdas I, Behan KC, Papadaki A, McRobbie DW, Aboagye EO. A phantom for diffusion-weighted MRI (DW-MRI). J Magn Reson Imag. 2013;38:173-9.
79. Pothayee N, Balasubramaniam S, Pothayee N, et al. Magnetic Nanoclusters with Hydrophilic Spacing for Dual Drug Delivery and Sensitive Magnetic Resonance Imaging. J Mater Chem B Mater Biol Med. 2013;1:1142–9.
80. Jain TK, Richey J, Strand M, Leslie-Pelecky DL, Flask C, Labhasetwar V. Magnetic Nanoparticles with Dual Functional Properties: Drug Delivery and Magn Reson Imag Biomater 2008;29:4012–21.
81. Singh A, Dilnawaz F, Mewar S, Sharma U, Jagannathan NR, Sahoo SK. Composite Polymeric Magnetic Nanoparticles for Co-Delivery of Hydrophobic and Hydrophilic Anticancer Drugs and MRI Imaging for Cancer Therapy. ACS Appl Mater Interfaces. 2011;3:842–56.

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REVIEW PAPER

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^{19}F MRI As a tool for imaging drug delivery to tissue and individual cells

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ABSTRACT

Over the past few decades, magnetic resonance imaging (MRI) has proven to be extremely successful in medical applications. More recently, the biomedical applications of MRI have been gaining more use in the field of clinical pharmacy. In 1977, perfluorocarbon compounds (PFC), which form emulsions that can carry drugs, were analyzed by ^{19}F MRI and emulsified PFC compounds have been investigated as potential blood substitutes since the early 1960s and now a wide variety of PFC compounds are currently available as ^{19}F MRI biomarkers. Molecules with ^{19}F substituents are particularly attractive for use in drug tracking by ^{19}F MRI due to 100% ^{19}F abundance, high ^{19}F MRI sensitivity (0.83 relative to ^{1}H MRI) and an impressively large chemical shift range (400 ppm). Another benefit in the use of ^{19}F MRI is a zero background signal in biological samples due to lack of endogenous fluorine. Therefore, drugs containing fluorine atom have potential for ^{19}F MRI imaging drug delivery to tissue. This article will review recent developments in the use of ^{19}F MRI in imaging drug delivery to tissue and individual cells.

Keywords. drug delivery, drug tracking, fluorine, magnetic resonance imaging

Introduction

In this review, we will cover recent publications on fluorine-19 (^{19}F) Magnetic Resonance Imaging and Spectroscopy, ^{19}F ralaxation time, contrast agents, drug delivery, oxygen concentration in tumor tissue and individual cells. Our interest is in review of ^{19}F MRI applications to study cancer cells and cancer drug efficacy *in vitro*. Our focus is to present study that trying to explore cellular aspect of cancer by ^{19}F MRI techniques, so that an anti-tumor drug containing ^{19}F can not only be delivered to the cell, but also released inside the cell itself and constantly monitored. Current drug targeting research shows posibility

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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of limiting the side effects of the drugs and ultimately improving cancer treatment. However, when MRI is used for *in vitro* cell culture studies will require a high concentration of cells (order of 10^8 cell/mL).

^{19}F MRI techniques are valuable for non-invasive and non-destructive monitoring of fluorine biopharmaceuticals. There is an urgent need for cost-effective, non-invasive diagnostic techniques for monitoring the location of anticancer drugs due to their high toxicity in non-diseased tissue. While contrast-enhanced MRI can provide high sensitivity for the detection, ^{19}F MRI can provide much higher contrast due to lack of tissue background. ^{19}F MRI can characterize the biophysical states of ^{19}F in tissue and tissue perfusion by relaxation time measurements. In studies on many drug interactions in the tissue, the application of ^{1}H MRI has been limited because of the large molecular mass of water results in very broad signals. However, ^{19}F MRI can be used to aid pharmacodynamics and pharmacokinetics of several medically important fluorinated drugs. A wide range of chemical shift for the ^{19}F nucleus ensures good separation of signals in different environments. ^{19}F MRI offers an quantitative way of *in vitro* imaging high ^{19}F MRI sensitivity (0.83 relative to ^{1}H MR), an impressively large chemical shift range (~400 ppm) and zero background signal in biological samples. ^{19}F MRI a reliable analytical tool for monitoring organofluorine compounds and their quantification, when an internal standard of known concentration is included without the need for separation and derivatization steps. Importantly, such ^{19}F NMR studies translate well into a clinical setting as ^{19}F MRI allows the noninvasive monitoring of drug metabolism and pharmacokinetics. With the aim to investigate drug ^{19}F MRI and MRS we search literature data bases to find innovative applications for our study. Both techniques, ^{19}F MRI and ^{19}F MRS, can be used to examine structural aspects of much higher molecular weight proteins, which traditional one- or multi-dimensional NMR experiments fail to resolve. Many medicines containing fluoride compounds are available on the market and they are still growing.[1] Attention should be drawn to the possibility of analyzing ^{19}F spectra of drugs in both *in vivo* and *in vitro* studies focusing on ^{19}F of physiological fluids such as blood plasma. Developing such a method would allow for the observation of drug metabolism and dosage control. Perfluorocarbons (PFCs) are chemical compounds in which hydrogen atoms replace fluorine atoms. Emulsion-forming PFC compounds are used as the drug carrier analyzed by ^{19}F MRI. A 5-fluorouracil (5-FU), was the first anticancer drug introduced in 1957.[2] 5-FU is an antimetabolite cytostatic and a thymine derivative where the C-5 methyl group is replaced by the ^{19}F atom. In 1977, PFC which are contrast emulsions that can carry drugs, were analyzed by ^{19}F MRI.[3] PFC compounds are an potential blood substitutes and they are investigated since the early 1960s.[4] In 2005, it was shown for the first time by Ahrens et al. that cells can be labelled with PFC emulsions and tracked *in vivo* by ^{19}F MRI.[5] Below is a graph showing the number of ^{19}F MRI publications in the PubMed database over the years starting from 1885.

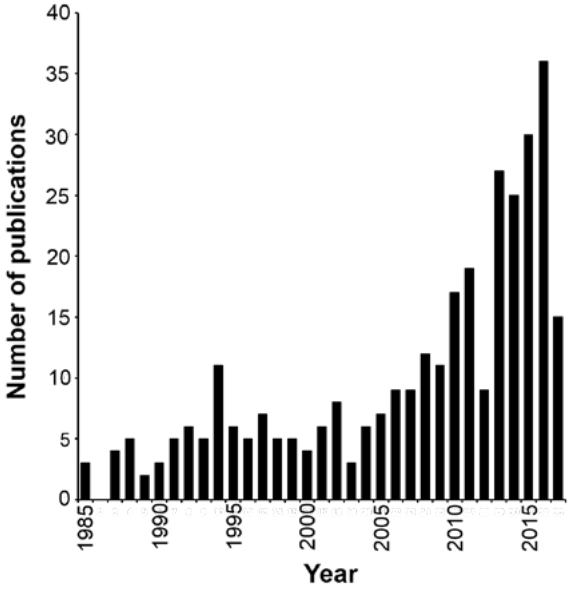

Figure 1. Number of publications on ^{19}F MRI collected from Library of National Center for Biotechnology Information (NCBI) PubMed Data Base

Among various types of imaging, ^{19}F MRI can provides a useful analytical tool to determine the localization of ^{19}F in the tissue. As the fluorine nuclei is normally absent in tissues, there is no natural abundance background. From all types of spectroscopy, ^{19}F MRS can provide signal and allows to quantify signal intensity. In MRS measurement of the spectrum is measured using a MRI scanner. The use of ^{19}F MRI and MRS has scientific and clinical significance. Both techniques can be used to measure specific drug concentrations, drug targeting and pharmacokinetics. Results obtained has helped to unveil how drugs work, why and which patients are non-responders, and aids modern individualized medicine. The most known examples of ^{19}F MRS are applications to measure the pharmacokinetics of drugs at their target sites are illustrated in many articles by human studies with 5-FU.[6] There is a direct relationship between tumor drug uptake and efficacy of therapy.[7]

Recently, micelles of tetraethylammonium perfluorooctanesulfonate (TPFOS, $C_8F_{17}SO_3N(C_2H_5)_4$, aqueous solutions of $C_9F_{19}COON(CH_3)_4$, fluoro- and hydrocarbon surfactants with different tail lengths and counterions ($^+N(CH_3)_4$, $^+N(C_3H_7)_4$ Li^+ and Na^+) have been studied by using ^{19}F MRS.[8] ^{19}F labeled phenolic compounds in early stages of oxidation of edible oils based on the formation of α-tocopheryl radicals initiated by oil-soluble vanadium complexes was studied.[9] The study by Hu and coworkers

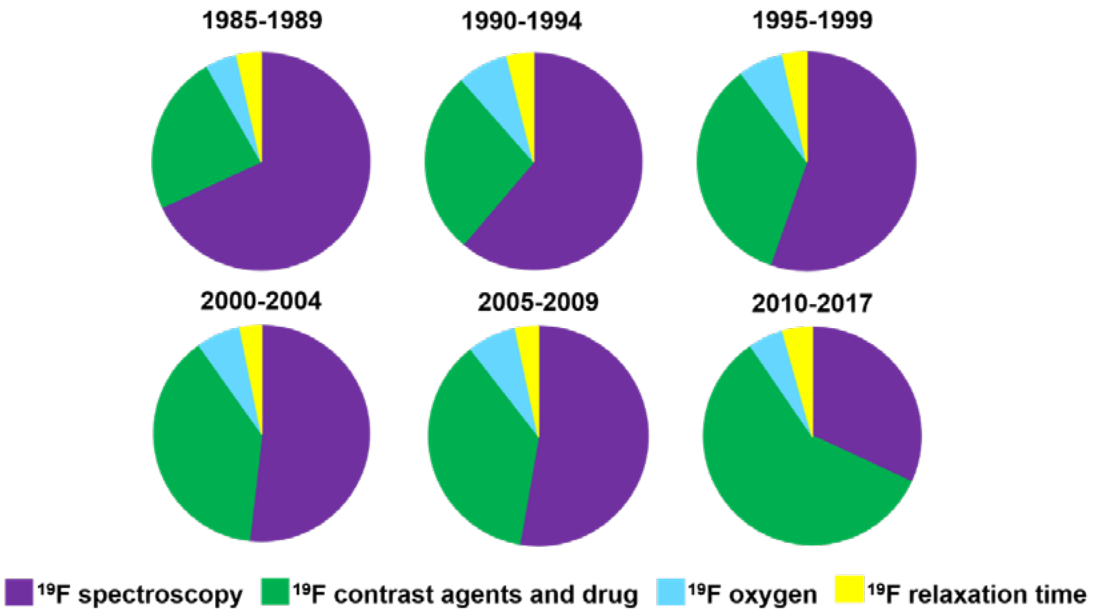

Figure 2. The statistic of ^{19}F research collected from Library of National Center for Biotechnology Information (NCBI) PubMed Data Base

have been demonstrated that ^{19}F NMR can be used as an alternative method to conventional radiolabeled studies for compounds containing fluorine without the need for radiolabeled synthesis.[10] Therefore, monitoring of tumor drug metabolism can separate responders form non-responders and thus prevent unnecessary toxicity. Possible to identify patients likely to respond to 5-FU. Capecitabine is designed as a pro-drug of 5-FU that can be taken orally; it is used as primary or adjuvant therapy in a range of cancers. Capecitabine metabolism in humans, and especially its heterogeneity, can be non-invasively assessed in the same manner. In addition, ^{19}F spectroscopy, ^{19}F contrast agents and drug ^{19}F oxygen and ^{19}F relaxation time were grouped. Figure 2 below shows the percentage distribution of topics in each year during 1985-2017.

^{19}F contrast agents and drugs

Fluoroorganic and fluoroinorganic compounds are very rare in nature and the few do occur in nature are highly toxic. The reaction to introduce ^{19}F to compounds is called fluorination. Examples of popular organofluoroxy reagents are acetyl hypofluorite which was used to fluorinate aromatic rings[11] and acetyl hypofluorite which has been intensively studied to use for addition to double bonds[12], fluorination of lithium enolates[13], and synthesis of α-fluorocarboxylic acid derivatives.[14] Another group the *N*-fluoropyrimidium triflates have been used to fluorinate aromatic rings, carbanions, enol ethers and their derivatives.[15]

As mentioned on above Figure 2, ^{19}F MRS has contributed to the development of agents modulating tumor drug metabolism. To date, the review of literature showed a research done with 5-FU[16], NaF[17-18], lescol[19], flunarizinum[20], mirenil[21], fevarin[22], sevofluran[23], cisprofloxacin[24], haloperidol[25], prozac[26], ciprinol flumetasone[27], perfluorodecalin[28] and trastuzumab[29]. To obtain a quantitative estimate of the drug, ^{19}F MRS is neccesary to study dynamic abundance of ^{19}F. In addition to the ^{19}F drugs, ongoing research is underway to find effective marker cells in ^{19}F MRI. Therapeutic cells are usually marked with MRI contrast agents, including gadolinium ions (Gd^{3+}), super paramagnetic iron oxides (SPIO) nanoparticles, or fluorinated compounds, to enhance the contrast.[30,31] PFCs, are inert and stable and are not part of any molecular pathway. Also, ^{19}F is known as a creator of a strong contrast signal *in vitro* and *in vivo* in background-free samples or tissue. *In vivo* ^{19}F MRS measurements of trifluorinated neuroleptics such as fluphenazine and trifluoperazine and antidepressants such as fluoxetine and fluvoxamine began in 1983. The experiment was performed using rats which have been treated with high oral doses of fluphenazine over a period of three weeks and imaged in 10 hours.[32] The reaction between perfluorotoluene and homocysteine thiolactone resulting in the formation of N-substituted homocysteine thiolactone derivative was studied.[33] Ahrens and coworkers describe the first clinical experience using a perfluorocarbon (PFC) tracer agent specifically engineered for ^{19}F MRI cell detection. Cells are labeled in culture using a PFC nanoemulsion formulation that is taken up by cells regardless of their phagocytic properties.[34] Optimal sensitivity can be achieved with dedicated ^{19}F compounds together with specifically adapted hardware and acquisition methods.[35] In general, the ^{19}F labels developed thus far consist of PFCs result-

ing in high ^{19}F density per molecule. PFCs have been proposed as blood substitutes for several decades.[36] ^{19}F MRI in the brains of living mice was published in 2005.[37] Two types of potential imaging agents were developed by using ^{19}F MRI they are ^{19}F-containing curcumin derivatives and ^{19}F-containing styrylbenzoxazole derivatives.[38] F-labeled proteins were used as a proof of concept and initial applications are presented for the HIV-inactivating lectin cyanovirin-N. Single F atoms were introduced at the 4-, 5-, 6- or 7 positions of Trp49 and the 4-position of Phe4, Phe54, and Phe80.[39] Liquid perfluorocarbon nanoparticle contains a high concentration of fluorine eg. 20 equivalent fluorine molecules.[40]

Herceptin efficacy in *ex vivo* cultures of MCF-7 breast carcinoma cells was analyzed.[41] Herceptin was used with perfluorooctyl bromide (PFOB) and conjugated with Lipoplex containing plasmid DNA and Lipofectamine (LipA) to allow ^{19}F MRI studies. Treatments such as Herceptin, Herceptin/PFOB and Herceptin/PFOB/Lipoplex were used for *ex vivo* targeting of MCF-7 cells cultured in three-dimensional (3D) geometry using hollow fiber bioreactor (HFB) device. The viability of MCF-7 cells after 72 h treatments decreased to 54±2%, 50±3% and 45±1% for Herceptin, Herceptin/PFOB and Herceptin/PFOB/Lipoplex, respectively. A significant correlation between the treatment concentration and efficacy was observed in MCF-7 cell cultures.[42] Using the calibration curves for ^{19}F SI, the ^{19}F SI of cells after treatments can be calculated and due to this we can calculate the number of cells targeted by emusions. The calibration curves allowed quantification of the number of fluorine particles bound to cells using the ^{19}F SI. Moreover, using this calibration, we can estimate number of free fluorine particles in media. The ^{19}F SI of PFCE not mixed with Herceptin and other compounds was considered as 100%. The new idea of the research is to synthetize dual probe, test cells labeling and monitor them with ^{19}F MRI/MRS and fluorescence. Drug delivery using dual probes into tumor cells can be accomplished in two steps: first, targeted delivery of drugs to the specific cell, and second, drug targeting inside the cell. The small size agents allow easier delivery to tumor cells as compared to larger or heavier agents. Perfluoropolyetheres (PFPE's) are examples of suitable fluorocarbon ^{19}F MRI agents [5]. Commonly used and useful are cyclic PFPE's such as perfluoro-15-crown-5-ether, perfluoro-18-crown-6-ether and perfluoro-12-crown-4-ether. These compounds can also include additional markers useful for e.g. Positron Emission Tomography (PET) and/or fluorescence. An interesting PFPE is also a linear PFPE. Linear PFPE can relatively easily conjugate a variety of functional entities to the terminal groups. For example, a fluorescent agent attached to the terminal group may be used to generate imaging agents that can be visualized with ^{19}F MRI and fluorescence microscopy.

There are the following 5 options to be considered as dual probes:

1. polyethylene glycols (PEG)+Linear PFPE+polyethyleneimine (PEI) (Figure 3)
2. Linear PFPE+dextran+Cy5.5+antibody (Ab) (Figure 4)
3. Linear PFPE+aminated dextran+Cy5.5+Ab (Figure 5)
4. Fluoresent (Cy5.5) - linear PFPE (Figure 6)
5. Biotinylated ^{19}F compound, FITC dextran, antibody and avidin (Figure 7)

Figure 3 shows the reaction of the terminal groups of polyethylene glycols (PEG) which may be exploited to prepare a range of conjugated imaging reagents. Hydrophilic and lipophilic moieties may be attached to a linear PFPE. PEG can be added to linear PFPE: A-[O--CF_2 (CF_2) x CF_2] n-B where x is an integer from 0 to 10, preferably 0-3, and n is an integer from 2 to 100, preferably 4–40, A–ester and B–amide, amine, forming water-soluble and lipid-soluble conjugates, respectively. Coupling PEG groups to PFPE ester will create a copolymer with water-soluble terminals. We expect that the PEG-PFPE polymer containing water-soluble fluorocarbon and hydrocarbon sections will form micelles with a PFPE core surrounded by PEG. Polyethyleneimine (PEI) can be added during the emulsification process to produce primary, secondary and tertiary amines. These amines in the form of nanoemulsion can be uptaken by tumour cells. The more PEI added into the emulsion, the greater the cellular uptake. ICG will be added during emulsification process.

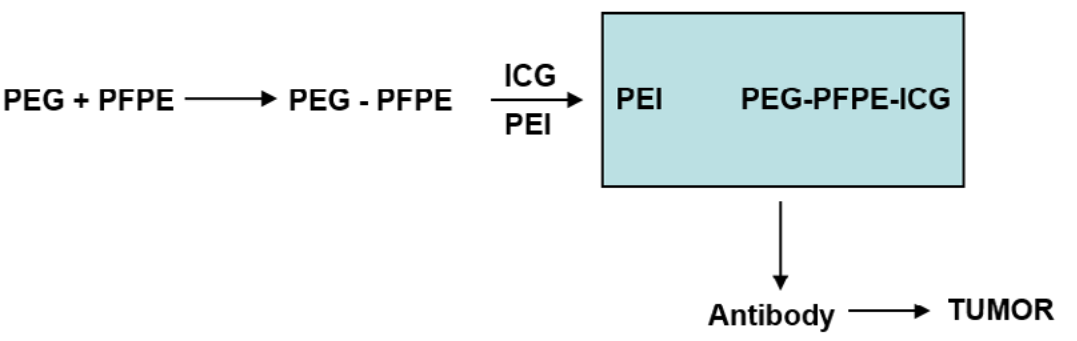

Figure 3. The synthetic scheme of polyethylene glycols (PEG) + Linear PFPE + polyethyleneimine (PEI)

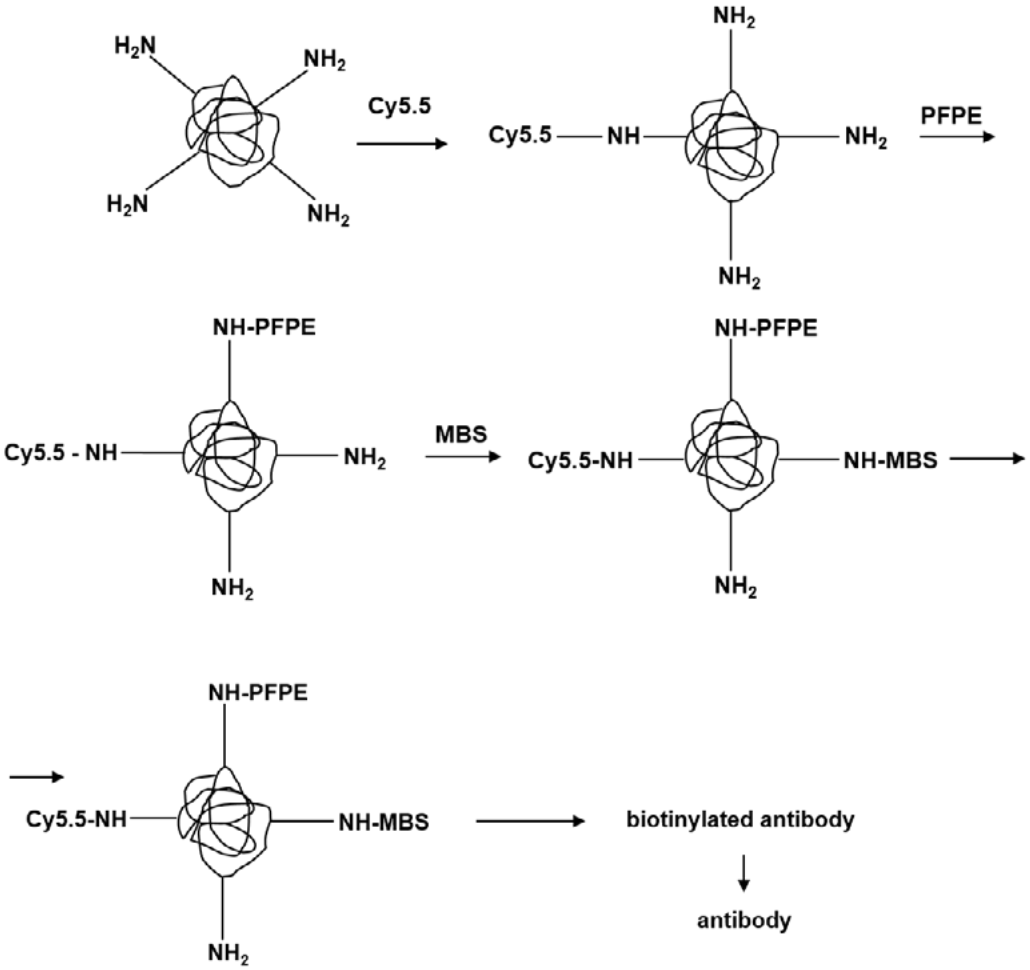

Figure 4. The synthetic scheme of linear PFPE+dextran+Cy5.5+Ab

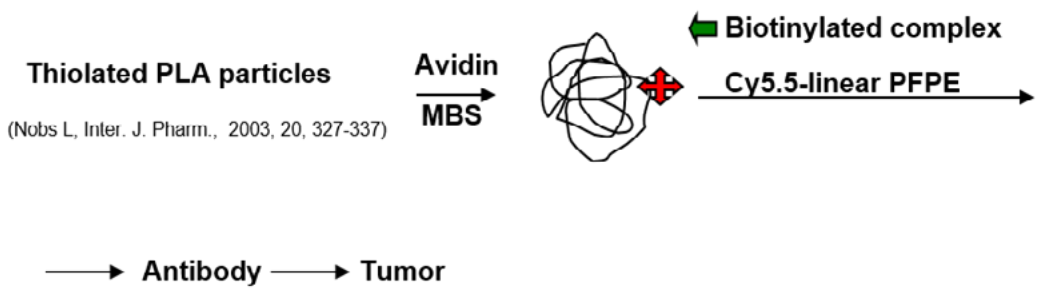

Figure 5. The synthetic scheme of linear PFPE + aminated dextran + Cy5.5+Ab

Figure 4 presents fluorine particles (FP's) consisting of linear PFPE, aminated dextran and Cy5.5. The probe can be modified with sulfo-m-maleimidobenzoyl-N-Hydroxyssicinimide ester (MBS). MBS offer two binding sites; one for primary amino group and one for thiols. Figure 5 shows addition of Avidin and antibody biotinylation.

Figure 6 presents a synthesis which is done by a carbodiimid method[43] followed by the coupling of cystamine dihydrochloride. Next, Avidin can be covalently bounded to the functional PLA particles via the sulfo-MBS. Next, fluoresent (Cy5.5) - linear PFPE particles will be prepared in the following 4 steps: 1) Cy5.5 will be conjugated with aminated dextran; 2) linear PFPE: A-[O--CF_2 (CF_2) x CF_2] n-B will added where x is an integer from 0 to 10, preferably from 0-3, and n is an integer from 2 to 100, preferably from 4 to 40 and A= ester (eg -C(O) O CH_2 CF_3), and B= amide, amine; 3) emulsion will be biotinylated; 4) functional PLA with avidin will be added; 5) Biotinylated antibody will be added and then fluorine particles labeled with avidin can be administrated. Bioti-

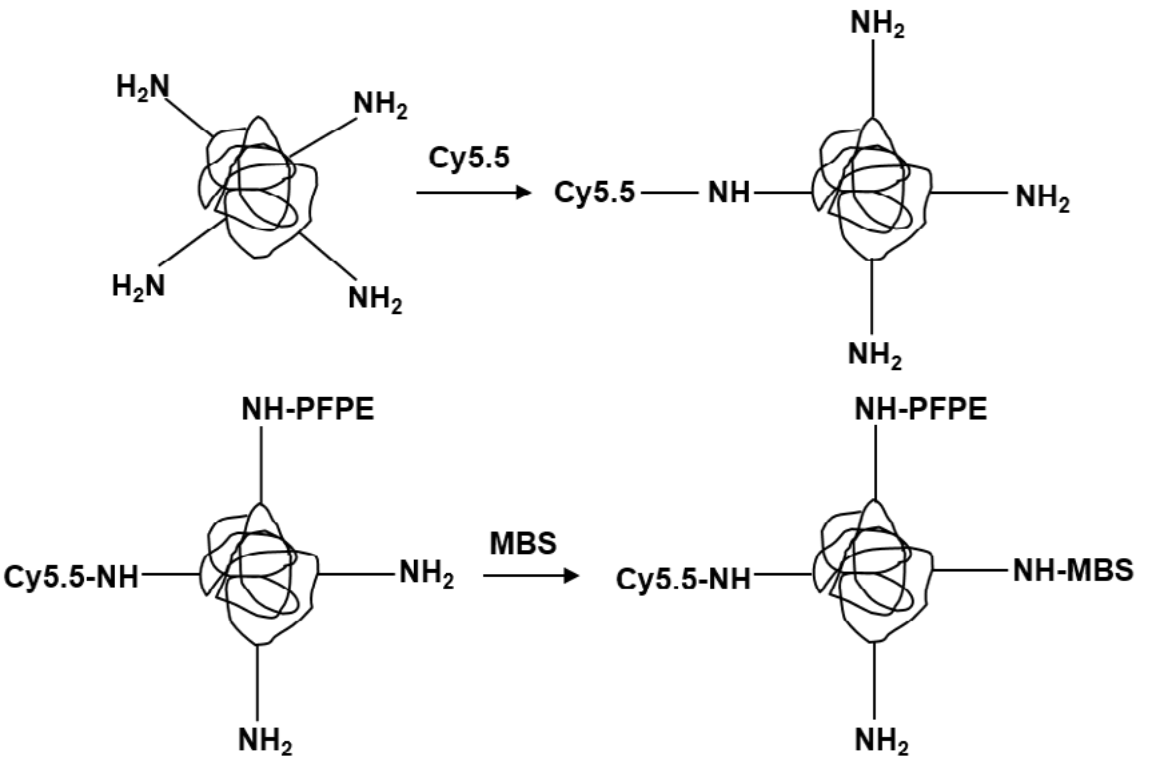

Figure 6. The synthetic scheme of fluoresent (Cy5.5) – linear PFPE

nylated ^{19}F compound, FITC dextran and antibody will be added to avidin.

^{19}F oxygen

Oxygen content in tumor tissue can be monitored with ^{19}F MRI. We hypothesize that the advantage of intracellular O_2 measurements will have an impact on the prophylaxis of cancer. We also expect that the results will provide information on therapeutic procedures involving specific antioxidants in order to improve cancer prevention. Many types of cancers are believed to result from DNA oxidative damage caused by free radicals. Our hypothesis is that antioxidant activity of tocopherols and tocotrienols are involved in the prevention of cellular damage.[44]

A recent clinical study among women using vitamin E supplements provided limited support for the hypothesis that antioxidant supplements may reduce the risk of breast cancer recurrence or breast cancer-related mortality. Antioxidants are substances that prevent damage of cells caused by free radicals.[45] Free radicals are oxygen atoms or chemical molecules with one or more unpaired electrons. Free radicals are usually very unstable and react rapidly with other cell compounds. These free radical reactions are responsible for cell and DNA damage causing possible development of cancer.[46] Vitamin E naturally occurs in four forms: alpha-, beta-, gamma- and delta-tocopherols and four corresponding unsaturated analogues, tocotrienols. The nutritional source of tocopherols and tocotrienols is either diet or vitamin supplements that are based on natural sources such as oil fraction of cereal grains, seeds or nuts. Palm oil is a particularly rich source of tocotrienols.[47] Tocopherols and tocotrienols are antioxidants and are believed to play a preventive role in diseases associated with oxidative stress including cancer.[48] The majority of conducted studies have been mostly focused on the role of alpha-tocopherol, thought to be the most biologically important form of vitamin E in breast cancer.[49] However, observational studies which investigated plasma or adipose tissue concentrations of alpha-tocopherol have failed to consistently support the theory that this isomer provides a protection against breast cancer. Other tocopherols, in particular gamma-tocopherol, have been associated with a reduced incidence of prostate cancer[50] and in a recent study an inhibitory effect on proliferation of prostate cancer cells was reported. Furthermore, delta-tocopherol was demonstrated, in one study, to have inhibitory effects on preneoplastic, neoplastic, and highly malignant mouse mammary epithelial cells whereas alpha- and gamma-tocopherol had no effect on cell proliferation.[51] The role of tocotrienols in cancer research is an important topic for investigators because it has been found that they are potentially active in prevention and treatment of breast, prostate and skin cancer. Recent data indicates that tocotrienols can inhibit proliferation in breast cancer cells. Tocotrienols were proven to protect intact DNA from oxidative stress by inhibition of lipid peroxidation and by binding of reactive free radicals to DNA.[52] The mechanism of action of tocotrienols,

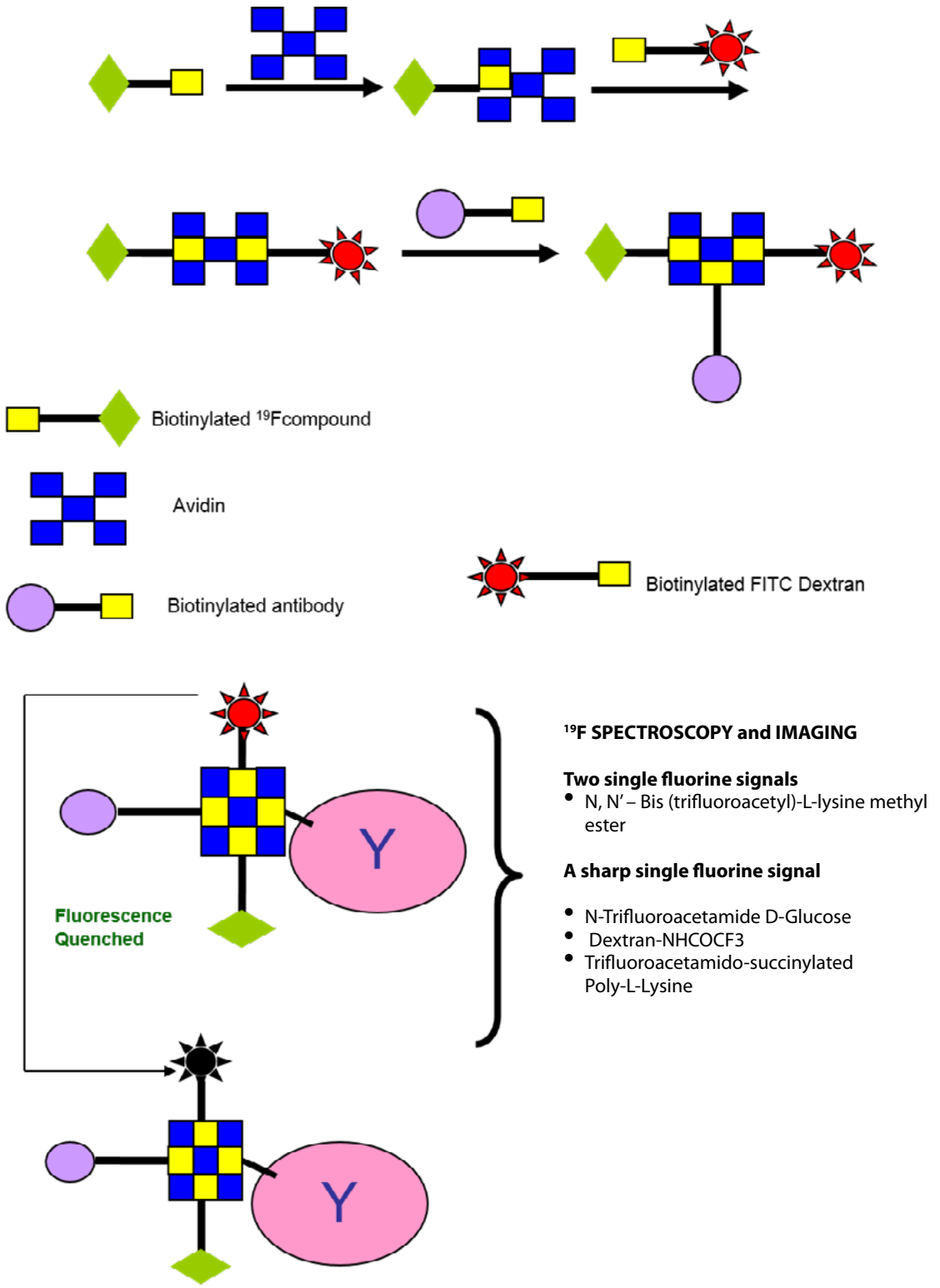

Figure 7. Biotinylated 19F compound constructed from FITC dextran, antibody and avidin

however, is still elusive. Some researchers postulate that the protective role of tocotrienols is not related to their antioxidant properties as shown in a recent study of the effect of tocotrienol-rich fraction from palm oil on gene expression in human breast cancer.[53] The antiproliferative activity of tocotrienols independent of antioxidant properties was also confirmed in a study with lung cancer cells.[54] Moreover, in a few studies, tocotrienols inhibited cancer cell proliferation in both estrogen receptor positive and negative human breast cancer cells, with slight difference in potency.[55] In a comprehensive study investigating the effect of alpha-, delta- and gamma- tocotrienols on

proliferation and apoptosis in preneoplastic, neoplastic and malignant mouse mammary cells it was found that tocotrienols inhibited cell growth in a following rank order potency: delta-tocotrienol ≥ gamma-tocotrienol >alpha-tocotrienol. Recently two extensive reviews of epidemiological studies were published on relationships between vitamin E and breast cancer, where vitamin E dietary intake, serum levels of vitamin E or alpha-tocopherol levels in adipose tissue were taken into consideration. It was concluded that natural alpha-tocopherol from dietary sources with or without presence of other tocopherols and tocotrienols may provide women a modest protection against breast cancer.[56]

However, up to today, there is still scarce information available about differences in individual tocotrienols levels in diseased and healthy women. Moreover, so far there were only a few studies simultaneously monitoring all forms of natural tocopherols and tocotrienols in women diagnosed with breast cancer. One study reported detection of all four tocopherols along with alpha- and beta-tocotrienol in breast adipose tissue from 10 women with cancer. Unfortunately, this study did not include control subjects, thus no data for comparison is available. In another study only alpha- and gamma-tocopherol were analyzed with no report on other vitamin E isomers. The newest study on the other hand, reports analysis of all tocopherols and tocotrienols except the β-isomer, in breast adipose tissue in benign and malignant breast lumps. Still, no control was included in research and no plasma values were determined. Despite an increasing number of reports on the influence of tocotrienols on breast cancer cells a question about which tocotrienol isomer is the most potent inhibitor of cell proliferation is still not answered. Moreover, an interrelationship between each individual tocopherol and tocotrienol with respect to their concentration in the breast adipose tissue/plasma is not yet established. In other words we are still lacking an overall study where all natural vitamin E isomers would be researched simultaneously in breast cancer. The ability to noninvasive monitor the oxygenation state of individual tumor cells would have important implications for planning and evaluation therapy. The presence of hypoxia limits is the success of radiotherapy in animal tumor. Hypoxic cells impair the effectives of because of their cell-cycle kinetics. Residual malignant cells protected from these therapeutic modalities by hypoxia may regroup to cause local recurrence of the disease. Consequently, knowledge of the oxygenation status of clongenic cells within solid tumors before and during treatment would be extremely valuable. The spin–lattice relaxation rate ($1/T_1$) of PFOB will increases linearly with increasing oxygen concentration. In work, they explored, for the first time, the uptake of chitosan-coated PLGA PFOB nanoparticles and their potential as contrast agents for ^{19}FMRI.[57]

A fluorinated cobalt(II) complex serves as a turn-on ^{19}F MRI tracer for reactive oxygen species including H_2O_2. Upon oxidation with H_2O_2, the complex converts from paramagnetic high spin Co^{II} to diamagnetic low spin Co^{III} resulting in a chemical shift change and enhancement in ^{19}F NMR signal. Further, the oxidation can be reversed in the presence of reductant $Na_2S_2O_4$. ^{19}F MRI presents a ~2-3 fold enhancement in signal.[58] ^{19}F MRI T_1 mapping with diffusion-based multispectral (MS) analysis was introduced.[59] Giraudeau and coworkers present in vivo quantitative measurements of O_2 in the liver and spleen using ^{19}F MRI.[60] The next study demonstrates that ^{19}F MRI of hexafluorobenzene offers a feasible tool to measure regional O_2 concentrations of brain, kidney, liver, gut, muscle, and skin during inhalation of both 30 and 100% oxygen *in vivo*, and that hyperoxia significantly increases O_2 of multiple organs in a rat model.[61]

Oximetry of the human T-Lymphoblastoid (CEM) cells was measured using ^{19}F magnetic resonance imaging ^{19}F MRI.[36] ^{19}F MRI was used to evaluate oxygen concentration in the cell suspension and thus its viability Human gland mammary adenocarcinoma (MCF-7).[38] Tumor oxygenation has been shown to be an important indicator of therapeutic response. The pO_2 spike was detected even though few ($\sim 4\times10^4$) T cells actually ingress into the CNS and with minimal tumor shrinkage.[62] In ^{19}F MRI oximetry, a method used to image tumour hypoxia, perfluorocarbons serve as oxygenation markers.[63] The pO_2 maps were generated after direct intratumoral administration of a fluorine compound (hexafluorobenzene) whose relaxation rate ($1/T_1$) is proportional to the % O_2.[64]

^{19}F relaxation time

The T_1 and T_2 relaxation times of ^{19}F are relatively short, while the chemical shift is large (1000 ppm). Therefore we selected fluorine to label the Abs .[65] To allow clinical studies, *in vivo* concentrations of fluorine–containing metabolites must be larger than about 200 mol/g tissue to be detectable in reasonable measurements times 5-15 min at B_0=1-2 T. For animal studies at 7 T amount of 50 nmol/g can be detected within 30 min. In *ex vivo* studies 5-10 nmol / g can be detected within 1-2 hrs at 11.7 T.

Herein we provide an overview of the methods available for relaxation measurements in MRI. While standard magnetic resonance imaging can provide basic information regarding tumour location, its size and spread, the quantified MRI can evaluate the effectiveness of therapy. Of particular interest are changes in cells relaxivity which are correlated with cancer cells death. Medical MRI resolution of a few millimeters is typically adequate for proper diagnosis, however cell samples require much higher resolution. At the same time valuable physiological information can be extracted from a small cluster of cells, while quantitative investigations of dynamics of drug delivery and drug effects *ex vivo* was shown to be crucial for the development of effective therapy Therefore, there is a growing interest in using MRI for examination of the treated cells.

Fluorine-19 MR spectroscopy was used to monitor the anti-depressant drug fluoxetine (and its metabolite norfluoxetine) *in vivo* in the human brain. The individual T_1*s* varied from 149 to 386 ms, which was attributed in part to interindividual differences based on the reproducibility of a phantom T_1. The individual T_1 correlated weakly with approximate brain concentration.[66] Initial cell labeling strategies for MRI made use of contrast agents that influence the MR relaxation times T_1, T_2, T_2* and lead to an enhancement T_1 or depletion T_2* of signal where labeled cells are present.[67] The observed MRI image intensities were related to the NMR longitudinal and transverse relaxation times, and were found to depend on polymer structure and method of micellization.[68] The value of the *in vivo* spin-lattice relaxation time T_1 of the fluoride ions naturally accumulated in bone mineral has been determined to be 2.00 .[69]

Insufficient contrast between normal and diseased tissues requires the use of contrast agents. Cross-linked iron oxide (CLIO) and other iron oxide formulations affect T_2 primarily and lead to a decreased signal.[70] Many commercial PFC emulsions have been found to be nontoxic or do not cause any health problems other than tissue swelling. Perfluoropolyethylene glycol (PFPE, MW 1750) is a linear PFC that contains a large number of ^{19}F atoms per molecule for enhancing sensitivity . The center CF_2 groups can generate a strong peak (>0.9 at -92 ppm) that dominates 90% of its ^{19}F signal, whereas the end CF_2 groups yield a weak signal (<0.1 at -79 ppm) that is below the *in vivo* MRI detection limit. [71]

Spin-lattice relaxation times T_1 and T_1rho of fluorine atoms in solid dispersions were determined at various temperatures (-20 to 150 °C). Correlation time (tauc), which is a measure of rotational molecular mobility, was calculated from the observed T_1 or T_1rho value and that of the T_1 or T_1rho minimum, assuming that the relaxation mechanism of spin-lattice relaxation of FLF fluorine atoms does not change with temperature.[72] MRI of the ^{19}F longitudinal relaxation time T_1 of an inert fluorinated gas at thermal polarization. In contrast to hyperpolarized noble gases, with very long T_1*s*, the T_1 of SF_6 in mammal lungs is 0.8-1.3 ms. T_1 imaging of a phantom consisting of four different SF6/air mixtures with known T_1 values validates the modified Look-Locker T_1 imaging. [73]

Conclusions

^{19}F MRI alows for monitoring of ^{19}F containing compounds *in vitro* and *in vivo*. Abnormal oxygen content in adipose tissue indicative of cancer disorder also can be monitored with ^{19}F MRI. The number of newly synthetized ^{19}F containing compounds with medical application is increasing.

References

1. Hagmann WK. The many roles for fluorine in medicinal chemistry. J Med Chem. 2008;51:4359-69.
2. Heidelberger C, Chaudhuri NK, Daneberg P, Mooren D, Griesbach L, Duschinsky R. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature. 1957;179:663-6.
3. Holland GN, Bottomley PA, Hinshaw WS. ^{19}F magnetic resonance imaging. J Mag Res. 1977;28:133-6.
4. Janjic JM, Ahrens ET. Fluorine-containing nanoemulsions for MRI cell tracking. Wiley Interdiscipl Rev Nanomed Nanobiotech. 2005;1:492-501.
5. Ahrens ET, Flores R, Xu H, Morel PA. *In vivo* imaging platform for tracking immunotherapeutic cells. Nature Biotechnol. 2005;23:983-7.
6. Ruiz-Cabello J, Barnett BP, Bottomley PA, Bulte JWM. Fluorine ^{19}F MRS and MRI in biomedicine. NMR in Biomed. 2011;24:114-29.
7. Kamm YJL, Heerschap A, van den Bergh EJ, Wagener DJT. ^{19}F-magnetic resonance spectroscopy in patients with liver metastases of colorectal cancer treated with 5-fluorouracil. Anti-Cancer Drugs. 2004;15:229-33.
8. Wang X, Chen J, Wang D, Dong S, Hao J, Hoffmann H. Monitoring the different micelle species and the slow kinetics of tetraethylammonium perfluorooctane-sulfonate by ^{19}F NMR spectroscopy. Advances in Colloid and Interface Science. 2017; DOI:10.1016/j.cis.2017.05.0169.
9. Drouza C, Dieronitou A, Hadjiadamou I, Stylianou M. Investigation of phenols activity in early stage oxidation of edible oils by electron paramagnetic resonance and ^{19}F NMR spectroscopies using novel lipid vanadium complexes as radical initiators. J Agric Food Chem. 2017;65:4942-51.
10. Hu H, Katyayan KK, Czeskis BA, Perkins EJ, Kulanthaivel P. Comparison between radioanalysis and ^{19}F Nuclear Magnetic Resonance Spectroscopy in the determination of mass balance, metabolism, and distribution of pefloxacin. Drug Metab Dispos. 2017;45:399-408.
11. Vints I, Gatenyo J, Rozen S. Fluorination of aryl boronic acids using acetyl hypofluorite made directly from diluted fluorine. J. Org. Chem. 2013;78:11794-7.
12. Gatenyo J, Hagooly Y, Vints I, Rozen S. Activation of a CH bond in polypyridine systems by acetyl hypofluorite made from F2. Organic & Biomol Chem. 2012;10:1856-60.
13. Liu Z, Shibata N, Takeuchi Y. Novel methods for the facile construction of 3,3-disubstituted and 3, 3-spiro-2H,4H--benzo[e]1,2-thiazine-1,1-diones: synthesis of (11S,12R, 14R)-2-fluoro-14-methyl-11-(methylethyl)spiro[4H-benzo[e]- 1, 2-thiazine-3,2'-cyclohexane]-1,1-dione, an agent for the electrophilic asymmetric fluorination of aryl ketone enolates. J Org Chem. 2000;65:7583-7.
14. Vora HU, Rovis T. N-Heterocyclic carbene catalyzed asymmetric hydration: direct synthesis of alpha-protio and alpha--deuterio alpha-chloro and alpha-fluoro carboxylic acids. J Am Chem Soc. 2010;132:2860-61.
15. Bennasar ML, Zulaica E, Juan C, Alonso Y, Bosch J. Addition of ester enolates to N-alkyl-2-fluoropyridinium salts: total synthesis of (+/-)-20-deoxycamptothecin and (+)-camptothecin. J Org Chem. 2002;67:7465-74.

16. Kamm YJ, Heerschap, van den Bergh EJ, Wagener DJ. ^{19}F-magnetic resonance spectroscopy in patients with liver metastases of colorectal cancer treated with 5-fluorouracil. Anticancer Drugs. 2004;15:229-33.
17. Stanosz M, Stanosz S, Puchalski A. An assessment of the influence of fluoride, modified transdermal replacement hormone therapy and supplement hormone therapy on unmanageable osteoporosis in postmenopausal women. J Elementol. 2009;14:545–51.
18. Lucas V, Sicre J, Laredo JD, Guérin C, Kuntz D, Dryll A. Spontaneous fracture of the femur neck in a female patient with osteoporosis treated with sodium fluoride, Rev Rhum Mal Osteoartic. 1990;5:545-8.
19. Zhang Q, Gladden L, Avalle P, Mantle M. In vitro quantitative ^{1}H and ^{19}F nuclear magneticresonance spectroscopy and imaging studies of fluvastatin™ in Lescol® XL tablets in a USP-IV dissolution cell. J Control Release. 2011;156:345-54.
20. White TE, Surles-Zeigler MC, Ford GD, et al. Bilateral gene interaction hierarchy analysis of the cell death gene response emphasizes the significance of cell cycle genes following unilateral traumatic brain injury. BMC Genomics. 2016;17:130.
21. Stolarczyk M, Apola A, Krzek J, Sajdak A. Validation of derivative spectrophotometry method for determination of active ingredients from neuroleptics in pharmaceutical preparations. Acta Pol Pharm Drug Res. 2009;66:351-6.
22. Bolo NR, Hode Y, Macher JP . Fluorine magnetic resonance spectroscopy measurement of brain fluvoxamine and fluoxetine in pediatric patients treated for pervasive developmental disorders. MAGMA. 2016:268-76.
23. Takeda T, Makita K, Ishikawa S, Kaneda K, Yokoyama K, Amaha K. Uptake and elimination of sevoflurane in rabbit tissues-an in vivo magnetic resonance spectroscopy study. Can J Anaesth. 2000;47:579-84.
24. Pablos AI, Escobar I, Albiñana S, Serrano O, Ferrari JM, de Tejada AH. Evaluation of an antibiotic intravenous to oral sequential therapy program. Pharmacoepidemiol drug safety. 2005;14:53–9.
25. Kane JM, Carson WH, Saha AR, et al. Efficacy and safety of aripiprazole and haloperidol versus placebo in patients with schizophrenia and schizoaffective disorder. J Clin Psych. 2002;63:763-71.
26. Sijens PE, Mostert JP , Irwan R , Potze JH , Oudkerk M, De Keyser J. Impact of fluoxetine on the human brain in multiple sclerosis as quantified by proton magnetic resonance spectroscopy and diffusion tensor imaging. Psychiatry Res. 2008;164:274-82.
27. Vakirlis E, Kastanis A, Ioannides D. Calcipotriol/betamethasone dipropionate in the treatment of psoriasis vulgaris. Ther Clin Risk Manag. 2008;4:141–8.
28. Lim YT, Cho MY, Kang JH, et al. Perfluorodecalin/[InGaP/ZnS quantum dots] nanoemulsions as ^{19}F MR/optical imaging nanoprobes for the labeling of phagocytic and nonphagocytic immune cells. Biomaterials. 2010;31:4964-71.
29. Bartusik D, Tomanek B, Lattová E, Perreault H, Fallone G. Combined treatment of human MCF-7 breast carcinoma with antibody, cationic lipid and hyaluronic acid using ex vivo assays. J Pharm Biomed Anal. 2010;51:192-201.
30. Ahrens ET1, Bulte JW. Tracking immune cells in vivo using magnetic resonance imaging. Nat Rev Immunol. 2013;13:755-63.
31. Managh AJ, Edwards SL, Bushell A, et al. Single cell tracking of gadolinium labeled CD4+ T cells by laser ablation inductively coupled plasma mass spectrometry. Anal Chem. 2013;85:10627-34.
32. Bartels M, Albert K. Detection of psychoactive drugs using ^{19}F MR spectroscopy. J Neural Transm Gen Sect. 1995;99:1-6.
33. Chubarova AS, Zakharovaa OD, Kovala OA, et al. Design of protein homocystamides with enhanced tumor uptake properties for ^{19}F magnetic resonance imaging. Bioorg Med Chem. 2015;23:6943–54.
34. Ahrens ET, Helfer BM, O'Hanlon CF, Schirda C. Clinical cell therapy imaging using a perfluorocarbon tracer and fluorine-19 MRI. Magn Reson Med. 2014;72:1696-701.
35. Amiri H, Srinivas M, Veltien A, van Uden MJ, de Vries IJ, Heerschap A. Cell tracking using 19F magnetic resonance imaging: Technical aspects and challenges towards clinical applications. Eur Radiol. 2015;25:726-35.
36. Waters EA, Chen K, Allen JS, Zhang H, Lanza GM, Wickline SA. Detection and quantification of angiogenesis in experimental valve disease with integrin-targeted nanoparticles and 19-fluorine MRI/MRS. J Card Magn Reson. 2008;10:43-5.
37. Higuchi M, Iwata N, Matsuba Y, Sato K, Sasamoto K, Saido TC. ^{19}F and ^{1}H MRI detection of amyloid beta plaques *in vivo.* Nat Neurosci. 2005;8:527-33.
38. Tooyama I, Yanagisawa D, Taguchi H, et al. Amyloid imaging using fluorine-19 magnetic resonance imaging ^{19}F MRI. Ageing Res Rev. 2016;30:85-94.
39. Matei E, Gronenborn AM. ^{19}F Paramagnetic Relaxation Enhancement: A Valuable Tool for Distance Measurements in Proteins. Angew Chem Int Ed Engl. 2016;55:150-4.
40. Neubauer AM, Caruthers SD, Hockett FD, et al. Fluorine cardiovascular magnetic resonance angiography in vivo at 1.5 T with perfluorocarbon nanoparticle contrast agents. J Cardiovasc Magn Reson. 2007;9:565-73.
41. Bartusik D, Tomanek B. Detection of fluorine labeled Herceptin using cellular ^{19}F MRI *ex vivo*. J Pharm Biomed Anal. 2010;51:894-900.
42. Bartusik D, Tomanek B, Siluk D, Kaliszan R. ^{19}F MRI of 3D CEM cells to study the effects of tocopherols and tocotrienols. J Pharm Biomed Anal. 2010;53:599-602.
43. Nobs L, Buchegger F, Gurny R, Allémann E. Surface modification of poly(lactic acid) nanoparticles by covalent attachment of thiol groups by means of three methods. Int J Pharm. 2003;250:327-37.
44. Bartusik D, Tomanek B, Siluk D, Kaliszan R, Fallone G. The application of ^{19}F magnetic resonance *ex vivo* imaging of

three-dimensional cultured breast cancer cells to study the effect of delta-tocopherol. Anal Biochem. 2009;387(2):315-7.
45. Clarkson PM. Antioxidants and Physical Performance. Crit Rev Food Sci Nutr. 1995;35:131-41.
46. Fleischauer AT, Simonsen N, Arab L. Antioxidant supplements and risk of breast cancer recurrence and breast cancer-related mortality among postmenopausal women. Nutr Cancer. 2003;46:15-22.
47. Meydani M, Evans WJ, Handelman G, et al. Protective effect of vitamin E on exercise-induced oxidative damage in young and older adults. Am J Physiol. 1993;264:992-8.
48. Buttner GR, Burns CP. Vitamin E slows the rate of free radical mediated lipid peroxidation in cells. Arch Biochem Biophys. 1996;334:261-7.
49. Hunter D A. prospective study of the Intake of Vitamins C, E, and A, and the risk of breast cancer. New Eng J Med. 1993;329:234-40.
50. Huang HY, Alberg AJ, Norkus EP, Hoffman SC, Comstock GW, Helzlsouer KJ. Prospective study of antioxidant micronutrients in the blood and the risk of developing prostate cancer. Am J Epidemiol. 2003;157:335-44.
51. Schwenke DC. Does lack of tocopherols and tocotrienols put women at increased risk of breast cancer? J Nutr Biochem. 2002;13:2-20.
52. Galli F, Stabile AM, Betti M, et al. The effect of alpha- and gamma-tocopherol and their carboxyethyl hydroxychroman metabolites on prostate cancer cell proliferation. Arch Biochem Biophys 2004;423:97-102.
53. Nesaretnam K, Ambra R, Selvaduray KR, Radhakrishnan A, Canall R, Virgill F. Tocotrienol-Rich Fraction from Palm Oil and Gene Expression in Human Breast Cancer Cells. Ann NY Acad Sci. 2004;1031:143-57.
54. Kashiwagi K, Harada K, Yano Y, et al. A redox-silent analogue of tocotrienol inhibits hypoxic adaptation of lung cancer cells. Biochem Biophys Res Commun. 2008;365:875-81.
55. Ditsch N, Mayer B, Rolle M. Estrogen receptor expression profile of disseminated epithelial tumor cells in bone marrow of breast cancer patients, Recent Results. Cancer Res. 2003;162:141-7.
56. McIntyre BS, Briski KP, Gapor A, Sylvester PW. Antiproliferative and apoptotic effects of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. Proc Soc Exp Biol Med. 2000;224:292-01.
57. Vu-Quanga H, Vinding MS, Xia D, et al. Chitosan-coated poly(lactic-co-glycolic acid) perfluorooctyl bromide nanoparticles for cell labeling in ^{19}F magnetic resonance imaging. Carbohydrate Polym. 2016;136:936–44.
58. Yu M, Xie D, Phan KP, Enriquez JS, Luci JJ, Que ML. A CoII complex for ^{19}F MRI-based detection of reactive oxygen species. Chem Commun. 2016;52:13885-88.
59. Shi Y, Oeh J, Eastham-Anderson J, et al. Mapping *in vivo* tumor oxygenation within viable tumor by ^{19}F MRI and multispectral analysis. Neoplasia. 2013;15(11):1241-50.
60. Giraudeau C, Djemaï B, Ghaly MA, et al. High sensitivity ^{19}F MRI of a perfluorooctyl bromide emulsion: application to a dynamic biodistribution study and oxygen tension mapping in the mouse liver and spleen. NMR Biomed. 2012;25:654-60.
61. Liu S, Shah SJ, Wilmes LJ, et al. Quantitative tissue oxygen measurement in multiple organs using ^{19}F MRI in a rat model. Magn Reson Med. 2011;66:1722-30.
62. Zhong J, Sakaki M, Okada H, Ahrens ET. *In vivo* intracellular oxygen dynamics in murine brain glioma and immunotherapeutic response of cytotoxic T cells observed by fluorine-19 magnetic resonance imaging. PLoS One. 2013;8:59479-83.
63. Baete SH, Vandecasteele J, De Deene Y. ^{19}F MRI oximetry: simulation of perfluorocarbon distribution impact. Phys Med Biol. 2011;56:2535-57.
64. Magat J, Jordan BF, Cron GO, Gallez B. Noninvasive mapping of spontaneous fluctuations in tumor oxygenation using ^{19}F MRI. Med Phys. 2010;37:5434-41.
65. Bartusik D, Tomanek B. Application of ^{19}F magnetic resonance to study the efficacy of fluorine labeled drugs in the three-dimensional cultured breast cancer cells. Arch Biochem and Biophys. 2010;493:234–41.
66. Komoroski RA, Newton JE, Cardwell D, Sprigg J, Pearce J, Karson CN. *In vivo* ^{19}F spin relaxation and localized spectroscopy of fluoxetine in human brain. Magn Reson Med. 1994;31:204-11.
67. Waiczies H, Guenther M, Skodowski J, et al. Monitoring dendritic cell migration using ^{19}F/^{1}H magnetic resonance imaging. J Vis Exp. 2013;73:e50251. doi: 10.3791/50251.
68. Peng H, Blakey I, Dargaville B, Rasoul F, Rose S, Whittaker AK. Synthesis and evaluation of partly fluorinated block copolymers as MRI imaging agents. Biomacromolecules. 2009;10:374-81.
69. Code RF, Harrison JE, McNeill KG, Szyjkowski M. *In vivo* ^{19}F spin relaxation in index finger bones. Magn Reson Med. 1990;13:358-69.
70. Chen J, Pan H, Lanza GM, Wickline SA. Perfluorocarbon nanoparticles for physiological and molecular imaging and therapy. Adv Chronic Kidney Dis. 2013:20:466-78.
71. Winter PM. Perfluorocarbon Nanoparticles: Evolution of a Multimodality and Multifunctional Imaging Agent. Scintifica. 2014; Article ID 746574, 1-10.
72. Aso Y, Yoshioka S, Miyazaki T, Kawanishi T. Feasibility of ^{19}F NMR for assessing the molecular mobility of flufenamic acid in solid dispersions. Chem Pharm Bull (Tokyo). 2009;57:61-4.
73. Adolphi NL, Kuethe DO. Quantitative mapping of ventilation-perfusion ratios in lungs by ^{19}F MR imaging of T_1 of inert fluorinated gases. Magn Reson Med. 2008;59:739-46.

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REVIEW PAPER

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Functional MRI – how does it work?

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ABSTRACT

Magnetic Nuclear Resonance (MRI) is a non-invasive tissue imaging method. This technique is based on the influence of a strong magnetic field and electromagnetic wave of strictly defined frequency on the nucleus of elements with non-zero spin. The study describes one of the variants of functional MRI, (fMRI), which has become a key technique in brain imaging. This technique has excellent spatial and temporal resolution and involves a changing signal intensity depending on the degree of oxygenation of the blood. Blood oxygenation levels are known to vary in accordance with neural activity and these differences can be used to detect brain activity. This is due to increased demand for energy and oxygen in the area of increased neural activity. The basis of this imaging is the so-called Blood Oxygenation-Level Dependent (BLOD) effect. The aim of this paper is to present the scope of fMRI as a diagnostic method in neurology and in neurosurgery. This paper presents the principles of fMRI, methods of application, research result development, and suggests areas of possible medical applications. The limitations of fMRI as a clinical tool in medical applications will also be addressed. Studies presented in this paper are based on clinical fMRI experience and a literature review.
Keywords. deoxyhemoglobin, oxyhemoglobin, Magnetic Resonance Imaging, functional Magnetic Resonance Imaging

FMRI as a method of brain research and more

Introduction

Imaging the human brain, the main component of the central nervous system, is critical for disease diagnosis. Although brain mass is estimated to be 2% of total body weight, it consumes 20% of the oxygen that passes through the body. The brain has an enormous number of neurons connected to each other by dendrites and axons that connect this supercomputer with its "outer world," which is the body. The number of neurons is in the order of 100,000,000,000, and each of them can create up to several thousand connections with other neurons. Each

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neuron has several thousand synapses which act as axon communication points. Synapses are of two main types, electrical and chemical, and they are distinguishable.

The brain has two hemispheres separated by a longitudinal slot and connected by the corpus callosum. Externally, the brain is covered by a gray matter or the cerebral cortex. Brain tasks include memory, speech, interpretation of stimuli, movement, association, and control of skeletal muscle movements. It is strongly folded and accounts for more than half of the total number of brain cells. The inner layer of the hemisphere is a white matter consisting essentially of axons connecting different areas of the brain. Anatomical and physiological studies have revealed that the individual patches on which each hemisphere divides perform different functions. The frontal lobe is responsible for, inter alia, action and interaction with the sensory area. Other important tasks are mating, as well as analysis and control of emotional states. The temporal lobe is the center of the, inter alia, hearing and smell centers. In the parietal lobe sensory cells, cells responsible for pain, spatial orientation, coordination of movement as well as spatial-motor coordination are located. The occipital lobe contains visual centers, color analysis, depth, and visual associations.

The brain has a complex connection structure and a central question is: how does it work? Historically, the only way to find out what role a particular part of the brain plays is observation of damaged or surgically removed parts of the brain and subsequent behavior. This indirect way of inference was to answer the question: what are the effects of the damage on a given part of the brain?

Today we have methods to observe the workings of the brain in a living person including electroencephalography[1], positron emission tomography (PET), functional cerebral resonance (fMRI) and magnetoencephalography (MEG).[2-6] Each of the above methods has both advantages and disadvantages. Below is a tabular summary of some non-invasive imaging methods (Table 1).

Magnetic resonance imaging

Magnetic Resonance, or Magnetic Resonance (MRI), is a phenomenon discovered by Isidor Isaac Rabi (1898-1988), an American physicist of Polish origin. This discovery was honored with the Nobel Prize in Physics in 1944 for the resonant method of observing the magnetic properties of nuclear nuclei. I.I. Rabi was born in Rymanów, Poland after which he and his parents emigrated to the United States. He studied chemistry at Cornell University, and physics at Columbia University where he obtained his doctorate in 1929. In 1937 he became a professor of physics. In 1952, Edward Mills Purcell (born August 30, 1912 in Taylorville, Illinois, March 7, 1997 in Cambridge, Massachusetts) and Felix Bloch (born October 23, 1905 in Zurich, September 10, 1983), also received the Nobel Prize in Physics for developing new methods of precision measurement of nuclear magnetism and for the development of new methods for precise measurements of the atomic nucleus.

The phenomenon of MRI is based on the interaction of nuclei of non-zero spin elements placed in a strong magnetic field with a strictly defined frequency electromagnetic wave. To approximate the phenomenon, let us assume that we put in a certain space a number of atoms with non-zero spin and that these atoms are hydrogen atoms because it is the most common element in our body and its' relative sensitivity is greatest. These two characteristics determine the choice of this element as the one by which the image of anatomical structures is created. Generally speaking, magnetic resonance is present in many nuclei, but only some have practical applications in medical imaging. In clinical practice, ^{1}H MRI is most often used, but is increasingly implemented with ^{13}C, ^{15}N, ^{19}F, and ^{31}P.

Positively charged protons in the nuclei of the atoms rotate around their own axis. They have their own momentum or spin. Spin is the basic property of particles like mass or charge. The movement of charge is accompanied by the formation of a magnetic field. So for the purpose of this discussion, we consider each of the protons as a small bar magnet. In cases where the protons are not subjected to a magnetic field, their magnetic momentum will be extinguished. A different situation occurs when the same atoms are placed in a strong magnetic field; protons having their own small magnetic field will be par-

Table 1. A summary of some non-invasive imaging methods

Parameter / method	fMRI	EEG	PET	MEG
Time resolution	5 s	0.001 s	60 s	0.001 ms
Spatial resolution	5 mm	10 mm	5 mm	50 mm
Disadvantages and limitations	Limitations of MR examination (claustrophobia, implants, etc), motion, expensive imaging systems	Examination of the cortex of the brain, interpretation difficulties	The need for isotopes, movement, difficulty in accessing the study, very expensive imaging systems	Poor spatial resolution, interpretation difficulties
Advantages	Functional analysis, non-invasive method	Easy patient access to the study, a method of cheap	Functional analysis	Action within deeper structures

allel or antiparallel to the main magnetic field lines. The setting of a given proton decides its energy state. A state that is privileged is a condition that requires less energy. The proton also performs a precession movement. To put it bluntly, it is a movement similar to that of a child's toy - a top. The speed of this movement, and hence the frequency, depends on the intensity of the magnetic field and the magnitude, which is different for different elements.

This frequency is defined by the Larmor formula:

$$\omega_0 = \gamma B_0$$

and

$$f = \frac{1}{2\pi}\gamma B$$

where
Ω - resonance pulsation, γ - γ-intercept, B - magnetic field induction.

To understand this phenomenon, we now place the entire sample in a three-dimensional coordinate system so that the Z axis coincides with the direction of the magnetic field force lines. The vector of the magnetic force of a single proton will have a certain value on the Z axis and a direction parallel to the field force lines. The component along the Y axis will be 0. Now looking globally across the whole sample, we will notice that these single vectors of parallel and antiparallel forces sum up to form a resultant vector of longitudinal magnetization. Its value will depend only on the very small number of protons aligned parallel to the force lines of the magnetic field, and thus on the lower energy level. The vast majority of parallel and antiparallel vectors will be abolished and will not participate in the experiment. This is where all the hardships in nuclear magnetic resonance research are hidden - a very poor signal is received from the sample. If the sample is now exposed to a strictly defined frequency called the resonant frequency, the energy will be absorbed by the protons and converted to higher energy levels. This affects the magnetization of the object. After switching off the RF signal, we will be dealing with the opposite phenomenon, namely the energy transfer by the protons going from the higher to the lower energy level. This will again be a resonant frequency electromagnetic wave. If now, for a permanent magnetic field, we add an additional three magnetic fields of the X, Y, Z axes. We are able to change the additional fields accordingly to establish magnetic resonance. It should be added that this frequency will depend on the magnetic field in a given voxel, which in turn will be the sum of the fixed field and the operation of the three fields of the gradient. Similarly, if you receive a frequency, you will receive a signal that is a sum of multiple frequencies. By analyzing Fourier's received signal, we divide it into individual components, which, combined with the knowledge of the magnetic field distribution inside the magnet, will allow us to show where the given frequency originates.

By manipulating the gradient of the magnetic field in the respective X, Y, Z axes, you can scan the volume of layer to be tested.

Principles of fMRI action

At the base of the method lies the assumption that the rate of metabolism of a given area of the brain depends on its activity. Seiji Ogawa was the first to observe this phenomenon in 1990.[7] Ogawa *et al.* Based on in vivo studies, blood oxygenated level dependent (BOLD) contrast can be used to map blood oxygenation in the brain.[8]

This method utilizes the physiologically occurring phenomenon of local increase in blood flow through the stimulated brain area and the magnetic properties of hemoglobin, which, as a result of metabolic changes, becomes a natural contrast agent. Hemoglobin is an oxygen carrier and when it passes through the capillaries of oxygenated lungs it takes the form of oxyhemoglobin. After release of oxygen to tissues it takes the form of deoxyhemoglobin. Both hemoglobin forms have different magnetic properties. Unlike structural imaging, where the source of the MR signal is the hydrogen nucleus, functional differences in signaling from the oxygenated or oxygenated hemoglobin content are used in functional imaging.[9] Performing specific activities (movement, memorization, speaking) is accompanied by stimulation of the areas of the brain responsible as active neurons have increased oxygen requirements. Local blood flow increases and hence the amount of oxyhemoglobin in a particular area allowing for a stronger MR signal from that region. The intensity of the signal from the degree of hemoglobin oxidation is determined BOLD.

The BOLD signal is therefore a reflection of the current activity of the neurons. With Echo Planar Imaging (EPI), in the stimulated areas of the brain, there is a discrete but measurable signal change in the range of 2-5% for scanners of 1.5 T and about 15% for very high 4 T field scanners.[10] This signal change is recorded and is the basis for further analysis. The fMRI study is characterized by high spatial resolution.[11] The invaluable advantage of this method is its non-invasiveness, reproducibility, and the possibility of widespread clinical use. The disadvantage is its relatively low temporal resolution despite EPI.

The fMRI study requires that the experimenter take into account the state and ability of the patient. This is crucial for success and can determine the outcome of the measurement scheme. It is based on the use of alternating control and activation blocks at regular intervals.[12] Figure 1a presents a fMRI block design paradigm. The received response signals contain activation regions, as shown in Figure 1b, and areas in which brain stimulation

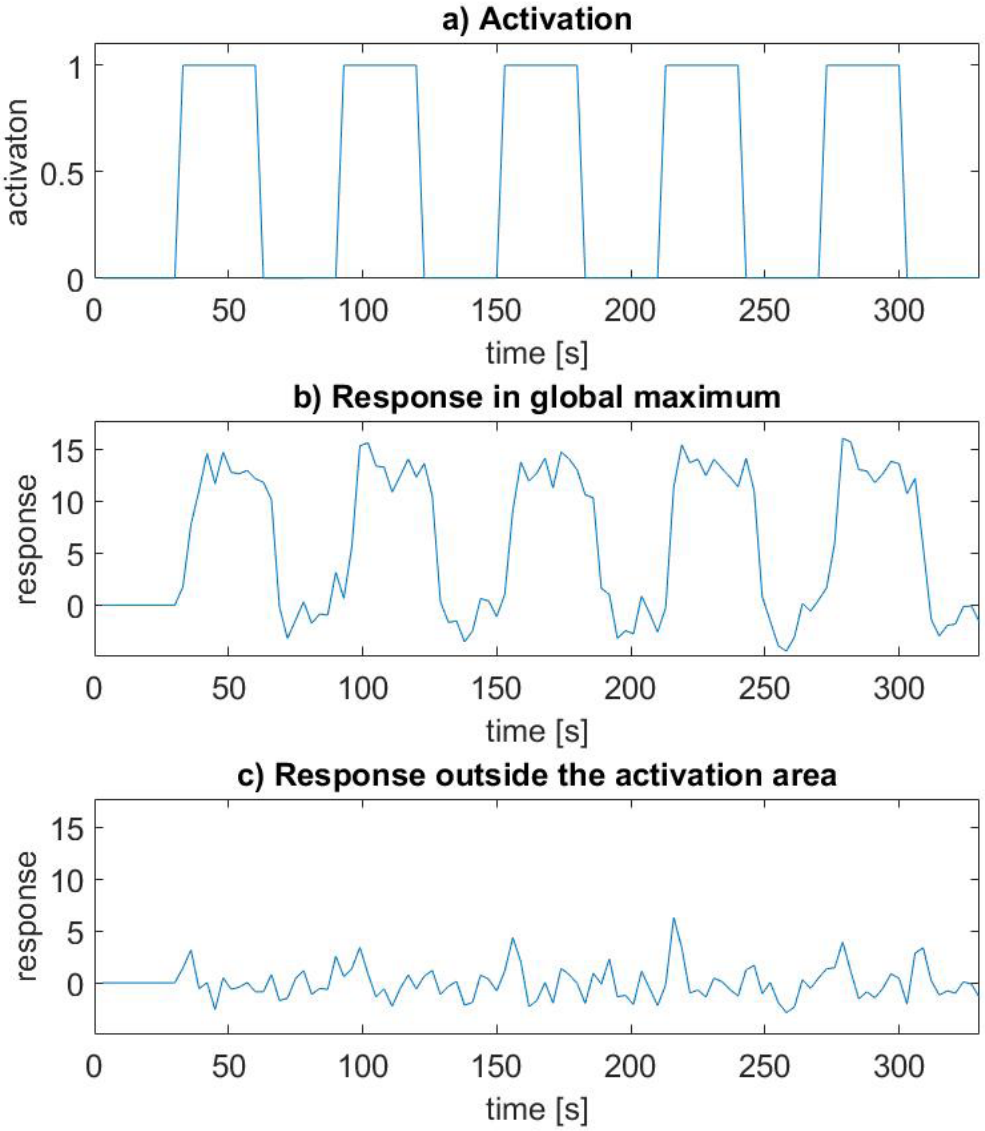

Figure 1. a) temporary activation, b) response at the global maximum, c) response from the place where activation did not take place

did not occur, as illustrated in Figure 1c. On this basis, the correlation can be clearly seen as well as the increase in the signal from the activated part of the brain with the activation signal.

Hardware and software

Performing functional tests with the fMRI method carries much higher technical and methodological requirements than MR imaging.[13] This study requires the use of additional equipment and specialized signal analysis software, which are not required in morphological studies.

The basic equipment of MR imaging equipment that performs fMRI studies is, inter alia, an auditory pacemaker device, a visual stimulation device, a stimulus response recording device, and a device for synchronizing a scanner with stimulation devices. Devices working in the scanner room should meet the very strict requirements of the PN-EN 60601-2-33 standard on the safety of magnetic resonance devices for medical diagnostics.[14]

The necessity to meet the standard can force the elimination of devices that may interfere with the tomographic signal. Hearing stimulation is performed by means of headphones, to which the sound wave is delivered with a sounder or by means of piezoelectric transducers. Visual stimuli are triggered by special goggles or, as a result of observation, in the mirror placed on the head coil of an image displayed on the screen with an image projector or special built-in monitors. Magnetic compatibility is achieved by the use of fiber optics, which can transmit patient response signals by pressing a button. In general, the specificity of equipment used in MR systems should be emphasized. These devices must be resistant to strong

magnetic fields and cannot themselves interfere with magnetic resonance.

Although manufacturers of magnetic resonance systems offer their own software, other signal analysis tools are also available. Because of the nature of the data and the way they are collected in time, the software should provide traffic correction, temporal and spatial data matching, and statistical analysis. One of the most popular software tools is the SPM (Statistical Parametric) using the MATLAB software package.[15,16] It allows you to perform a complete processing of the signal coming from DICOM scanners and their graphical representation.

Experiments

The fMRI study requires the preparation of both patient and staff. The paradigm and method of stimulation must be selected both for the purpose of the experiment and for the state of the patient. It may turn out that the person being tested due to his or her medical condition is not able to perform the instructions. Before the test the instructor should be carefully prepared in both the purpose and the test method. It is advisable to practice with the person taking part in the study and, in particular, with the patient before the experiment. This will allow you to avoid jittery or incorrectly executed commands, which can ultimately undermine the test itself. For example, in a simple experiment involving words that start with a particular letter, it is important that the patient does not try to speak because he activates the centers of the facial muscles movement and also causes head movement. This will lead to additional changes in other areas of the brain. In some cases, especially when the researcher is looking for answers to the question "which brain regions are responsible for the task", such additional changes may cause interpretation errors.

The study itself involves placing the patient in a magnet, performing a location scan, and then selecting layers in the area of interest to make an EPI sequence during which an experimental condition is generated to stimulate the investigated center. In addition, a sequence is created to obtain an accurate picture of the structural brain.

The first sequence with EPI, due to its speed and sensitivity, allows the BOLD signal to be recorded, while the other is used to obtain very good quality anatomical images. The results of the analysis of statistical images of EPI sequences are, in a sense, overlapping.

Signal Analysis

A separate discussion requires analysis of the signal from the experiment. A series of hundreds or even thousands of digital images are produced. The series is a time series showing the changes taking place in the brain. Owing to its nature and amplitude, very specific and sophisticated measures are needed to catch interesting changes. Pre-treatment of the signal is intended to minimize the effects of interference that affect the image. The most important sources of interference are involuntary movements of the head or whole body. It is important to note that the patient is required to remain completely immobilized during the study period, which is difficult or impossible for people suffering from various diseases although currently produced MR systems have motion compensation but are unable to completely remove this artifact. Flow artifacts occurring near large blood vessels, noise generated by the non-patient system itself related to receiving systems, amplifiers, ADC converters, mathematical processing, disturbances caused by the presence of metal elements in the patient's body, of which dental implants are a good example. Sometimes this artifact can completely distort the image and make it impossible to perform the test. Interference with MR findings and its effects on the patient, such as the impact of noise generated by the EPI sequence on the BOLD signal in the auditory cortex.

The most important methods for detecting functional activity include: correlation analysis, frequency analysis, multivariate analysis, analysis of the main components, and analysis of independent components. It should be emphasized that a poorly-selected mathematical apparatus that allows data to be developed can lead to serious errors and even to quite the opposite conclusion. It is not enough to get a large amount of data from a high-end measuring system, but it is important to properly develop research results. A leading example of data misconception is the experiment with dead salmon.[17] Conducted with all the rigors of scientific work, the experiment showed that the dead brain tissue of Atlantic salmon responds to a series of images presented. When analyzing the data, the researchers were astounded as they noticed the activity of two groups of nerve cells, which should not have been the case. The error was in the statistical methods used by neuroscientists. EPI is the fastest method of fMRI data acquisition that gives one image in 100 ms. However, it causes random noise that may be mistakenly identified as nerve cell activity. In the second step, the researchers used additional mathematical tools to help control the data from accidental noise and recalculate everything and the result turned out to be in line with the actual situation. Signal analysis of on-screen results may be misinterpreted. For example, let's use a fMRI experiment, which allegedly argued that rejection by others causes "psychological pain." The test participant participated in the virtual game of throwing a ball to other players mapped on the screen. At a time specified by the investigator, other "players" stopped throwing the ball to the test subject resulting in activation of a specific area of the brain. This result was interpreted as evidence of "internal pain". Unfortunately, this part of the brain activates, among others cognitive conflict, misunderstanding, and fatigue.[18] The more likely interpretation of the results was: "wondering what to do in the unexpected," as confirmed later.

Areas of application

The fMRI study is a constantly evolving research method. It finds its application to a large number of medical specialties associated with the brain. The use of functional cerebrovascular resonance in areas where it may not seem to apply, namely rehabilitation, forensics, or marketing research, is also being observed. Here are some examples to illustrate the enormous potential of functional cerebral resonance.

The pre-operative fMRI study of patients with brain tumors allows identification of important centers (mostly motor-sensory and speech) adjacent to tumors, as well as the possibility of complete resection, postoperative risk assessment and presumptive neurological deficits.[19] Analysis of these factors makes it possible for a neurosurgeon to make a rational decisions to optimize the planning of the surgery and to save important cortical centers. In neuroscience, this method is used to study emotions, decision making, speech and addictions.[20-26]

In psychology this method is also used, among others. For detecting lies.[27,28] The image of cortical activation in the course of speaking lies is well illustrated in fMRI. Lying requires more engagement of cognitive resources than telling the truth, and thus more intense brain work. This explains the fact that activation of the brain is greater in the course of lying than in telling the truth, and in particular the activation of the prefrontal cortex, which is the neuroanatomical substrate of memory. There was no significant difference in patients with schizophrenia compared to healthy subjects with pre-cortical activation. The presence or absence of delusions did not alter these results.[29]

A. Bryńska in his work entitled "In Search of the Causes of Autism Spectrum Disorders - Functional Neuroimaging (Part II)" describes the use of FMRI in the study of disorders in the spectrum of autism.[30] With fMRI you can also diagnose the occurrence of certain diseases. Studies have been performed on the risk of Alzheimer's disease.[31,32] Some apolipoprotein carriers of ApoE4 have seen increased levels and intensity of activation of relevant regions compared with ApoE3 carriers, which was associated with progressive memory loss, observed two years later.

A very interesting application is the study of the effectiveness of rehabilitation and plasticity of the brain. Having more knowledge about brain plasticity mechanisms is conducive to the development of new therapies for people with various brain injuries. In the case of patients experiencing a stroke, it is now possible to accurately plan rehabilitation taking into account which brain region has been damaged by cutting off the blood supply and selecting a set of specific exercises for recruiting new synapses.[33]

Another interesting area of fMRI applications is marketing research. It analyzes the impact of product advertising on the activity of each brain region and on the decisions a customer makes.

Summary

The paper describes a method of study called functional magnetic resonance. In such a short discussion it is impossible to include the full spectrum of possibilities offered by this imaging method. The method allows you to peek into not only the brain but also the awareness of human reactions to the surrounding world. Certainly in the coming years we will see the development of this method and its new applications.

References

1. Tarotin IV, Ivanitsky GA. Central EEG rhythm associated with movement and EEG rhythm associated with spatial reasoning: are they homologous? Zh Vyssh Nerv Deiat Im I P Pavlova. 2014;64:615–26.
2. Lu S, Xia Y, Cai TW, Feng DD. Semi-supervised manifold learning with affinity regularization for Alzheimer's disease identification using positron emission tomography imaging. Conf Proc IEEE Eng Med Biol Soc. 2015;2015:2251–4.
3. Sen B, Bernstein GA, Tingting Xu. Classification of obsessive-compulsive disorder from resting-state fMRI. Conf Proc IEEE Eng Med Biol Soc. 2016;2016:3606–9.
4. Leibovich T, Ansari D. Accumulation of non-numerical evidence during nonsymbolic number processing in the brain: An fMRI study. Hum Brain Mapp. 2017;38:4908-21.
5. Ioannides AA, Liu L, Poghosyan V, Kostopoulos GK. Using MEG to Understand the Progression of Light Sleep and the Emergence and Functional Roles of Spindles and K-Complexes. Front Hum Neurosci. 2017;11:313.
6. Salustri C, Tecchio F, Zappasodi F. Sensorimotor Cortex Reorganization in Alzheimer's Disease and Metal Dysfunction: A MEG Study. Int J Alzheimers Dis. 2013;2013:638312.
7. Moser E, Stadlbauer A, Windischberger C, Quick HH, Ladd ME. Magnetic resonance imaging methodology. Eur J Nucl Med Mol Imaging. 2009;36:30–41.
8. Ogawa S, Lee TM, Kay AR , Tank DW. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proc Natl Acad Sci USA. 1990;87:9868–72.
9. Aronen HJ, Korvenoja A, Martinkauppi S, Perkiö J, Karonen J, Carlson S. Clinical Applications of Functional Magnetic Resonance Imaging. International J Bioelectromagn. 1999;1:23–34.
10. Hutter J, Price AN, Cordero-Grande L, et al. Quiet echo planar imaging for functional and diffusion MRI. Magn Reson Med. 2017;doi: 10.1002/mrm.26810.
11. Polimeni JR, Renvall V, Zaretskaya N, Fischl B. Analysis strategies for high-resolution UHF-fMRI data. Neuroimage. 2017;doi: 10.1016/j.neuroimage.2017.04.053.
12. Pagani E, Bizzi A, Di Salle F, De Stefano N, Filippi M. Basic concepts of advanced MRI techniques. Neurol Sci. 2008;29:290-5.
13. Kozub J, Urbanik A, Chrzan R, Karcz P. Przedoperacyjne badanie funkcjonalne mózgu MR (fMRI). Przegl Lek. 2010;67:326–9.

14. Bogorodzki P, Piątkowska-Janko E, Orzechowski M, et al. Sprzęt i oprogramowanie do obrazowania czynności mózgu techniką rezonansu magnetycznego. Elektronika : konstrukcje, technologie, zastosowania. 2008;49:128–35.
15. Dale AM, Liu AK, Fischl BR, et al. Dynamic Statistical Parametric Mapping: Combining fMRI and MEG for High-Resolution Imaging of Cortical Activity. Neuron. 2000;26:55–67.
16. Acton PD, Friston KJ. Statistical parametric mapping in functional neuroimaging: beyond PET and fMRI activation studies. Eur J Nucl Med. 1998;25:663–7.
17. Zyzik R. O czym myśli martwy łosoś? Zdechła ryba w fMRI. Granice nauki. https://www.granicenauki.pl/o-czym-mysli-martwy-losos-zdechla-ryba-w-fmri-26011. Accessed October 5,2012.
18. Winkielman P. Psychologia społeczna a neuronauki: dominacja, separacja czy satysfakcjonujący związek? Psychol Społeczna. 2008;31:11–22.
19. Hou BL, Bradbury M, Peck KK, Petrovich NM, Gutin PH, Holodny AI. Effect of brain tumor neovasculature defined by rCBV on BOLD fMRI activation volume in the primary motor cortex. NeuroImage. 2006;32:489–97.
20. Northoff G, Richter A, Gessner M, et al. Functional Dissociation between Medial and Lateral Prefrontal Cortical Spatiotemporal Activation in Negative and Positive Emotions: A Combined fMRI/MEG Study. Cereb Cortex. 2000;10:93–107.
21. Britton JC, Phan KL, Taylor SF, Welsh RC, Berridge KC, Liberzon I. Neural correlates of social and nonsocial emotions: An fMRI study. NeuroImage. 2006;31:397–409.
22. Ivanoff J, Branning P, Marois R. fMRI Evidence for a Dual Process Account of the Speed-Accuracy Tradeoff in Decision-Making. PLoS ONE 2008;3:2635.
23. Johanna P, Ville O, Taina A, et al. Primary auditory cortex activation by visual speech: an fMRI study at 3 T. Neuroreport. 2005;16:125–8.
24. Riecker A, Mathiak K, Wildgruber D, et al. fMRI reveals two distinct cerebral networks subserving speech motor control. Neurology. 2005;64:700–6.
25. Asensio S, Romero MJ, Palau C, et al. Altered neural response of the appetitive emotional system in cocaine addiction: an fMRI Study. Addiction Biology. 2010;15:504–16.
26. McClernon FJ, Kozink RV, Lutz AM, Rose JE. 24-h smoking abstinence potentiates fMRI-BOLD activation to smoking cues in cerebral cortex and dorsal striatum. Psychopharmacology. 2009;204:25–35.
27. Spence SA. Playing Devil's advocate: The case against fMRI lie detection. Legal Criminoll Psychol. 2008;13:11–25.
28. Langleben, DD, Loughead JW, Bilker WB, et al. Telling truth from lie in individual subjects with fast event-related fMRI. Human Brain Mapping. 2005;26:262–72.
29. Lass P, Sławek J, Sitek E, Szurowska E, Zimmermann A. Diagnostic imaging of lying. Psych Polska. 2013;47: 65–74.
30. Bryńska A. Seeking the aetiology of autistic spectrum disorder. Part 2: functional neuroimaging. Psych Polska. 2012;46:1061–71.
31. Matthews PM, Jezzard P. Functional magnetic resonance imaging. Journal of Neurology Neurosurg Psych. 2004;75:6–12.
32. Bookheimer SY, Strojwas MH, Cohen MS, et al. Patterns of brain activation in people at risk for Alzheimer's disease. N Engl J Med. 2000;343:450–6.
33. Sikorski W. The mechanism of neuroplasticity and significance for psychotherapy and the evaluation of its effectiveness. Psychoterapia. 2016;177:43–56.

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REVIEW PAPER

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Magnetic Resonance Elastography – noninvasive method to assess liver disease

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ABSTRACT

Currently, liver disease is widespread and the awareness of these diseases is low. Early symptoms of liver disease do not necessarily indicate problems with this organ and patients are usually informed of their problems when the stage of the disease is already advanced. Invasive biopsies are the clinical diagnostic method most commonly used in the evaluation of liver disease. A biopsy is associated with a high risk of false results and additional complications. Finding new non-invasive imaging methods has led to the discovery of a new method called Magnetic Resonance Elastography (MRE). This technique allows one to evaluate the mechanical properties of tissues and to distinguish between pathological states. Testing using this technique can be performed on a conventional magnetic resonance system by using few additional components and properly prepared software. Studies have shown that there is a strong correlation between MRE-measured liver stiffness and the degree of fibrosis. MRE is also useful in characterizing liver tumors. Studies show that this technique is highly credible in both health volunteers and patients with liver fibrosis. MRE has tremendous diagnostic potential. The described technique is not currently widely used and has the potential to serve as a safe and accurate alternative in clinical diagnostics in the future.

Keywords. fibrosis, liver stiffness, MRE, magnetic resonance imaging

Introduction

The frequent occurrence of liver disease is a common problem in society. Nationwide polish studies of the elderly showed that over 37 % of people had Non-Alcoholic Fatty Liver Disease (NAFLD), and the incidence of advanced fibrosis in the general population was 7.79 %.[1] In most cases, people with liver disorders and pathologies are not aware of them at all. The most common diseases affecting the flesh of the liver are acute and chronic hepatitis B and C. Also prevalent are liver cirrhosis such as alcoholic or nonalcoholic fatty liver as well as various types of tumors.[2-5] NAFLD is the most prevalent dis-

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ease.[6,7] Liver cirrhosis associated with liver fibrosis is a serious and irreversible disease whose development (in case of early detection and use of appropriate treatment) can be slowed.[8,9] Liver cirrhosis is a progressive fibrosis of the liver parenchyma which leads to destruction of its structure.[10-12] Early symptoms of liver disease do not indicate problems with this organ. Often, sick people learn about their problem when the stage of the disease is already very high.

Currently, the most popular method for assessing liver disease is invasive biopsy. This method is associated with a high risk of complications.[13,14] The advantages of biopsy are a direct assessment of the degree of fibrosis and other pathological lesions (presence of fat, iron in the liver, biliary tract disease).[15,16] A limitation of biopsy is that cirrhosis may not be detected if the liver biopsy specimen is insufficient (too small) or taken from a healthy segment.[17,18] Biopsy is also associated with a high risk of false positives and additional complications. Another disadvantage is the high cost of performing a liver biopsy. Biopsies require a trained physician and hospitalization is often required.[19-22]

The search for new non-invasive imaging methods led to the discovery of a new method called Magnetic Resonance Elastography (MRE).[23] This technique allows one to evaluate mechanical properties of the tissue in vivo and as such, can used to provide quantitative imaging of stiffness of the liver.[24-26] This technique was introduced in clinical practice in 2007 and is currently used in more than 600 sites worldwide.[27] MRE is currently available in several centers in Poland including the Center for Innovation and Transfer of Natural Sciences and Engineering Knowledge, the University of Rzeszow and the Department of Radiology and Diagnostic Imaging, Nicolaus Copernicus University in Bydgoszcz. This review describes the MRI technique and its clinical use in liver testing. The topics covered will be NAFLD, liver fibrosis, hepatitis B and C.

MRE of the liver - technique and operation of the system

The liver MRE is a technique that lasts for several minutes and takes place in a lying position. This technique can be used on a conventional MR system. MRE requires the installation of additional equipment to generate mechanical waves and special software. MRE can be divided into three stages: (1) generating mechanical waves in the tissues of interest; (2) use of a special sequence of motion-encoding gradients (MEGs); (3) processing of wave images by means of an automated inversion algorithm and the formation of quantitative images, called elastograms, that depict the stiffness of tissue. In the liver MRI technique, mechanical waves with a frequency of 60 Hz are used.[23] The mechanical properties of liver tissue can be assessed since waves dissipate faster in stiffer tissue than in a normal liver.

Mechanical shear waves are generated by an acoustic driver outside the magnet space. Then the waves are transmitted through the connecting Tube to a disc-shaped passive driver that is placed in contact with the patient body over the liver (Figure 1). An elastic strap is used to ensure continued contact of the passive driver with patient body. Source of elastic waves (active driver) is synced to a MRI sequence from the scanner as described by Venkatesh *et al.*[28] The vibrations used in the liver MRE technique are well tolerated and do not affect the patient's comfort.

Measuring liver tissue motion by a passive driver with MRE is based on an MR imaging technique called phase-contrast MRI.[29] MR imaging is performing during continuous harmonic motion in the liver tissue, and MEG

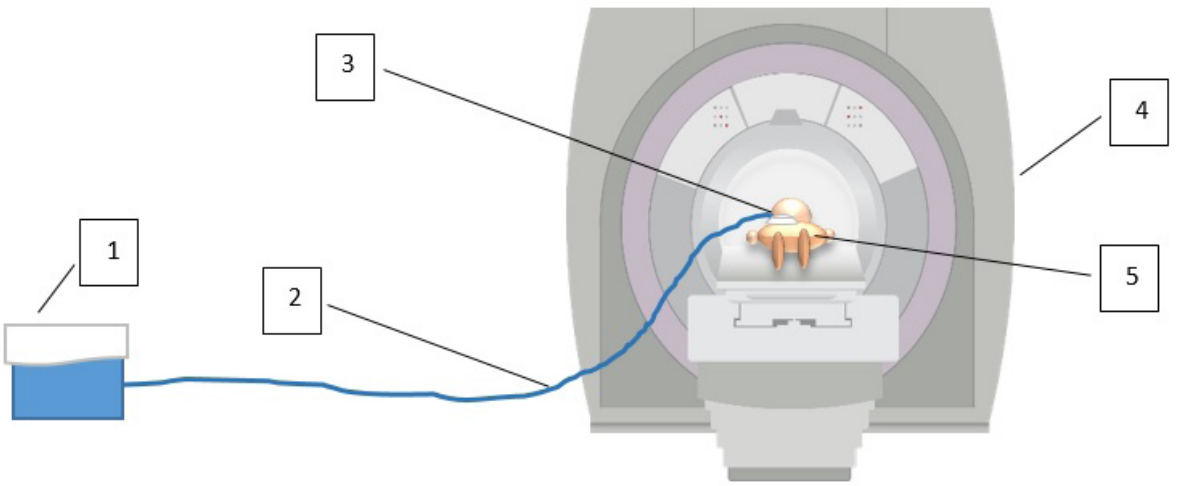

Figure 1. Scheme of a liver MRE system. 1 – Active Driver (source of mechanical waves placed outside the MR room); 2 – Connecting Tube (plastic tube transmitting acoustic vibration); 3 – Passive Driver (non-metallic element with flexible membrane, located on the liver); 4 – magnet; 5 – patient

oscillating at the same frequency. A special MR_Touch sequence is used in the MRE study. In this sequence, 4-8 layers of 6-10 mm thickness are used to produce liver stiffness maps. Each segment/layer requires a breath hold for about 16 seconds. During the breath-holding sequence, images of the pattern of propagating waves in the liver are generated. Upon completion of the study, wave images are automatically processed using an algorithm called an inversion algorithm to generate elastograms which are images of tissue stiffness. These images represent stiffness in units of pascals (Pa). Elastograms can be displayed in a color scale (range 0-8 kPa or 0-20 kPa) or in grayscale.

Stiffness of the liver is assessed by determination of a region of interest (ROI's) on elastograms. The ROI should be placed in the liver area taking into account the offset from the edge of the liver. Stiffness of a healthy liver should not exceed 2.93 kPa.[24,26,30] In the case of cirrhotic liver the shear waves are longer than the shear waves in the normal liver on wave images. MRE liver also serves as a good tool for characterizing liver tumors. In studies by Venkatesh et al., malignant neoplasms have been shown to be significantly more rigid than benign tumors.[31]

The liver MRE can be made in the majority of patients meeting the requirements of conventional MRI.[28,32,33] The study should be performed in fasting status, especially in patients with chronic liver disease.[34]

Detection of Liver Fibrosis using the MRE method

Hepatic fibrosis is easy to recognize using MRE. If the measured stiffness of the liver is greater than 2.93 kPa then more stages of fibrosis can be determined with high sensitivity and specificity.[26,35] Over the past ten years, more than 1,500 publications have been published describing the use of MRE for the detection of liver fibrosis (Fig. 2).

Liver fibrosis causes mechanical changes in the liver which are characterized by increased stiffness.[22] In further advanced stages of fibrosis, the stiffness of the liver parenchyma increases as described in a study by Yin M. et al.[26] MRE makes it possible to differentiate patients with advanced degrees of fibrosis from patients with less fibrosis.[25,26,30,35,36] In addition, this method allows detection of elevated stiffness caused by liver fibrosis, while the use of conventional imaging techniques shows no morphological changes or other anatomical features of liver fibrosis.[37] This is an important feature of this method, which can help in a successful diagnosis allowing for early treatment in chronic liver disease. Starting treatment at an early stage often prevents the development of the disease. In studies by Yin et al. from the Mayo Clinic, where 1377 subjects were examined, a high technical efficiency of 94.4 % was demonstrated.[38] In addition, mean liver rigidity was significantly higher in patients with advanced fibrosis (stage F3, F4 in METAVIR scale) than in patients with mild to moderate fibrosis (stage F0 to F2). Increased liver stiffness may be caused by conditions unrelated to liver fibrosis. Increased stiffness can affect, among others, acute hepatitis or acute biliary obstruction. Research conducted by Arena et al. have confirmed that acute inflammation of the liver without presence of fibrosis affects liver stiffness.[39]

Fatty liver, chronic hepatitis and other liver pathology in MRE technique

Nonalcoholic fatty liver disease is becoming more prevalent throughout the world and is often the main cause of chronic liver disease.[40] This disease is associated with many characteristics such as obesity, insulin resistance, hypertension and diabetes.[41] Many studies have confirmed that MRE liver can accurately distinguish simple fatigue from nonalcoholic steatohepatitis (NASH) and steatohepatitis with fibrosis.[42-44] In addition, the MRE technique is

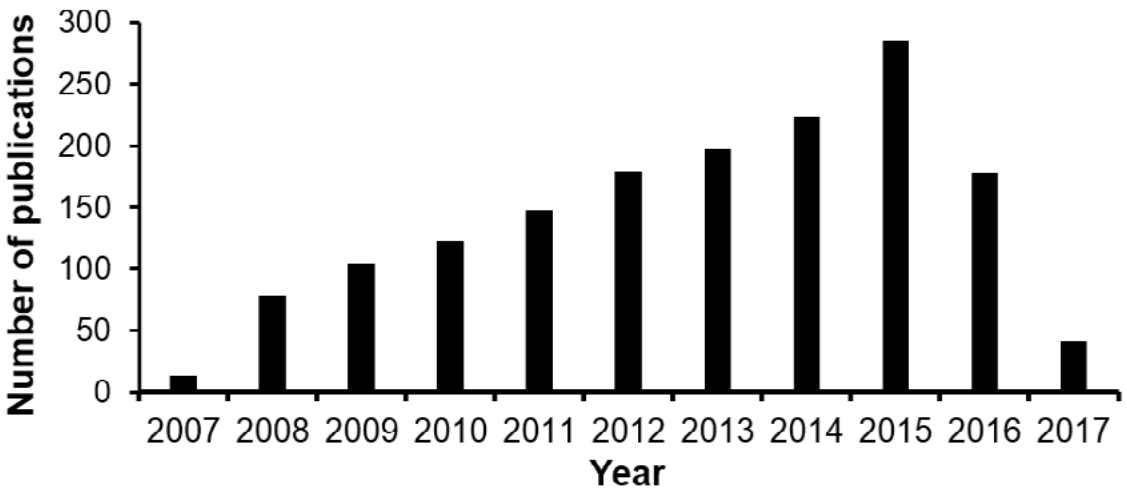

Figure 2. Number of publications in the PubMed National Center for Biotechnology Information (NCBI) for "magnetic resonance elastography liver fibrosis" from 2007 to 2017

helpful in evaluating and treating metabolic disorders. In NAFLD patients, lipid stiffness alone does not have a significant effect on liver stiffness, as shown by Yin M. *et al*.[26] However, studies by Chen J. *et al*. show that if the disease progresses to inflammation, the MRE technique allows the stiffness of the liver to be assessed before the onset of fibrosis.[42]

The Atsushi Nakajima team conducted a study of 142 patients with NAFLD to determine the accuracy of MRE and Transient Elastography (TE) in the classification of fatty liver and liver fibrosis in NAFLD patients.[45] These studies have shown that the diagnostic accuracy of MRE for liver fibrosis was found to be higher than that of clinical scoring systems and TE. In the study, 7.1 % of patients with advanced fibrosis (F3-F4 stage) were diagnosed by TE as "mild fibrosis" among 127 NAFLD patients, while only 2.8 % patients with advanced fibrosis were diagnosed using MRE as "mild fibrosis" Out of 142 patients with NAFLD. The results show that TE may misclassify patients with advanced fibrosis as compared with MRE.

MRE works very well for patients with chronic hepatitis C. This technique allows you to evaluate the liver's response to treatment. The hepatic stiffness measured by MRE in a patient with chronic hepatitis C can reduce to normal, suggesting proper drug therapy has been used.[46] Conducting cyclic liver MRE studies for patients with chronic hepatitis allows for the definition of progression or regression of disease.

Liver research conducted by Rohit Loomba et al. using MRE in patients with NAFLD has shown that a reduction in body mass index (BMI) of at least 5 % has a significant effect on the reduction of liver stiffness.[47]

MRE studies may be useful in the characterization of liver tumors. Studies conducted by Venkatesh *et al*. in liver examinations using MRE showed that benign liver tumors exhibited lower or similar stiffness to normal hepatic tissue. Malignant lesions, in the majority of cases, are more rigid than those of benign hepatic tumors. In each of 29 patients with malignant tumors, tumor stiffness was more than 5 kPa.[48] Other studies conducted at the Mayo Clinic, in turn, aimed to establish a correlation of tumor stiffness with histopathology features in patients with hepatocellular carcinoma (HCC). In this study, 21 patients with confirmed HCC were examined using MRE. The obtained data showed that tumor stiffness by MRE may be differentiated by HCC tumor grade.[49]

Summary

MRE is a non-invasive method for evaluating tissue stiffness. In liver MRE studies, it is important to know the presence of conditions that increase stiffness in order to properly interpret the results of MRE in the patients studied. Another important feature of MRE is the possibility of creating a spatial map of liver fibrosis that may be helpful in biopsy planning.[32] Numerous publications have shown that MRE is beneficial as a clinical tool for the diagnosis of hepatic fibrosis. MRE can be particularly important for patients taking medication to inhibit liver fibrosis. This technique is useful in the treatment of chronic liver disease. MRE allows to characterize focal lesions and estimate liver fibrosis. The enormous diagnostic potential of this method, which is outlined in the cited papers, is capable of providing a significant improvement in the diagnosis of liver disease. Continuous research using this technique is aimed at refining it and optimizing this method for subsequent clinical use. In conclusion, more and more evidence indicates that the MRE technique, thanks to the information it brings, can become an important element in the detection and characterization of cancer and the diagnosis of disease.

References

1. Hartleb M, Barański K, Zejda J, Chudek J, Więcek A. Non-alcoholic fatty liver (NAFL) and advanced fibrosis in the elderly: results from a community-based Polish survey. Liver Int. 2017; DOI:10.1111/liv.13471.
2. Craxi A, Antonucci G, Camma C. Treatment options in HBV. J Hepatol. 2006;44:77–83.
3. Liaw YF, Sung JJ, Chow WC, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med. 2004;351:1521–31.
4. Dienstag JL, McHutchison JG. American gastroenterological ascoication technical review on the management of hepatitis C. Gastroenterology. 2006;130:231–64.
5. Fernandez-Yunquera A, Rincón D, Salcedo M, Bañares R. Update on the use of direct-acting antiviral agents for the treatment of chronic hepatitis C virus infection. Rev Esp Quimioter. 2013;26:189-92.
6. Lonardo A, Byrne CD, Caldwell SH, Cortez-Pinto H, Targher G. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence and outcomes. Hepatology. 2016;64:1388-9.
7. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64:73-84.
8. Fowell A, Iredale J. Emerging therapies for liver fibrosis. Dig Dis. 2006;24:174–183.
9. Zoubek ME, Trautwein C, Strnad P. Reversal of liver fibrosis: From fiction to reality. Best Pract Res Clin Gastroenterol. 2017;31:129-41.
10. Koyama Y, Xu J, Liu X. New Developments on the Treatment of Liver Fibrosis. Dig Dis. 2016;34:589-96.
11. Friedman SL. Liver fibrosis- from bench to bedside. Journal of hepatology. 2003;38:38–53.
12. Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Clin Gastroenterol. 2011;25:195–206.
13. Bravo AA, Sheth SG, Chopra S. Liver biopsy. The New England journal of medicine. 2001;344:495–500.

14. Ratziu V, Charlotte F, Heurtier A, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology. 2005;128:1898–906.
15. Hernando D, Levin YS, Sirlin CB, Reeder SB. Quantification of liver iron with MRI: State of the art and remaining challenges. J Magn Reson Imaging. 2014;40:1003–21.
16. Reeder SB, Cruite I, Hamilton G, Sirlin CB. Quantitative assessment of liver fat with magnetic resonance imaging and spectroscopy. J Magn Reson Imaging. 2011;34: 729–49.
17. Maharaj B, Leary WP, Naran AD, et al. Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. Lancet. 1986;327:523–5.
18. Pagliaro L, Rinaldi F, Craxi A, Di Piazza S, Filippazzo G. Percutaneous blind biopsy versus laparoscopy with guided biopsy in diagnosis of cirrhosis. Dig Dis Sci. 1983;28:39–43.
19. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD. Liver biopsy. Hepatology. 2009;49:1017–44.
20. Eisenberg E, Konopniki M, Veitsman E, Kramskay R, Gaitini D, Baruch Y. Prevalence and characteristics of pain induced by percutaneous liver biopsy. Anesth Analg. 2003;96:1392–6.
21. Fernandez-Salazar L, Velayos B, Aller R, Lozano F, Garrote JA, Gonzalez JM. Percutaneous liver biopsy: patients' point of view. Scand J Gastroenterol. 2011;5521:1–5.
22. Tang A, Cloutier G, Szeverenyi NM, Sirlin CB. Ultrasound elastography and MR elastography for assessing liver fibrosis: Part 2, diagnostic performance, confounders, and future directions. Am J Roentgenol. 2015;205:33–40.
23. Muthupillai R, Lomas D, Rossman P, et al. Magnetic resonance elastography by direct visualization of propagating acoustic strain waves. Science. 1995;269:1854–7.
24. Rouvière O, Yin M, Dresner M, et al. MR elastography of the liver: preliminary results. Radiology. 2006;240:440–8.
25. Huwart L, Peeters F, Sinkus R, et al. Liver fibrosis: non--invasive assessment with MR elastography. NMR Biomed. 2006;19:173–9.
26. Yin M, Talwalkar J, Glaser K, et al. Assessment of hepatic fibrosis with magnetic resonance elastography. Clin Gastroenterol Hepatol. 2007;5:1207–13.
27. Tan C, Venkatesh S. Magnetic Resonance Elastography and Other Magnetic Resonance Imaging Techniques in Chronic Liver Disease: Current Status and Future Directions. Gut Liver. 2016;10:672-86.
28. Venkatesh SK, Yin M, Ehman RL. Magnetic resonance elastography of liver: technique, analysis, and clinical applications. J Magn Reson Imaging. 2013;37:544–55.
29. Moran PR. A flow velocity zeugmatographic interlace for NMR imaging in humans. Magn Reson Imaging. 1982;1:197–203.
30. Huwart L, Sempoux C, Vicaut E, et al. Magnetic resonance elastography for the noninvasive staging of liver fibrosis. Gastroenterology. 2008;135:32–40.
31. Venkatesh S, Yin M, Glockner J, et al. MR Elastography of Liver Tumors. Proceedings 16th Scientific Meeting, International Society for Magnetic Resonance in Medicine; Toronto. 2008;a.p.2781.
32. Lee V, Miller F, Omary R, et al. Magnetic resonance elastography and biomarkers to assess fibrosis from recurrent hepatitis C in liver transplant recipients. Transplantation. 2011;92:581–6.
33. Binkovitz LA, El-Youssef M, Glaser KJ, et al. Pediatric MR elastography of hepatic fibrosis: principles, technique and early clinical experience. Pediatr Radiol. 2012;42:402–9.
34. Yin M, Talwalkar JA, Glaser KJ, et al. Dynamic postprandial hepatic stiffness augmentation assessed with MR elastography in patients with chronic liver disease. AJR Am J Roentgenol. 2011;197:64–70.
35. Asbach P, Klatt D, Schlosser B, et al. Viscoelasticity-based staging of hepatic fibrosis with multifrequency MR Elastography. Radiology. 2010;257:80–6.
36. Wang J, Malik N, Yin M, et al. Magnetic resonance elastography is accurate in detecting advanced fibrosis in autoimmune hepatitis. World J Gastroenterol. 2017;23(5):859-68.
37. Rustogi R, Horowitz J, Harmath C, et al. Accuracy of MR elastography and anatomic MR imaging features in the diagnosis of severe hepatic fibrosis and cirrhosis. J Magn Reson Imaging. 2012;35:1356–64.
38. Yin M, Glaser K, Talwalkar J, Chen J, Manduca A, Ehman R. Hepatic MR Elastography: Clinical Performance in a Series of 1377 Consecutive Examinations. Radiology. 2016;278:114-24.
39. Arena U, Vizzutti F, Corti G, et al. Acute viral hepatitis increases liver stiffness values measured by transient elastography. Hepatology. 2008;47:380–4.
40. Loomba R, Sanyal A. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol. 2013;10:686–90.
41. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55:2005–23.
42. Chen J, Talwalkar J, Yin M, Glaser K, Sanderson S, Ehman R. Early detection of nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease by using MR elastography. Radiology. 2011;259:749-56.
43. Loomba R, Wolfson T, Ang B. Magnetic resonance elastography predicts advanced fibrosis in patients with nonalcoholic fatty liver disease: a prospective study. Hepatology. 2014;60:1920-8.
44. Kim D, Kim W, Talwalkar J, Kim H, Ehman R. Advanced fibrosis in nonalcoholic fatty liver disease: noninvasive assessment with MR elastography. Radiology. 2013;268: 411-9.
45. Imajo K, Kessoku T, Honda Y, et al. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. Gastroenterology. 2016;150:626–37.

46. Venkatesh S, Yin M, Ehman R. Magnetic Resonance Elastography of Liver: Clinical Applications. J Comput Assist Tomogr. 2013;37:887–96.
47. Patel N, Hooker J, Loomba R, et al. Weight Loss Decreases Magnetic Resonance Elastography Estimated Liver Stiffness in Nonalcoholic Fatty Liver Disease. Clinical Gastroenterology and Hepatology. 2017;15:463–4.
48. Venkatesh S, Yin M, Glockner J, et al. MR elastography of liver tumors: preliminary results. AJR Am J Roentgenol. 2008;190:1534–40.
49. Thompson S, Wang J, Chandan V, et al. MR elastography of hepatocellular carcinoma: Correlation of tumor stiffness with histopathology features—Preliminary findings. Magnetic Resonance Imaging. 2017;37:41-5.

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REVIEW PAPER

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Clinical application of advanced neuroimaging techniques – Magnetic Resonance Spectroscopy

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ABSTRACT

Continuous scientific research and the increasing saturation of the medical market in Poland implements the possibilities of using advanced MR techniques including MRS in everyday practice. This method, which has so far been used primarily for research purposes, can bring measurable benefits to patients not only in terms of clarifying diagnosis and narrowing differential diagnosis, but also monitoring the course of various diseases and their treatment. Here we present the basic principles of performing and interpreting spectroscopic spectra and possible clinical applications and development prospects of MRS. The literature reviewed both Polish and foreign articles both historically and in the past 10 years. The paper presents methodological issues related to the proper performance of magnetic resonance spectroscopy (MRS) and spectral composition and the role of major metabolites, as well as current clinical applications and directions of MRS development.

Keywords. spectroscopy, MR, SVS, CSI

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Introduction

The dynamic development of magnetic resonance over the past 30 years has led to the tumultuous development of diagnostic methods based on medical imaging. In the present era, we are no longer limited to structural imaging, showing anatomy and enabling detection of pathological changes in the central nervous system, mainly for CT and MR, but we also have the possibility (unfortunately not widely used) of imaging processes below the actual spatial MR resolution, based on advanced techniques such as Magnetic Resonance Spectroscopy (MRS), Diffusion Weighted Imaging (DWI), Diffusion Tensor Imaging (DTI), Perfusion Weighted Imaging (PWI), Susceptibility Weighted Imaging (SWI), and Functional Magnetic Resonance Imaging (fMRI). MRS is a non-invasive method of testing both *in vitro* and *in vivo* compounds in normal and pathological tissues, allowing for a semi-quantitative assessment of their metabolic composition and thus allowing for a biochemical evaluation of the various processes occurring in the body such as neoplastic lesions, degenerative processes, ischemic changes and many others.[1]

The basic phenomenon used in MRS is the so-called "chemical shift" and signal splitting associated with the shielding of hydrogen nuclei (^{1}H) and the local chemical environment respectively. The intensity of the signal corresponds to the amount of chemical compound present. The frequency differences are directly proportional to the magnitude of the external magnetic field B_0. The chemical shift of a peak measured in parts per million (ppm) reflects shielding factors in the magnetic environment of the molecule which may shift the position of the peak. The chemical shift in ppm is a dimensionless quantity obtained by dividing frequency in Hertz (Hz) by the field strength which makes it independent of the magnitude of the applied magnetic field. MRS uses signals not only of the ^{1}H nuclei, but also other nuclides such as ^{13}C, ^{15}N, ^{19}F, ^{23}Na and ^{31}P. Compared to ^{1}H MRS (hydrogen spectroscopy), other spectrometers such as those designed to study nucleic acids are characterized by lower sensitivity and require a significant prolongation of study time due to the significantly lower concentrations of these elements in the tissues. ^{1}H MRS is performed in standard MR systems with conventional RF coils, and additional spectrum detection and transmission equipment is required to obtain spectrum from ^{13}C, ^{19}F and ^{31}P. In practice, the most commonly used is ^{1}H MRS due to the key role of hydrogen in living organisms, although obtaining satisfactory spectra is associated with a rather complicated process and requires appropriate equipment, at least 1.5 T MR and appropriate software. As a result of the MRS study, we obtain the resonance spectra of the area of interest in coordinates (amplitude of the signal/shift) composed of so-called bands or peaks that have a location and shape specific to the metabolite, and the field size under each peak determines the amount of metabolite.[2] MRS allows you to study both simple and complex chemical compounds and, consequently, cells in both physiological and pathological states.[3] Practical use of MRS requires the use of a method for recording the spectrum from a selected region, referred to as "voxel" or more precisely as VOI (volume of interest). We have two location methods: SVS (single voxel spectroscopy); CSI (multi-voxel chemical shift imaging) or MVS (multi voxel spectroscopy) - multiple voxels - 2D/3D spectroscopy. The VOI should be adjusted so that it does not go beyond the test area. In SVS, the voxel can cover an area such as a small lesion and are limited if used in areas that undergoes changes in size (<1 cm), near bone, sinuses, fluid reservoirs, or blood since the results of the study are distorted. By using the SVS method, you can change the intensity of a solid magnetic field in a position-dependent manner, allowing you to record the spectrum from the selected voxel. The advantage of SVS is the high homogeneity of the test area, the ease of selectively in suppressing the water signal, the high signal to noise ratio (S/N ratio) and the short test time (4-8 min.). The sequences used in the SVS include: ISIS (Image-Selected *in vivo* Spectroscopy), STEAM (Stimulated Echo Acquisition Mode), PRESS (Point-Resolved Localized Spectroscopy).[4]

Multi-voxel CSI allows for the simultaneous recording of spectra from many adjacent voxels, allowing for spatial mapping of individual metabolites in the examined organ layer.[5] CSI allows for a large-scale assessment of a large area of both tumor and uncertain zones and edema and normal brain (tumor border evaluation), and is also important in biopsy planning. The advantage of CSI is the smaller voxel volume, larger coverage area, and higher spatial resolution. The need for long echoes (TEs) limits the information obtained to three major spectral spikes (N-acetyl aspartate - NAA, choline compounds - Cho, creatine and its derivatives - Cr). Also, adjacent voxel image contamination, and lower signal-to-noise are disadvantages of multi-voxel CSI.

Fundamental to achieving high sensitivity and resolution, the MRS study selects appropriate echoes and repetition times (TE and TR) and voxel size and position. TE spectrum time is recorded at different values (most commonly used are 20 ms, 30 ms, 135 ms, 144 ms and 270 ms). MRS spectra recorded at short TEs contain signals from most metabolites, whereas long TEs are used when lipid and intercellular signal is required, but the MRS spectrum is limited to NAA, Cr and Cho signals (**Figure 1**). Levels of metabolites and their ratios recorded with short and long TEs can vary. Fundamental to the high sensitivity and resolution of the MRS study is the selection of suitable TE and TR times and the size and position of the voxel.

The size of voxel in practice is usually 2×2×2 cm i.e. 8 cm^3. A small voxel volume gives better spectral resolution and field homogeneity, but increases test time (smaller vol-

ume → weaker signal → greater repetition). The greater voxel volume results in greater heterogeneity of the field associated with averaging the spectrum of healthy and pathologically altered tissues, which interferes with the proper proportions of metabolites and may falsify the results.

The strongest signal in the ^{1}H MRS spectrum is derived from water protons, which determines the need to suppress it so that it can record signals from other compounds. A similar problem applies to areas where fatty tissue predominates. The purpose is the CHESS (Chemical Shift-Selected Sequence) sequence. It is necessary to use a variety of correction techniques for FID (Free Induction Decay), undesirable signals, noise and deformation associated with acquisition to obtain the correct spectral spectrum. The stages of processing the resonant time domain signal (prior to Fourier transformation) are: offset correction (removal of the constant electrical current generated by the electronic circuit while the FID signal has disappeared to zero), zero filling (the signal is extrapolated by the addition of zero points, which improves resolution of the spectrum), apodization (signal multiplication by appropriate signal-to-noise correction functions). Phase conversion of the resonance signal in the frequency domain (after Fourier transform) is comprised of: phase correction (to obtain pure absorption spectrum), baseline correction (elimination of device artifacts and spectrum of large non-mobile molecules that underline baseline outlines). Calculation of the surface area under the resonance bands of metabolites (by calculating the value of the surface area under the resonance band curve) is the most direct and fast quantitative spectrum analysis method, however, this requires a lat baseline and well separated bands. Determining the molar concentrations of a substance on the basis of integrals of the corresponding resonant bands requires calibration, which encounters significant difficulties in *in vivo* studies. Because of these problems, the quantitative MRS results are most often used as the quotient of the intensity of the resonant signals of individual metabolites.[6] Calculation of absolute amounts of metabolites is possible in dedicated programs such as the LC Model.[7]

Proton spectra (^{1}H MRS) consists of peaks representing the most commonly occurring chemical compounds at a predetermined position – ppm: [1]

1. NAA - N-Acetyl Aspartate (2.02 ppm)
2. Cr + PCr - Creatine and Phosphocreatine (3.03 ppm, 3.93 ppm)
3. Choline compounds (3.20 ppm, 3.22-3.23 ppm)
4. I / m - Inositol, Myo-inositol (3.56 ppm, 4.06 ppm)
5. Glx - Glutamates, GABA, Glucose (2.0-2.45ppm, 3.60-3.80 ppm)

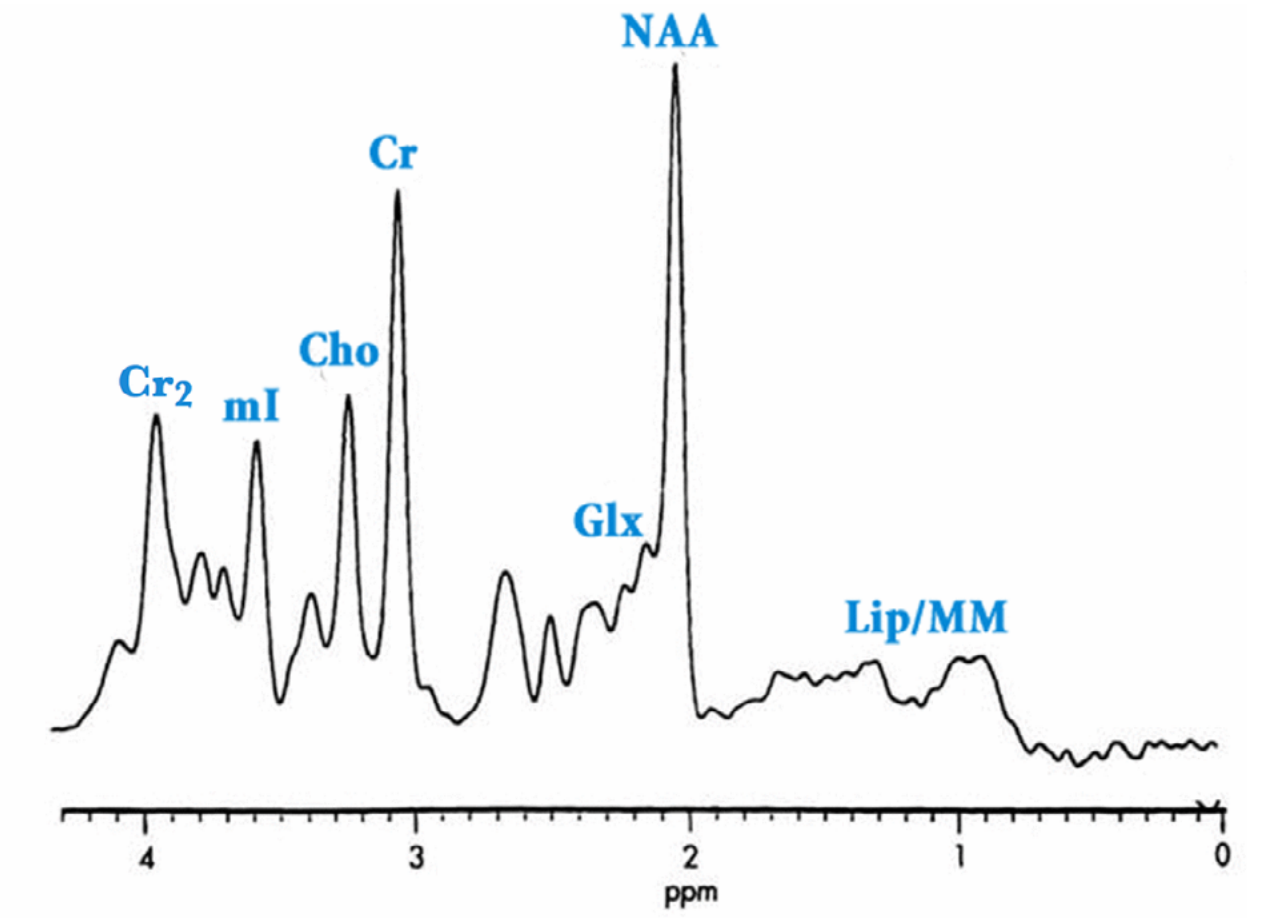

Figure 1. MRS spectrum sample

6. Gly-glycine (3.56 ppm)
7. Ala - alanine (1.48 ppm)
8. Tau - taurine (3.21 ppm, 3.28 ppm, 3.35 ppm)
9. Lac - lactic acid (1.30 ppm)
10. Lip-lipids (0.90 ppm, 1.30 ppm, 2.00 ppm)

NAA (N-acetyl aspartate) is a marker of nerve cells (axons). N-acetyl aspartate participates in the process of neuronal protein synthesis, metabolism of neurotransmitters, and participation in the synthesis of fatty compounds in the myelin sheath. It is present in a healthy cerebral cortex and in glial tumors. Its level is lower in most diseases except Canavan disease. It does not occur in non-electrocardiomous tumors such as metastases, meninges, neuroblastomas, striatum and the central part of glial tumors and malignant lymphomas.

Cr + PCr (creatine and phosphocreatine) is a marker of cellular energy processes and an indicator of the proper functioning and use and storage of energy in a cell. It is a reservoir for high energy phosphate groups, maintains a normal ATP/ADP ratio and is used as an additional energy carrier between mitochondria and other cellular structures. The concentration of Cr is stable, hence it is used to calculate ratios with other metabolites for objective spectral estimation. The total Cr concentration decreases in all brain tumors compared to normal tissue, although it is relatively higher in neuroectodermal tumors.

Cho (choline) is a marker of the metabolism of cell membranes and myelin. The increase in its concentration may result from the breakdown of cell membranes (inflammation, demyelination, infarction, tumor necrosis) or their increased synthesis (tumor cell proliferation). It is a precursor of acetylcholine and phosphatidylcholine. The dominant spike in the brain of a healthy newborn, decreases in concentration with age. Pathological changes cause large fluctuations in the choline peak. The Cho / NAA ratio is particularly important when the level of Cr decreases as a result of pathological processes.[8]

I/mI (inositol, phosphatidylinositol, polyphosphoric inositol and monophosphoric inositol, myo-inositol) is a marker of astrocytic asthma. The suggested role is regulation of osmosis and maintenance of normal cell volume. The mI band is well visible in the brain of a healthy newborn. Increased concentration means astrocytoma astrocytosis (post-glial or post-inflammatory glare, benign astrocytomas or neuroblastoma). The highest concentration of I is in neuroblastomas, hence the possibility of neuroblastoma differentiation in ^{1}H MRS.

Glx (glutamine, glutamate, glutamic acid, γ-aminobutyric acid, glucose) are considered markers of focal ischemia and hypoxia (nerve cell death markers). Glutamine is a major neurotransmitter that stimulates the brain and plays a role in mitochondrial metabolism. Glutamate concentrations increase during periprocedural stroke, appear rapidly in cells devoid of oxygenation, in epileptic foci. Changes in glutamic acid and glutamate levels are also observed in neurodegenerative diseases such as Alzheimer's disease.

Gly (glycine) is considered to be a marker of glial tumors that is observed in neuroanatomical tumors such as glioblastoma, medulloblastoma, ependymoma. It is important in the differential diagnosis of glial tumors with metastatic tumors.

Ala (alanine). Alanine levels increase in meninges, gliomas and pituitary adenomas.

Tau (taurine) is an amino acid, neurotransmitter and has cytoprotective properties. It occurs only in PNET (primitive neuroectodermal tumor), lacking in other tumors and healthy brain tissue.

Lac (lactic acid) is a marker of anaerobic processes and does not occur under physiological conditions. It appears in the foci of cell necrosis (tumors, ischemia) and in infectious processes (abscesses), as well as in diffuse lesions of the axons and mitochondrial metabolic disorders.

Lip (lipids) also do not occur under physiological conditions. They appear in pathological changes associated with acute destruction of myelin and in cell necrosis sites.

Clinical applications of proton spectroscopy - ^{1}H MRS:
1. In brain cancer[9-14]
- assessment of histological character of gliomas,
- assessment of the extent and borders of the brain tumor,
- determining the degree of malignancy of glial tumors,
- assessment of malignant transformation of benign glial tumors,
- differentiation of primary brain tumors and metastases,
- differentiation of tumors,
- differentiation of low grade gliomas with ischemic lesions,
- planning stereotactic biopsy of brain tumors,
- evaluation of the completeness of surgery,
- assessment of tumor recurrence,
- differentiation of recurrence with radiation necrosis,
- evaluation of radio- and tumor chemistry and monitoring of oncological therapy,

2. In degenerative brain diseases
3. In multiple sclerosis
4. In infectious diseases of the brain (abscess, ADEM, HIV, toxoplasmosis)
5. In metabolic diseases

6. In brain trauma
7. In ischemic brain diseases
8. In epilepsy
9. In psychiatry

Clinical applications of ^{1}H MRS in the assessment of histological characterization of gliomas[10,12]

Astrocytoma - decreased concentrations of N-acetyl aspartate (NAA) and NAA/Cr ratio, high choline content (Cho) and increase in Cho/NAA and Cho/Cr ratio, lipids (Lip) and lactate (Lac) may appear, although not very high. Elevated myo-inositol concentration (ml) - peak mI is inversely proportional to tumor grade.

Oligodendroglioma shows decreased concentrations of N-acetyl aspartate (NAA) and NAA/Cr ratio, high choline content (Cho) and an increased Cho/NAA and Cho/Cr ratio. Lipid and lactate content (Lac) may be selected but not high. It can be differentiated with other gliomas quantitatively on the basis of elevated glutamine and glutamate metabolites (Glx) using short TE time.

Ependymoma shows decrease of N-acetyl aspartate (NAA) and NAA/Cr ratio, choline growth (Cho) and Ch/NAA, Cho/Cr ratios, myo-inositol (mI), glycine (Gly) and taurine (Tau).

Glioblastoma Multiforme (GBM) shows significant decreased N-acetyl aspartate (NAA) and NAA/Cr ratio, very high choline levels (Cho) and elevated Cho/Cr and Cho/NAA ratios. Dominant high peaks of free lipids and lactate (Lip+Lac), in tumors with extensive necrosis foci. High concentrations of choline (Cho) and Cho/NAA are indications of the degree of malignancy of the tumor. Glioblast multiforme is a disease of the entire brain, and changes in MRS may also occur outside the maximum contrast enhancement areas of the tumor. Assessment of the extent and boundaries of GBM is essential for operative and radiotherapy. It has been demonstrated that structural MR examination is in some types of tumors (GBM) insufficient in assessing their limits, hence the need to test in the IHMRS environment around the tumor strengthening region for evaluation of choline (Cho) and N-acetyl aspartate (NAA) (MRI fusion map with CM and ^{1}H MRS-CSI).

Clinical application of ^{1}H MRS in determining the degree of malignancy of glial tumors[10,11]

^{1}H MRS allows non-invasive determination of the type and degree of malignancy of the glial tumor on the basis of comparison of metabolite concentrations in the tumor and the white matter of the opposite brain tissue. In general, N-acetyl aspartate (NAA) and creatine (Cr) concentrations, cholecystolone (Cho) and Cho/NAA and Cho/Cr ratios, myo-inositol (mI) and glycine (Gly) and lactate (Lac) and Lipids (Lip), typically occur in the areas of necrosis. The intensity of these changes correlates with the degree of tumor malignancy, particularly choline growth (cell proliferation and membrane degradation) and lipids (disintegration), and the decrease in N-acetyl aspartate (nerve cell loss). The NAA/Cho ratio of less than 1.6 is a high grade malignant glioma. In the G3-G4 glioblastoma necropsy area, Cr also decreased and Cho/Cr increased (G1-2=2.05±0.18, G3=2.58±0.11, G4=5.1±0.89). Lipid (Lip) growth is observed in G3-G4 gliomas. The tendency to increase Cho/Cr and Ch/NAA ratios in tumor surrounding tissue was also noted in case of its malignant nature. MRS spectroscopy makes it possible to differentiate between G2 and G4, but it is more difficult to differentiate G2 from G3 and G3 from G4.

Clinical application of ^{1}H MRS in evaluation of malignant malignant tumors[10-12]

The decrease in N-acetyl aspartate (NAA) and high choline growth (Cho) and the appearance of lactate (lac) and lipids (Lip) as well as the decrease in myo-inositol (mI) levels suggest that they are malignant.

Clinical application of ^{1}H MRS in differentiating glial tumors and metastases

Glucose levels (Cho) and Cho/Cr and Cho/NAA levels increase due to environmental stimulation, and N-acetyl aspartate (NAA) level decreases with G3-G4. In the context of metastasis due to angioedema, the Cho/NAA ratio is preserved. Metastases are characterized by the growth of free (moving) lipids (Lip) and the absence of other brain metabolites. Increasing lactate concentration (Lac) is not a pathogenic phenomenon in this case. Diagnostic problems may arise with differentiation of GBM-derived metastases in which necrosis and disintegration are prevalent, especially if SVS voxel in the GBM carries necrotic tissue, and in the metastases will be fragments of healthy tissues.

Clinical application of ^{1}H MRS in differentiation of other tumors[13]

Tumors have low or zero N-acetyl aspartate (NAA) because the tumor is of mesenchymal origin. In sequences with short TE 20-30 ms visible glutamate growth (Glx). In sequences with short and long echoes (TE 20-30 ms and 135-136 ms), high choline concentration (Cho) and alanine peaks (Ala) and Lactates (Lac) are observed. Syphilis - high levels of the compound inositol (I/mI), which makes it possible to differentiate with mucin.

PNET (Primitive NeuroEctodermal Tumor) - high concentration of choline (Cho), lipid (Lip) and lactate (Lac), visible taurine (Tau), which is pathognomonic for PNET.

Central Neurocytoma - high concentration of choline (Cho) and glycine (Gly).

Gliomatosis cerebri - increases in choline (Cho) and myosinolytic (mI) and high creatine (Cr) and conse-

quently decreases the Cho/Cr ratio, as opposed to Low Grade Glioma (LGG) (Cho) and increased Cho/Cr ratio.

Based on the ^{1}H MRS-CSI maps, especially in the case of non-contrast-enhancing stereotactic biopsies, we estimate where the highest Cho/Cr and Cho/NAA levels exist. We evaluate the completeness of surgery based on ^{1}H MRS-CSI maps, evaluate the tissue for residues of high choline concentration (Cho) and Cho/NAA. The assessment of tumor recurrence and differentiation with radiation necrosis and tumor radioscopy and oncological monitoring are transposed on the basis of the following criteria: resumption of Cho/Cr and Cho/NAA ratios; Necrosis - markedly low values of N-acetyl aspartate (NAA), choline (Cho) and creatine (Cr) compared to healthy tissue. For recurrence, choline concentration (Cho) and N-acetyl aspartate (NAA) decrease, but in early changes after radiotherapy also increases choline (Cho) concentration. The fall of lactate (Lac) and "moving" lipids (lipids) during radiotherapy is a good indicator of brain tumor radioactivity. The presence of blood, air and fluid in the post-operative box results in distortion of ^{1}H MRS results.

Clinical applications of ^{1}H MRS in cerebral degenerative diseases and differentiation of dementia syndromes[15,16]
AD (Alzheimer Disease) - increase in myo-inositol (mI) and decrease of N-acetyl aspartate (NAA), a significant ratio of these values in the hippocampus and temporo-parietal cortex.
FTD (Fronto-Temporal Dementia) - decrease of N-acetylasparaginate (NAA) and Glutamate (Glx) and increase of myosinolytic (mI) in the gray matter of the temporal-parietal and central region.
PD (Parkinson Disease) - decrease in NAA/Cr ratio in the temporo-parietal cortex, black essence, basal nucleus, striatum and occipital lobe.
HD (Huntington Disease) - decrease in N-acetyl aspartate (NAA) and increased lactate (Lac) levels in the striatal, occipital and frontal cortex, NAA/Cho ratio decrease in basal and brain cortex.

Clinical applications of ^{1}H MRS in multiple sclerosis[17,18]
In the acute phase, demyelinating MSs show a slight decrease in N-acetyl aspartate (NAA), which over time returns to normal, and contrast-enhanced MSs also exhibit choline (Cho) and lipid (Lip) growth. In the phase of chronic MS focus hyposensitivity in T_1 dependent images show a decrease in N-acetyl aspartate (NAA) and increase in myo-inositol (mI). It has now been demonstrated that white matter with normal MR signal may exhibit elevated choline (lipid) and lipid (Lip) and N-acetyl aspartate (NAA) levels in MS, which correlates better with functional damage than MS in T_2 dependent images. ^{1}H MRS seems to be helpful in assessing axonal lesions and demyelination, and together with DTIs, it can better monitor the evolution of MS lesions.

Clinical applications of ^{1}H MRS in cerebral infectious diseases[19]
Chunks - Very low concentration or absence of choline (Cho), creatine (Cr) and N-acetyl aspartate (NAA), lipid and lactate levels (Lip, Lac), and alanine peak (Ala).
ADEM - normal choline levels (Cho) and lipid level (Lip) and N-acetyl aspartate (NAA) drop, which returns to normal after treatment.
HIV - decline in NAA/Cr ratio and increase in Cho/Cr and mI/Cr ratios (ability to monitor HIV treatment).
Toxoplasmosis - increase in lipid and lactate concentrations (Lip, Lac) and no other metabolites.

Clinical applications of ^{1}H MRS in metabolic diseases[14]
Mitochondrial diseases (Leigh, Kearns-Sayre Syndrome, Mitochondrial Encephalopathy, lactic acidosis, MELAS, MERRF) associated with cellular respiratory disorders lead to anaerobic glycolysis and lactate accumulation in the brain and in ^{1}H MRS we can prove the presence of a lactate (Lac) normal brain.
Peroxisomal diseases (Adrenoleukodystrophy, Zellweger's syndrome) lead to nerve cell damage, which is manifested in ^{1}H MRS by N-acetyl aspartate (NAA) and glutamate (Glx) and choline (Cho) growth. ^{1}H MRS plays a special role in discriminating patients for bone marrow transplantation in the early stages of disease before the onset of neurological symptoms, and can also be used to screen for early demyelination.
Phenylketonuria - phenylalanine peaks in ^{1}H MRS with short TE time at 7.37 ppm can be demonstrated; other metabolites are normally normal.
Disease "Maple Syrup" - can be shown to increase the leucine peaks, isoleucine and valine at 0.9 ppm.
Canavan disease - damage to the enzyme aspartoacylase leads to an increase in the concentration of N-acetyl aspartate (NAA).
Alexandra's disease - leads to a decrease in the concentration of N-acetyl aspartate (NAA) and an increase in lactate concentration (Lac).

Clinical applications of ^{1}H MRS in brain injury [14]
As a result of spilled axonal injury and depression of nerve cell metabolism, N-acetyl aspartate (NAA) declines. In fact, the white brain after N-acetyl aspartate (NAA) falls back to normal NAA levels. Contrary to this, in fact, the gray matter of the brain decreases the concentration of NAA is progressing. Likewise, the concentration of choline (Cho) increases after trauma and its level is maintained in elevated gray matter affected. In adult patients with normal-looking white matter, the NAA/Cr ratio correlates with the severity of injury and is a predictor of neurological damage.

Clinical applications of ^{1}H MRS in ischemic diseases[14]
At the acute and subacute stage of the ischemia, the level of N-acetyl aspartate (NAA) gradually decreases, while the concentration of choline, lactate and glutamate (Cho, Lac and Glx) is increasing. Lacs (lac) grow shortly after the infarction within a few hours and may persist for as long as the chronic phase. In the chronic phase, a very large decrease in N-acetyl aspartate (NAA) and myosinosis (mI) is evident. Phosphoric spectroscopy (^{31}P MRS) in ischemia decreases the ratio of phosphocreatine to inorganic phosphorus (PCr/Pi).

Clinical applications of ^{1}H MRS in epilepsy[20]
N-acetyl aspartate (NAA) drop and drop in NAA/Cr ratio and Glx concentration (glutamine/glutamate complex). Lactation increases (Lac) is observed in the epilepsy camp and can last several hours. During laparoscopic, lactate elevation (Lac) is also used to evaluate lateralization of epileptic activity.

Clinical applications of ^{1}H MRS in psychiatry[21-24]
Schizophrenia - decrease in NAA/Cr ratio in the prefrontal cortex, hills and hippocampus, glutamate elevation (Glx) in the initial stage of the disease and fall in the chronic stage.
Depression - increase of Cho/Cr ratio in the real white frontal lobe, subcortical nucleus, myo-inositol decrease (mI) in the prefrontal cortex, Glx level (glutamate) decreased in the prefrontal cortex and increased in the occipital lobes.
Cyclophrenia - increase of myoinositol concentration (mI) in the frontal and frontal cortex of the rump, decrease of N-acetyl aspartate (NAA) in the prefrontal cortex and hippocampus, glutamate increase (Glx) in the prefrontal cortex during mania and choline increase in course of depression.

Due to device limitations and economic aspects, the use of Phosphor Spectroscopy ^{31}P MRS is currently largely limited to research, although in the near future this method will probably be more widely used in the evaluation of the musculoskeletal, cardiac and liver systems.[25,26] Spectra obtained from brain tissue consists of 7 peaks:
γ-ATP - gamma adenosine triphosphate
Pi - Inorganic phosphate
α-ATP - alpha adenosine triphosphate
PDE - phosphodiester
β-ATP - beta adenosine triphosphate
PME - phosphomonoesters
PCr - phosphocreatine

Analysis of ATP, PCr and Pi peaks allows us to determine the concentration of high energy intracellular compounds and the intensification of energy transformation processes. PCr/Pi is an indicator of the oxygen potential of the cell. The chemical shift Pi relative to PCr determines the intracellular pH. The mutual position of phosphates γ and α in ATP allows the calculation of intracellular magnesium concentration.

Current trends in spectroscopy are: finding new clinically important markers (eg GABA, taurine, glucose), applying dynamic and 3D spectroscopy, developing a metabolite mapping program (CSI), and assessing responses to pharmacological treatment such as lithium (mI) antiepileptic (GABA level).

Although proton MR spectroscopy in many cases is not able to answer the clinical questions in many cases, and in most brain pathologies, NAA (except Canava disease) and choline growth are likely to be affected. MR studies widen the possibilities of predicting histological origin and degree of malignancy of tumor processes and intensification or differentiation of other diseases of the nervous system. MR spectroscopy, based on other elements, is still an active research area with hopes to expand the possibilities of disease differentiation in clinical applications in the near future.

References

1. Bertoldo D, Watcharakorn A, Castillo M. Brain proton magnetic resonance spectroscopy. Introduction and overview. Neuroimag Clin N Am. 2013;23:359-80.
2. Jansen JFA, Backes WH, Nicolay K, Kooi ME. ^{1}H MR spectroscopy of the brain : absolute quantification of metabolites. Radiology. 2006;240:318-32.
3. Posse S, Otazo R, Dager SR, Alger J. MR spectroscopis imaging : principles and recent advances. J Magn Reson Imaging. 2013;37:1301-25.
4. Moonen CT, von Kienlinvan M, van Zijl PC, et al. Comparison of single-shot localization methods (STEAM and PRESS) for in vivo proton NMR spectroscopy. NMR Biomed. 1989;2:201-7.
5. Maudsley AA, Darkazanli A, Alger JR, et al. Comprehensive processing, display and analysis for in vivo MR spectroscopic imaging. NMR Biomed. 2006;19:492-503.
6. Klose U. Measurement sequences for single proton MR scpectroscopy. Eur J Radiol. 2008;67:194-201.
7. LC Model website. http://lcmodel.ca. Assessed November 18 2016, Updated January 9, 2017.
8. Sobiecka B, Urbanik A. The role of choline (Cho) in the diagnostics and differentiation of brain tumours with H MRS technique. Pol J Radiol. 2009;74:7.
9. Oz G, Alger R, Barker PB, et al. Clinical proton MR spectroscopy in central nervous system disorders. Radiology. 2014;270:658-79.
10. Herminghaus S, Dierks T, Pilatus U, et al. Determination of histopathological tumor grade in neuroepithelial brain tumors by using spectral pattern analysis of in vivo spectroscopic data. Journal of Neurosurgery. 2003;1:74-81.

11. Julià-Sapé M, Griffiths JR, Tate RA. Classification of brain tumours from MR spectra: the INTERPRET collaboration and its outcomes. NMR in Biomedicine. 2015;28:12,1772-87.
12. Jaskólski DJ, Fortuniak J, Majos A, et al. Magnetic resonance spectroscopy in intracranial tumours of glial origin. Neurol Neurochir Pol. 2013;47:438-49.
13. Jaskólski DJ, Fortuniak J, Stefańczyk L, et al. Differential diagnosis of intracranial meningiomas based on magnetic resonance spectroscopy. Neurol Neurochir Pol. 2013;47: 247-55.
14. Semantic Scholar - An academic search engine for scientific articles. https://pdfs.semanticscholar.org. Updated January 12, 2017.
15. Magierski R, Sobów T, Kłoszewska I. Spektroskopia MR w chorobach zwyrodnieniowych ośrodkowego układu nerwowego. Post Psych Neurol. 2005;14:155-163.
16. Urbanik A. Diagnostyka otępienia przy pomocy protonowej spektroskopii MR. Przegl Lek. 2010;67:237.
17. Szafirska M, Urbanik A, Róg T. Zmiany metaboliczne w obrębie blaszek u chorych na stwardnienie rozsiane - ocena w technice protonowej spektroskopii MR (1HMRS). Prz Lek. 2013;70:328-34.
18. Szafirska M, Urbanik A. Rola rezonansu magnetycznego w diagnostyce stwardnienia rozsianego. Ogólnopol Prz Med. 2012;3:48-53.
19. Podsiadło L, Urbanik A, Garlicki A, et al. 1H MRS application in the evaluation of CNS in the patents with AIDS. Pol J Radiol. 2003;68:5-9.
20. Michalska J, Kociemba W, Steinborn B, et al. Rola spektroskopii rezonansu magnetycznego w padaczce. Neur Dziecięca. 2008;34:61-4.
21. Silverstone PH, McGrath BM, Kim H. Bipolar disorder and myo-inositol: a review of the magnetic resonance spectroscopy findings. Bipolar Disord. 2005;7:1-10.
22. Renshow PF, Lafer B, Babb SM, et al. Basal ganglia choline levels in depression and response due to fluoxetine treatment: in vivo proton magnetic resonance spectroscopy study. Biol Psychiatry 1997;41:837-43.
23. Rachel W, Siwek M, Dudek D, et al. Protonowa spektroskopia rezonansu magnetycznego w zaburzeniach afektywnych. Neuropsychiatria i Neuropsychologia. 2008;3:29.
24. Herman-Sucharska I, Werewka-Maczuga A, Urbanik A, et al. Ocena przydatności protonowej spektroskopii MR w diagnostyce i monitorowaniu leczenia choroby afektywnej jednobiegunowej Przegl Lek. 2010;67:4.
25. Karcz P, Urbanik A. Spektroskopia protonowa mięśni podudzia przed i po wysiłku fizycznym. Przegl Lek. 2013;70:286-92.
26. Zaleska T, Walecki J, Michalak M, Michalak E. Spektroskopia magnetycznego rezonansu w kardiologii. Medical Science Review - Diagnostyka Obrazowa. 2002;1:20-4.

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REVIEW PAPER

Dorota Bądziul [1(AFG)], Agnieszka Banaś-Ząbczyk [1(AF)], Jacek Tabarkiewicz [2(AF)]

An overview of the preclinical and clinical studies of the effects of tumor treating fields on malignant glioma cells

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ABSTRACT

Anaplastic astrocytoma (AA, WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV) are malignant tumors of the brain. The average survival time of patients with GMB is approximately one year and two years in the case of anaplastic astrocytoma with standard therapy based on surgical tumor resection followed by chemotherapy or radiotherapy. High invasiveness of gliomas, the ability of rapid division and so-called diffusive infiltration of tumor cells into normal brain tissue, which prevents complete surgical removal, are hallmarks of theses tumors. Therefore, new specific therapies for eliminating cancer cells are needed to treat this tumors. Recently, it has been demonstrated that alternating electric field, also known as tumor treating fields (TTFields) has a unique mechanism of destroying glioma cells. TTFields applies electromagnetic energy frequency-dependent and intensity-dependent and disrupts cancer cell replication as they undergo mitosis. Futhermore, TTFields turn out to act comparably to conventional chemotherapeutics, lacking numerous side adverse associated with chemotherapy. The authors provide an up-to-date review of the mechanism of action as well as preclinical and clinical data on TTFields.

Keywords. anaplastic astrocytoma, glioblastoma multiforme, brain tumor, tumor treating fields, tumor therapy

Introduction

The term malignant gliomas comprises WHO grade III tumors (e.g. anaplastic forms of astrocytomas, oligodendroglioma, and oligoastrocytoma) and WHO grade IV tumors, such as glioblastoma multiforme (GBM). Glioblastoma multiforme is the most frequent and the most devastating primary malignant glioma. Most patients diagnosed with a GBM survive less than a year despite intensive treatment, which may include maximal safe surgical resection, radiation and chemotherapy. The prognosis for patients with anaplastic astrocytoma (grade III) is somewhat better. Due to the slower growth of cancer, the average survival time is about 2 years. The hallmarks of gliomas include diffusive invasion into normal brain tissues, high proliferation rate, aggressive growth pattern and microvascular proliferation.[1] From the molecular stand-

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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point, several signaling pathways responsible for regulation, proliferation, differentiation and survival have been found to be differentially activated or silenced, hence gliomas typically do not respond to currently available therapies and what is worse is most of the therapeutic options have been exhausted.[2-4] Therefore, development of new methods for gliomas treatment are particulary important. Recently much attention in this regard is paid to alternating electric fields referred to here as Tumor Treating Fields (TTFields), currently widely discussed in terms of cell biology effects, physical properties and clinical trial data. Compared with historic cancer treatment modalities, TTFields have an innovative mechanism of action, and more importantly do not have sufficient energy to induce mutagenic damage to DNA and cannot cause the cellular damage usually associated with cancer initiation.[5] A series of publications present experimental studies conducted for potential genotoxicity of electric and magnetic fields which have shown negative results provided strong support for this view.[6-7] The biological effects of TTFields were first observed during in vitro experiments by analyzing the values of the electric field that affect proliferation and viability of cancer cells in culture. These experiments revealed a tight range of cytotoxic effect, inducing prolonged or completely arrested mitotic phase of treated cells leading to cell death, generated by TTFields at a frequency range between 150 and 200 kHz, and was not observed at frequencies <50 kHz and >500 kHz. Furthermore, these analysis allowed also to observed that the effect of TTFields is also dose-dependent and the inhibitory effect of TTFields starts at 1 V/cm and increases with increasing intensity of the field. According to the ability of electric field to kill cancer cells mentioned above, a pioneering technology has been developed, described and referred as Tumor Treating Fields.[8-10]

Molecular Targets of TTFields

In order to analyze a mechanism of action of TTFields, a systematic review of the literature data was performed. Exposure of multiple cancer cell lines, e.g. glioma, lung, prostate, breast, to TTFields reveals exertion of a strong growth inhibitory effect by inducing cell cycle arrest and in consequence, apoptosis, while no effect was induced on non-dividing cells.[8-13] Disruption of cells by TTFields during mitosis suggest that they exert forces or movement on definable molecular targets, the functions of which are critical to a mitotic process or processes.[14] Cells treated with TTFields exhibited a variety of abnormalities indicative of mitotic catastrophe and aberrant mitotic exit, including cells in polyploidy prophase, rosettes, multi-spindled metaphase, single-spindled metaphase, and asymmetric anaphase.[8] Indeed, cells exhibit violent membrane blebbing as they enter anaphase and attempt to divide which results in aberrant mitotic exit and subsequent cell death *in vitro*.[15] The inhibitory effect of TTFields of proliferation inhibition is largely manifested in malfunction in the mitotic spindle apparatus. That is why molecular targets of TTFields includes proteins characterized by high dipole moments such mitotic septin complex and the α/β-tubulin monomeric subunit of microtubules.[8,14-16] The dipole moments of such proteins will align within an electric field to orient towards the oppositely charged pole of the fields. Therefore, the re-polarization of the alternating field will induce a re-alignment of the protein dipoles within the field. Thus, such proteins would be expected to experience rotational forces within TTFields. α/β-Tubulin form the building blocks for microtubules. The functional subunit of microtubules is a heterodimer consisting of α- and β-tubulin, which possesses a high predicted dipole moment of 1660 Debyes (D).[17-19] Therefore, it is possible that TTFields interfere with a critical mitotic function performed by microtubules, including the formation of the metaphase and anaphase spindles and their respective mechanical functions, or the astral microtubules that help regulate the cytokinetic cleavage furrow (CCF).[8,9,15,20,21] Septins, in particularly septin 2, 6 and 7, characterized by an extremely large dipole moment of 2711 Debyes, oligomerize into a heterotrimer and is active in mitosis.[8,19] The main function of this complex is to regulate contractile function within the cytokinetic furrow, and it is likely to provide tensile strength needed within the submembranosus cortical cytoskeleton to restrain the hydrostatic pressure within the cytoplasm during cell division. Once it is recruited, it then oligomerizes and organizes contractile elements within the cytokinetic furrow above the equatorial cleavage plane by binding to F-actin filaments and spatially regulates myosin activation. The perturbation of the septin complex is particularly enticing because of its known roles in the regulation of CCF function and actin bundle cross-linking and organization of structures such as the cellular submembranous actin cytoskeleton that is required for its rigidity.[22,23] Short hairpin RNA-driven depletion of septin 7 of the heterotrimer results in mitotic bleebing similar to that seen when cells attempt to divide in the presence of TTFields.[8,23] Therefore, perturbation of either α/β-Tubulin or septins may perturb microtubule function.[15,24] These observations strongly suggest a mechanism of action were TTFields perturb mitosis by interfering with normal septin localization and function during mitosis, leading to membrane blebbing and aberrant mitotic exit.

Implications of TTFields in therapy

A series of publications provided evidence supporting preclinical studies pointing at the applicability range of TTFields in a various of in vitro and in vivo cancer models, either alone or in combination with standard chemotherapy.[9,25] Animal models of various tumors, including i.a. glioblastoma, non–small cell lung cancer, pancreatic cancer, and

malignant melanoma confirmed the inhibition of tumor growth and moreover metastatic seeding when TTFields were delivered externally at the appropriate frequencies.[16] TTFields were administered to the animal organisms by using a noninvasive single electrically insulated transducer array located on the head or torso surrounding the region of the tumor. As an example, an experimental model of rats with intracranially inoculated GBM cells treated with TTFields at a frequency of 200 kHz over 6 days showed smaller tumors in comparison to untreated animals. The inhibitory effect was significantly increased when at least two or three directional fields were delivered.[9,10,25] Importantly, synergistic antitumor activity was discovered when TTFields were applied in combination with commonly used chemotherapeutic agents such paclitaxel, doxorubicin, cyclophosphamide, or dacarbazine; the sensitivity to chemotherapy was increased one-fold to three-fold by adjuvant TTFields. Hence, TTFields may acts as an animitotic agent and a chemotherapy sensitizing agent.[19,26]

In October 2015, the FDA approved TTFields for use in newly diagnosed GBM patients. To date, two crucial randomized clinical trials for the safety and efficiacy of TTFields therapy have been reported. In patients with recurrent glioblastoma, currently indicated by FDA (U.S. Food & Drug Administration) as the only one for the TTFields therapy, the trial has not demonstrated improved outcome compared with best physicians choice chemotherapy (BPC). However, when TTFields were delivered as a part of the initial treatment in newly diagnosed patients a consistent prolongation of progression-free survival (PFS) and overall survival (OS) has been noted. From the other point of view, TTFields applied in early stage of disease allows for prolonged exposure, and moreover synergy with Temozolomide (TMZ), a standard chemotherapeutic agent commonly used in glioblastoma therapy, observed in vitro may further enhance its efficacy. The average treatment time of patients with recurrent disease was only 2.3 months compared with 9 months in the case of patients with newly diagnosed GBM. Still, TTFields alone in recurrent disease have shown objective responses in 14% of patients, consistent or even numerically higher than that observed in other trials using chemotherapeutic agent Lomustine[27,28] or Temozolomide.[29] It is worth noting that the best results with this novel treatment modality have been achieved when TTFields were administered in the early stage of disease in combination with standard maintenance TMZ therapy[30], similar to that shown 10 years ago when TMZ was combined with standard radiotherapy. The very important issue is to ensure an adequate treatment effect; the values of TTFields intensity and frequency must be adapted to the tumor type and cell properties. The optimal frequency to maximize the antitumor effect is inversely correlated with cell size and when the incident angle of the electrical field is perpendicular to the mitotic plate. As the cell division may occur at any time, prolonged exposure to the electrical fields is required for maximal effect. For the precise delivery of TTFields, a special portable and battery-powered device has been constructed. The electric field is applied to the brain through 4 transducer arrays with 9 insulated electrodes each and continuous temperature sensing fixed to the patient's shaved scalp.[8,26]

Summary

The ability of TTFields to block the mitotic cell cycle results in cell cycle arrest or delays in cell division and interfere with organelle assembly, particularly the spindle apparatus. The consequences are inadequate cell division and unequal chromosome distribution, and ultimately cell death. The possibility of using this action has become promising. TTFields therapy is currently being tested in gliobalstoma patients and provides more evidence supporting the use of TTFs as an efficacious, antimitotic treatment with minimal toxicity in patients with newly diagnosed and recurrent glioblastoma. Nevertheless, additional studies are needed to further optimize patient selection, determine cost-effectiveness, and assess the full impact on quality of life.[30,31] Moreover, there is a need to integrate this novel TTFields treatment approach with the current standard of care. At this moment, TTFields is the one of the most promising therapeutic methods because of its locoregional action, which allow extension to other types of tumors and metastatic diseases such as brain metastase, ovarian carcinoma, mesothelioma or pancreatic tumors, and trials are currently ongoing.[26] If those trials confirm the positive effects observed in GBM patients, a truly new cancer treatment modality will be born and will find multiple useful indications alone or in combination with other established standard of treatment or new therapy methods.

References

1. Merz F, Gaunitz F, Dehghani F, et al. Durante M, Bechmann I. Organotypic slice cultures of human glioblastoma reveal different susceptibilities to treatments. Neuro Oncol. 2013;15:670-81.
2. Hasller MR, Sax C, Flechl B, et al. Thialomide as palliative treatment in patients with advanced secondary glioblastoma. Oncology. 2015;88:173-9.
3. Marlon GS, Santoshi K. Efficiacy and safety of trating glioblastoma with tumor-treating fields therapy. Clin J Oncol Nurs. 2016;20:9-13.
4. Omuro AM, Faivre S, Raymond E. Lessons learned in the development of targeted therapy for malignant gliomas. Mol Cancer Ther. 2007;6:1909-19.
5. Hester GL. Electric and magnetic fields: managing and uncertain risk. Environment. 1992;34:7-31.
6. McCann J, Dietrich F, Rafferty C, Martin AO. A critical review of the genotoxic potential of electric and magnetic fields. Mutat Res. 1993;297:61-95.

7. McCann, Dietrich F, Rafferty C. The genotoxic potential of electric and magnetic fields: an update. Mutat Res. 1998;411:45-86.
8. Kirson ED, Gurvich Z, Schneiderman R, et al. Disruption of cancer cell replication by alternating electric field. Cancer Res. 2004;64:3288-95.
9. Kirson ED, Dbalý V, Tovarys F, et al. Alternating electric fields arrest cell proliferation in animal tumor models and human brain tumors. Proc Natl Acad Sci U S A. 2007;104:10152
10. Tailibert S, Le Ruhn E, Chamberlain M. Tumor trating fields: a new standard treatment for glioblastoma? Curr Opin Neurol. 2015;28:659-64.
11. Kirson ED, Schneiderman RS, Dbalý V, et al. Chemotherapeutic treatment efficiacy and sensitivity are increased by adjuvant alternating electric field (TTFields). BMC Med Phys. 2009;9:1.
12. Pless M, Droege C, von Moos R, Salzberg M, Betticher D. A phase I/II trial of tumor treating fields (TTFields) therapy in combination with pemetrexed for advanced non-small cell lung cancer (NSCLC). Lung Cancer. 2013;81:445-50.
13. Pless M, Weinberg U. Tumor treating fields: concept, evidence and future. Exp Opin Invest Drugs. 2011;20:1099-106.
14. Swanson KD, Lok E, Wong ET. An overview of alternating electric fields therapy (NovoTTF Therapy) for the treatment of malignant glioma. Curr Neurol Neurosci Rep. 2016;16:8.
15. Gera N, Yang A, Holtzman T, Lee SX, Wong ET, Swanson KD. Tumor treating fields perturb the localization of septins and cause aberrant mitotic exit. PLOS One. 2015;10:e0125269. doi: 10.1371/journal.pone.0125269.
16. Giladi M, Schneiderman RS, Voloshin T, et al. Mitotic spindle disruption by alternating electric fields leads to improper chromosome segregation and mitotic catastrophe in cancer. Cells Sci Rep. 2015;5:18046:2–16.
17. Yvon AM, Wadsworth P, Jordan MA. Taxol suppresses dynamics of individual mictrotubules in living human tumor cells. Mol Biol Cell. 1990;10:947-59.
18. Lowe J, Li H, Downing KH, Nogales E. Refined structure of alpha beta-tubulin at 3.5 A resolution. J Mol Biol. 2001;313:1045-57.
19. Felder CE, Prilusky J, Silman I, Sussman JL. A server and database for dipole moments of proteins. Nucleic Acids Res. 2007;35:512-21.
20. Albertson R, Cao J, Hiseh TS, Sullivan W. Vesicles and actin are targeted to the cleavage furrow via furrow microtubules and the central spindle. J Cell Biol. 2008;181:777-90.
21. D'Aviono PP, Savoian MS, Glover DM. Cleavage furrow formation and ingression during animal cytokinesis: a microtubule legacy. J Cell Sci. 2005;118:1549-58.
22. Gilden J, Krummel MF. Control of cortical rigidity by the cytoskeleton: emerging roles for septins. Cytoskeleton (Hoboken). 2010;67:477-86.
23. Gilden JK, Peck S, Chen YC, Krummel MF. The septin cytoskeleton facilitates membrane retraction during motility and blebbing. J Cell Biol. 2012;196:103-14.
24. Bowen JR, Hwang D, Bai X, Roy D, Spiliotis ET. Septin GTPases spatially guide microtubule organization and plus end dynamics in polarizing epithelia. J Cell Biol. 2011;194:187-97.
25. Gutin PH, Wong ET. Noninvasive application of alternating electric fields in glioblastoma: a fourth cencer treatment modality. Am Soc Clin Oncol Educ Book. 2012:126-31.
26. Hottinger A, Pacheco P, Stupp R. Tumor treating fields: a novel treatment modality and its use in brain tumors. Neuro Oncol. 2016;18:1338-49.
27. Wick W, Puduvalli VK, Chamberlain MC, et al. Phase III study of enzastaurin compared with lomustine in the treatment of recurrent intracranial glioblastoma. J Clin Oncol. 2010;28:1168–74.
28. Batchelor TT, Mulholland P, Neyns B, et al. Phase III randomized trial comparing the efficacy of cediranib as monotherapy, and in combination with lomustine, versus lomustine alone in patients with recurrent glioblastoma. J Clin Oncol. 2013;313212–18.
29. Yung WK, Albright RE, Olson J, et al. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. Br J Cancer. 2000;83:588–93.
30. Stupp R, Taillibert S, Kanner AA, et al. Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: a randomized clinical trial. JAMA. 2015;314:2535–43.
31. Stupp R, Wong ET, Scott C, et al. NT-40 interim analysis of the EF-14 trial: a prospective, multicenter trial of NOVOTTF-1ooA together with temozolomide compared to temozolomide alone in patients with newly diagnosed GMB. Neurooncology. 2014;16:167.

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REVIEW PAPER

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ASL (Arterial Spin Labeling) – historical and current perfusion MR methods

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ABSTRACT

Despite continuous scientific and technological advances in MR imaging, MR perfusion methods have not yet been widely deployed for routine clinical diagnostics. This is especially true for ASL (arterial spin labelling) methods used to evaluate cerebral perfusion. This method does not require a contrast agent, as new discoveries about gadolinium accumulation in the cerebellum and brain nucleus appear to be a valuable asset and provide the opportunity to be more widely deployed in clinical practice. The aim of this paper is to present the historical determinants of the development of MR perfusion techniques, the disadvantages and advantages and possible clinical applications and prospects of ASL development. Both historical articles published on MR in the 1990s and current research between 2006-2016 have been reviewed. The authors present in the work the MR perfusion method focusing on issues related to arterial spin labeling (ASL). Historically CASL (continuous ASL) and PCSL (pulsed ASL) techniques have been described and the pseudocontinuous ASL (pseudocontinuous ASL) 3D technique presents its technical and methodological considerations, advantages and disadvantages over previous methods. The methods of test protocol optimization and accompanying artifacts, as well as possible clinical applications and development perspectives, have been described.

Keywords. perfusion, MR, ASL

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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Introduction

In Magnetic Resonance Imaging Tomography, typical functional morphologies can be obtained by using a variety of spin echoes and gradient echoes. Functional imaging shows processes far beyond the actual spatial MR resolution. Among the advanced MR imaging techniques, in addition to the diffusion imaging that has been shown to be important in the early detection of cerebral ischemia already implemented in the 1980s, we can also list methods for evaluating cerebral perfusion, whose use and clinical relevance go beyond the assessment of cerebrovascular disease and are increasingly widespread with recognized application in the evaluation of oncological processes taking place not only in the brain.

Arterial spin labelling (ASL) is a MR perfusion technique that is used to measure cerebral blood flow (CBF). ASL utilizes the ability of MRI to label arterial blood by spin inversion of innate water with radiofrequency pulses and noninvasively image without the use of contrast agents. In ASL, water serves as the natural biomarker for imaging arterial blood flow.

Among the perfusion techniques used in MR imaging, we can distinguish: DSCI (dynamic susceptibility contrast imaging), DCE (dynamic contrast enhanced) and ASL (arterial spina labeling). DSC most commonly used in clinical practice is based on the evaluation of the first pass of the contrast agent (gadolinium chelate) by the capillary bearing. DCE is a method based on the assessment of a change in the longitudinal relaxation time of T_1 induced by contrast agents, and ASL uses a method of marking arterial blood spins.

Advantage of ASL perfusion imaging is that no contrast agent is required, therefore, the study may be performed in patients with renal impairment, in allergic patients or in patients who do not agree to use contrast media. The other advantages are the possibility of multiple repetitions in a short time (under 24 hours), lack of geometric distortion Images and the fact that CBF (cerebral blood flow) is measured in ml/100g/min and does not depend on the type of coil.

ASL or AST (arterial spin tagging) is one of the MR perfusion methods that uses intrinsic water molecules as a marker, and unlike DSC and DCE, no contrast agent or other external marker is required. This technique for marking arterial blood spins was introduced in 1992 in MR research by John A. Detre and co-workers. Among the scholars who dealt with the development of ASL in perfusion studies, the names of Robert R. Edelman and David C. Alsop and Weiying Dai should also be noted. The MR technique of MR perfusion has evolved since the early 1990s through further methodological improvements from initial studies in one layer over very long acquisition times to the current high-quality 2D and 3D image of perfusion of the entire cerebral area within 5-6 minutes. ASL is currently available in most new MR devices and its reproducibility has been confirmed in numerous multicenter studies.

The basic principles and sequential steps for MR imaging in ASL are as follows:

1. Checkpoints of interest area (brain) in GE (gradient echo) or SE (spin echo) sequences.
2. Signaling of RF (radiofrequency) blood spins (water molecules) at the neck level through their saturation or inversion.
3. After post-label delay (PLD), marked spins water molecules in the arterial blood affect the interest (brain) and behave as a freely diffusing marker, changing the tissue magnetization by about 1-2%.
4. The area of interest (brain) is re-scanned (as in point 1), and the data obtained is subtracted from the control image, which allows calculation of parametric rCBF (regional cerebral blood flow) images, as local changes in tissue magnetization depend on blood flow parameters.

Under ASL perfusion, various techniques have been developed[1-3]:

— CASL (continuous ASL) with continuous RF pulse combined with electromagnetic field gradients applied to flowing blood,
— PASL (pulsed ASL) using intermittent RF pulse,
— EPISTARE (first PASL sequence - asymmetric spin marking),
— PICORE (asymmetric marking of water spins),
— FAIR (symmetrical marking of water spins),
— pCASL (pseudo-continuous ASL) - hybrid method,

CASL (Continuous Arterial Spin Labeling)

The first real ASL technique described by Detre et al. in 1992 used a constant, low-amplitude RF pulse in combination with electromagnetic field gradients causing inversion of blood flow perpendicular to the imaging layer.[4] Spine infiltration reduced the intensity of the stationary tissue signal to a lesser degree, which was possible due to subtraction.[5] This technique was abandoned in the late 1990s due to technical difficulties with its implementation in MR scanners due to the need for an additional RF pulse transmitter which resulted in a significant increase in tissue temperature. In addition, the inversion pulses used in CASL caused the phenomenon of magnetization transfer (MT), which, although marginal and non-specific, could undermine perfusion-related images. All ASL techniques must take into account the MT effect caused by the inversion impulse, which reduces the signal from the imaged area. To eliminate or reduce it, two solutions were proposed:

1. Apply the second inversion pulse during control imaging on the other side of the imaging area, symmetrically. The MT effect is then identical in the imaged layer to the labeled and control images, and during the subtraction is eliminated.

2. Inversion Impulse during control imaging is divided into two parts by amplitude modulation. When both parts of the pulse have only half the amplitude and are very close together, the MT effect disappears when the images are subtracted.

PASL (Pulsed Aretrial Spin Labeling)

The first PASL (Pulsed Aretrial Spin Labeling) technique introduced in the mid-1990s by Edelman - EPISTAR (Echo-Planar Imaging-Based Signal Targeting by Alternating Radiofrequency Pulses) was based on sequential application[6]:

1. 90 slice selective pulse and intermittent gradients for the initial saturation of the magnetization in the imaging layer.
2. A broad pulse of 180° proximal to induce inversion of incoming blood spins.
3. A control sequence in which repeated pulse 90° interrupted saturation of static tissues in the imaging layer and followed the mirror image spaced distances from the layers of imaging impulse 180°.
4. Subraction, which endured the undesirable effect of magnetization transfer.

EPISTAR gave the image of MR perfusion in a single layer and subsequent techniques (STAR, PULSAR, QUASAR) were already multilayer.[7]

PICORE (asymmetric marking of water spins) and FAIR (symmetrical marking of water spins)

The next PASL techniques developed are PICORE (Proximal Inversion with Control of Off-Resonance Effects), an asymmetric multi-layer PASL-like EPISTAR-like technique in which the tagging sequence was identical to EPISTAR, and the control sequence was different in that the re- Equivalent to the impulse in the tagging sequence but without the spatial gradient and FAIR (Flow-sensitive Alternating Inversion Recovery). FAIR was slightly different in symmetry with respect to the imaged marking technique. The marking sequence was started spatially bound to the area of the selected layer with an inversion pulse of 180° and in the control sequence the same inversion pulse 180° but without a layer selection gradient[8] was applied. The differences between the EPISTAR, PICORE and FAIR techniques depend in part on the geometry of the expected blood flow to the test volume (imaging area). PICORE marks only blood on one side and is therefore a sensible choice for axial imaging of cerebral perfusion, where all the incoming blood comes from the neck area. FAIR is more suitable when blood flow occurs from many directions (e.g. cerebral perfusion imaging or liver perfusion). FAIR is also more susceptible to signal pollution associated with unwanted venous infiltration and more difficult to implement in multilayer mode. In some situations, EPISTAR is preferable to other sequences, due to fewer artifacts due to balanced gradation gradients in labeled and control sequences.

pCASL (pseudo-continuous ASL)

pCASL (Pseudo-continuous Arterial Spin Labeling) is a Hybrid method proposed in 2008 by Dai *et al*., Using a narrow marking level (similar to CASL), which facilitates flow-dependent spin inversion. Marking of spins is just below the imaged volume and minimizes the loss of tagged blood. A series of very short RF pulses are used here, which imitate a single continuous pulse (as in CASL), but show much less energy in the imaging area and less on the RF power cycle. Compared to PASL, pCASL offers a higher SNR (signal to noise ratio) and is less susceptible to scattering and is highly sensitive to flow volume, compared to CASL for higher marking efficiency and can be used in modern MR scanners.[9,10]

From the point of view of the correct execution of the test and its optimization in the ASL technique the following values of parameters and rules[2,3] are taken into account:

1. PLD, that is, the spin time from their marking to the level of the imaged area should typically be between 1.5 and 2.5 seconds in healthy individuals. Longer PLD times should be considered in the elderly, patients with vascular pathology or low cardiac output, and in children, the PLD time should be shorter and should be about 1.0-1.5 s. This parameter is critical and directly affects the quality of the ASL test, where the marking and PLD depends on field strength, ASL technique and flow volume. For the brain in the 1.5 T field, the typical marking time for PASL is 700 ms, for pCASL about 1500ms, and the PLD is about 1800 ms. In the 3.0 T field the PLD times should be somewhat longer.
2. All ASL techniques perform better when imaging occurs in a transverse plane perpendicular to the direction of marked blood flow. Relevant symmetric neck and head alignment is important from the point of view of preparation.
3. ASLs are dependent on SNR, so it is best to use the MR with the highest available field strength, i.e. 3.0 T or possibly 1.5 T, and multi-channel coils are mandatory.
4. Repeat times (TR) should be greater than 3500ms and minimum echo time (TE).
5. Spatial resolution due to SNR should be low. For 2D ASL, the typical matrix is 64×64 or 128×128 with a 4-6mm layer thickness.
6. For maintaining a prudent SNR and a test time of 5 minutes, multiple averaging signals are required: for 2D techniques between 30 and 50 averaging signals at 3.0 T, and even more at 1.5T, and for 3D 2-4 signal averaging.

7. Background suppression is required for 3D image segmentation, which uses single excitation during TR, less effective for 2D multi-layer techniques.

Like other MR methods, ASL also exhibits the presence of artifacts associated with magnetic susceptibility, flow or movement, although some of them manifest themselves differently in ASL.[1,11] The most common are:

1. Artifacts of magnetic susceptibility.

Artifacts of magnetic susceptibility occurring at the level of the labeled layer may cause distortion of the CBF measurement. Artifacts of magnetic susceptibility at the level of the test layer (a test layer is a separate control image acquired without prior labeling of arterial spins) may mimic stroke.

2. Artifacts related to the sensitivity of the coil.

Spatial differentiation of the sensitivity of the receiving coil may mimic hyper areas or hypopituitarism in ASL images. This artifact can be minimized by image filtering and other postprocessing techniques.

3. Traffic related artifacts.

The movement of the patient between the marking phase and the control phase causes a rim ("halo") around the imaged area. Motion correction (patient stabilization) reduces the artifact. There may be differences in the brightness of this artifact between the imaging layers.

4. Artifacts related to downstream signal loss.

The common feature of ASL techniques is the reduction of the signal in the test layers away from the marking area. This is due to the longitudinal relaxation of T_1-labeled water protons between their inversion and the reading in the test layer. This phenomenon is more evident at 1.5 T field strength than in 3.0 T due to shorter T_1 blood pressure lowering times. This artifact can be reduced with the use of 3D sequences in the reading phase and parallel imaging techniques to shorten the acquisition time.

5. Intravascular signal artifact.

Delayed flow of labeled blood proton into the imaging area and lack of time to spread in tissues may cause them to be present during re-scan in the vascular bed. This artifact may also be due to a lack of accurate demarcation of the inversion pulse when marking blood proton, excessive shortening of PLD time or pathological delayed arrhythmia. In order to reduce high vascular signals, large bipolar gradients are often used along several axes just before the reading phase. Sometimes, despite the fact that these artifacts persist, they may be a significant clinical symptom reflecting the delayed arterial flow associated with vascular disease.

6. Artifacts related to unsuccessful saturation in the background.

Since the differences between the labeled and ASL control sequences are small (about 1%), all lesions in static tissues subjected to imaging can damage the perfusion images. This is particularly true in the ASL 3D method, and several types of background suppressions within the imaged volume are used to remedy this. If background saturation during the ASL test does not take place in postprocessing, the layers covered by this error may be removed, resulting in the failure to read the test.

Gadolinium significantly shortens the longitudinal relaxation time of T_1 blood proton, so between the inverse and read sequences, there is an almost complete return of longitudinal magnetization T_1. Thus, labeled and controlled images do not differ from each other, which, after subtracting them (image acquisition area of labeled spins subtracting from the control image), gives a picture in which there are no perfusion signals. After gadolinium administration, wait at least 8-12 hours before performing ASL imaging with normal renal function, and for accurate rCBF measurement, this time should be prolonged to 24-48 hours.[1]

Guidelines for proper ASL performance:

1. Do not perform ASL imaging with contrast agent, as there is no signal to create an image (CBF map).
2. Do not perform ASL if the images at the neck level are distorted due to the risk of false diagnosis e.g. occlusion of the carotid artery.
3. The neck placement of the patient should be accurate, without side deviation due to the possibility of the "right" or left hemisphere "shadow" artifact.
4. Properly delay the acquisition after spin marking should be used - the PLD value should be adjusted appropriately for age: in children 1.0-1.5 sec, in healthy patients 1.5-2.5 sec, in older patients and in stroke 2.5-3.0 sec.

Like other MR perfusion techniques, quantitative blood flow calculations are desirable but difficult to achieve. Differences in signal intensity data in the inversion and read phase allow for perfusion-dependent images. Translating these raw data into absolute blood flow measurement requires three steps: processing and filtering images to remove artifacts, acquiring separate maps in PD or T_1 images depending on the intensity of the signal, selecting data for the mathematical model to calculate the pixel blood flow.

Buxton's general kinetic model is the most widely used, and the final equation for calculating blood flow depends on many measurements and parameters, as well as the use of precision ASL pulse sequences.[10,12]

Areas of currently used and possible uses of ASL for cerebral perfusion are:[5,13-16]

1. Neurological diseases: vascular diseases (TIA, stroke, migraine, vascular malformations), brain tumors, neurodegeneration
2. Developmental and genetic effects
3. Functional and pharmacological MR imaging
4. Neuromodulation

In addition, it is possible to use selectively regional ASL (selective ASL region). This technique, by using a single 180° wide inversion impulse and applying thin-film impulses to the main blood supply vessels to the brain, is able to visualize separately the supply areas of each of the major cerebral blood vessels.

ASL is a very sensitive imaging technique (more than DSC), but not very specific.[15] An elevated signal on CBL mapping in ASL may be due to a wide range of vascular pathologies such as: decreased arterial flow, increased cortical flow, arteriovenous leakage. For accurate localization of signal disturbances in ASLs, image fusions with other more specific 3D sequences like TOF and SWAN are used. ASL is a sensitive method for evaluating perfusion mainly in the area of the cerebral cortex whereas, in fact, the white rate is limited to a low level compared to the gray matter and the MTT.[17] The use of 3D FSE ASL sequences enhances diagnostic efficacy in patients with aneurysms, clips, coils, and those with brain heme transformation as FSE sequences tend to reduce artifacts from magnetic susceptibility, as opposed to EPI sequences.[12,18]

Conclusions

In the literature, ASL perfusion has reached over 1,000 publications and is now of great interest, hence the desire to popularize this technique of MR perfusion on native soil. Numerous authors, apart from technical and methodological aspects, are concerned with the possibilities of implementing this technique in clinical practice, which is not easy due to its limitations and lack of popularization, due to the fact that it has only been available for several years in modern MR scanners. Nevertheless, the current work shows similar possibilities as in MR perfusion with DSC and comparable with CT perfusion assessment of cerebral perfusion and have wide application possibilities, also in functional MR imaging.[12,15,19] In Poland, few authors have attempted to assess the clinical relevance of ASL.[20] There is a 3D ASL technology available at the Medical Research Center of the University of Rzeszow, where the 1.5 T system is installed. Hopefully, in good cooperation with the Clinical Departments in Rzeszow, our experience and research will be documented. Due to the currently known side effects of the use of paramagnetic contrast agents in patients with chronic renal failure (NSF) and newly discovered brain gadolinium accumulation, the interest in "old" MR-ASL perfusion will increase.[21,22] Continuous technological progress is not difficult to imagine that this technique in the near future may be dominant in the assessment of cerebral perfusion in many clinically important areas.

References

1. Diebler AR, Pollock JM, Kraft RA, et al. Arterial spin-labeling in routine clinical practice, Part 1: techniques and artifacts. AJNR Am J Neuroradiol. 2008;29:1228-34.
2. McGehee BE, Pollock JM, Maldjian JA. Brain perfusion imaging: how does it work and what should I use? J Magn Reson Imaging. 2012;36:1257-72.
3. Essig M, Shiroishi MS, Nguyen TB, et al. Perfusion MRI: the five most frequently asked technical questions. AJR Am J Roentgenol. 2013;200:24-34.
4. Aslop DC, Detre JA. Multisection cerebral blood flow MR imaging with continuous arterial spin labeling. Radiology. 1998;208:410-6.
5. Detre JA, Rao H, Wang DJJ, et al. Applications of Arterial Spin Labeled MRI in the Brain. J Magn Reson Imaging. 2012;35:1026–37.
6. Edelman RR, Chen Q. EPISTAR MRI: Multislice mapping off cerebral blood flow. Mag Reson Med. 1998;40:800-5.
7. Golay X, Peterson ET, Hui F. Pulsed Star Labeling of Arterial Regions (PULSAR):a robust regional perfusion technique for high field imaging. Magn Reson Med. 2005;53:15-21.
8. Kim SG. Qunatification of relative cerebral blood flow change by Flow-sensitive Alretnating Inversion Recovery (FAIR) technique:application to functional imaging. Magn Reson Med. 1995;34:293-301.
9. Dai W, Garcia D, de Bazelaire C, Alsop DC. Continuous flow driven inversion for arterial spin labeling using pulsed radiofrequency and gradient fields. Magn Reson Med. 2008;60:1488-97.
10. Petersen ET, Lim T, Golay X. Model-free arterial spin labeling quantification approach fo perfusion MRI. Magn Reson Med. 2006;55:219-32.
11. Amukotuwa SA, Yu C, Zharchuk G. 3D Peseudocontinuos Arterial Spin Labeling in routine clinical practice: a review of clinically significant artifact. J Magn Reson Imaging. 2016;43:11-27.
12. Alsop DC, Detre JA, Golay X, et al. Recommended implementation of arterial spin-labeled perfusion MRI for clinical applications: A consensus of the ISMRM perfusion study group and the European consortium for ASL in Dementia. Magn Reson Med. 2015;73:102-16.
13. Chen J, Licht DJ, Smith SE, et al. Arterial Spin Labeling Perfusion MRI in Pediatric Arterial Ischemic Stroke: Initial Experiences. J Magn Reson Imaging. 2009;29:282–90.
14. Bokkers RPH, Hernandez DA, Merino JG, et al. Whole--brain Arterial Spin Labeling Perfusion MRI in Patient With Acute Stroke. Stroke. 2012;43:1290-4.
15. Wang DJJ, Alger JR, Qiao JX, et al. Multi-delay multi-parametric arterial spin-labeled perfusion MRI in acute ischemic stroke - Comparison with dynamic susceptibility contrast enhanced perfusion imaging. NeuroImage: Clinical. 2013;1-7.
16. Qiao XJ, Salamon N, Wang DJJ, et al. Perfusion Deficits Detected by Arterial Spin-Labeling in Patients with TIA with Negative Diffusion and Vascular Imaging. Am J Neuroradiol. 2013;34:2125-30.
17. van Osch MJP, Teeuwisse WM, van Walderveen MAA. et al. Can Arterial Spin Labeling Detect White Matter Perfusion Signal? Magn Reson Med. 2009;62:165-73.

18. Alsop DC, Detre JA. Reduced Transit-Time Sensitivity in Noninvasive Magnetic Resonance Imaging of Human Cerebral Blood Flow. J Cerebral Blood Flow and Metab. 1996;16:1236-49.
19. Nael K, Meshksar A, Liebeskind DS, Wang DJJ, et al. Periprocedural Arterial Spin Labeling and Dynamic Susceptibility Contrast Perfusion in Detection of Cerebral Blood Flow in Pateints with Acute Ischemic Syndrom. Stroke. 2013;44:664-70.
20. Sprężak K, Urbanik A. Ocena przydatności metody znakowania spinów krwi tętniczej (arterial spin labeling, ASL) w rezonansie magnetycznym w diagnostyce obrazowej udaru niedokrwiennego mózgu. Przeg Lek. 2013;70: 319-27.
21. Redbruch A, Weberling LD, Kieslich PJ, et al. Gadolinium retention in the dentate nucleus and globus pallidus is dependent on the class od contrast agent. Radiology. 2015;275:783-91.
22. Malayeri AA, Brooks KM, Bryant LH, et al. National Institutes of health perspective on reports of gadolinium deposition in the brain. J Am Coll Radiol. 2016;13:237-41.

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REVIEW PAPER

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Neural tube defects: risk factors and prevention

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ABSTRACT

Neural tube defects are abnormalities that can occur in the brain (anencephaly, encephalocele), spine (spina bifida, myelomeningocele, myelodysplasia), both brain and spine (craniorachischisis) or spinal column of a developing embryo that are present at birth. They arise when the neural tube, the embryonic precursor of the brain and spinal cord, fails to close during neurulation. Many cases of neural tube defects occur worldwide each year in more than 300,000 newborn babies and are a significant cause of infant death and lifelong disability. Most neural tube defects are preventable. The prevalence of these abnormalities has decreased in the past 20 to 30 years due to periconceptional folate supplementation, food fortification and decreased exposure to environmental factors. Women who are planning to conceive should be informed about the importance of folic acid in fetal development and encouraged to take 400 µg/day of folic acid supplements. Numerous research studies have shown that taking this dosage of folic acid before and during early pregnancy significantly reduces the risk of neural tube defects. For that reason it is important to increase the awareness of women in childbearing age about the necessity of primary prevention and folate intake which is a strong factor that has wide implications in public health in reducing the mortality and morbidity of offspring.

Key words. neural tube defects, NTD, anencephaly, spina bifida

Introduction

Neural Tube Defects (NTD) are one of the most common and most serious developmental disorders of the fetus and the newborn. The total prevalence of NTD in Europe is 9.1 (3.34 identified as anencephaly and 4.63 by spina bifida) per 10,000 births. In Poland, with data only from Wielkopolska, this condition affects 9.25 children per 10,000 births (1.61 anencephaly and 6.59 spina bifida).[1]

The neural tube is a transient structure formed during embryonic development of the central nervous system which consists of the brain and spinal cord. Neural tube closure (NTC) or neurulation is one of the most complex morphogenetic events that occurs during embryogene-

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sis. The process begins in humans at a very early stage of fetal development, approximately day 17 postfertilization. The entire neurulation process requires 10 days and occurs during weeks 3 and 4 postfertilization. During the neurulation process, the neural plate is folded, elevated, apposed, closed and fused at the dorsal midline, thereby separating non-neural ectodermal tissue from the neural tube.[2,3] Neural tube closure is initiated by signals from the primitive node (the organizer) and notochord that cause the overlying epiblast to become thickened forming the flat neural plate.[4] By day 19, the plate has lengthened and the edges in the cranial region begin to elevate on either side, forming the neural groove in the midline. Soon the edges of the folds begin to elevate further, rolling into a tube to meet each other and fuse. Fusion begin at the level of the fifth somite and proceeds in cranial and caudal directions. The cranial and caudal openings of the tube, created by the initiation of fusion, are known as neuropores. Closure of the cranial neuropore occurs on day 25, followed by closure of the caudal neuropore on approximately day 27.[3,5] Disruption of this dynamic and complex process can caused neural tube defects. The process of NTC is affected by various genetic and non-genetic factors. More than 200 genes are known to cause NTD in mice. These genes are involved in folic acid metabolism, glucose metabolism, retinoid metabolism, and apoptosis.[6]

Most nonsyndromic NTD is thought to be of multifactorial origin with influence of both genetic and environmental factors.

Risk factors

Nutritional

Folic acid (folate) is one of the water-soluble B vitamins that plays a significant role in the occurrence or recurrence of NTD. Folic acid is necessary for normal cell growth and replication, production and maintenance of new cells, DNA synthesis and RNA synthesis through methylation, and for preventing changes to DNA. Folate serves as a 1-carbon donor for the synthesis of purines and thymidine as well as in the remethylation cycle of homocysteine to methionine.[7,8] There are many studies that have confirmed the crucial role of folic acid supplementation during conception on decreasing prevalence of NTD. The first study was published in 1964. 1484 women under obstetric care when folic acid deficiency was quite common had been investigated. It was shown that insufficient folic acid levels resulted in congenital anomalies including NTD, megaloblastic anemia, and abruptio placentae, and emphasized that true prophylaxis should be started prior to conception in every woman.[9–11] It must be noted that folic acid is not a panacea to prevent occurrence in all cases, and that about 30% of the NTD recurrence is not folic acid-preventable which suggests that a proportion of NTD are resistant to folic acid. Fortunately, new studies pointed out another nutritional agent, myo-inositol, one of inositol isomers which is cyclic sugar alcohol obtainable from vegetables, citrus fruits, cereal grains and so forth, as a potential factor preventing spinal and cranial NTD.[12–14] Calcium formate also has been shown to have preventive effects on NTD in mice but evidence is not yet forthcoming in prevention of human NTD.[15]

Mother's age

In a meta-analysis, a correlation between maternal age and higher risk for NTD was investigated. The authors stated that mothers over 40 years old and less than 19 years have an increased risk of having a child with NTDs and the risk was greater for spina bifida than for anencephaly.[16]

Socio-economics factors

Studies on the influence of socio-economic factors indicated that mothers with higher education and higher social status are less likely to have children with NTD, but this finding may be partially explained by the fact that these mothers are more likely to use folic acid in preconception period and during the neural tube closure.[17]

Ethnic groups

Centers for Disease Control and Prevention report that spina bifida and other NTD are more common in certain ethnic groups such as the Hispanic living in America. Women of African American and Asian descent seem to have the lowest prevalence of spina bifida. However, the society of United States is increasingly multicultural and diverse, making more difficult to categorize individuals into distinct ethnic/racial group.[18]

Chemical factors

Parents with exposure to organophosphorus agents have an increased risk of having progeny with NTD. **(8?)** The correlation between maternal exposure to pesticides and neural tube defects (NTD) in offspring was evaluated in 184 Mexican women living in the USA and 225 women in a control group. If the mothers were exposured to pesticides, the risk of NTDS also increased, especially when pesticides were used in the perinatal period within 0.25 miles from home.[19] Spina bifida (SB) is a common congenital developmental defect in southeastern Mexico. Parents of children with SB reside in areas where frequent pesticide use and agricultural activities are high, suggesting potential exposure to pesticides. Paraoxonase 1 (*PON1*) is the enzyme responsible for deactivation of organophosphates in the central nervous system. Polymorphisms of *PON1* genes influence the catalytic activity and plasma protein level of the enzyme. Results suggest that *PON1* polymorphisms are relevant risk factors for having offspring affected with SB.[20] Recent studies have suggested that mothers employed in the chemical industry as cleaners or generally, which have contact with chemical substances (e.g. nursing, process-

ing food and beverages, farming, textile dye and leather industries, spraying pesticides) have a higher risk of having children with congenital defects including CNS malformations.[21–23]

Hyperthermia

Hyperthermia (HT) is a well-studied teratogenic factor that induces serious developmental defects, including NTD. Hyperthermia is defined as a temperature of at least 1.5 degrees C over normal core body temperature The teratogenicity of hyperthermia was first recognized in the laboratory during animal research (Edwards, 1966), and subsequent epidemiological and clinical studies have shown that HT is also a potential teratogen in humans.[24,25] Enhanced core body temperature appears to interfere with several critical developmental events such as cell proliferation, migration, differentiation and apoptosis. The sources of hyperthermia in humans might be febrile illnesses, sauna bathing, hot tub use and excessive physical exercise in a warm and humid environment.[26] The central nervous system is especially susceptible to damage induced by too high body temperature.[27,28] The mechanism of HT-induced NTD is not well understood. Studies have shown that hyperthermia induces apoptosis by activation of the mitochondrial apoptosis pathway, which is characterized by the release of cytochrome C and the subsequent activation of caspases, cleavage of poly ADP-ribose polymerase, DNA fragmentation and activation of the p53 protein- transcription factor called "the guardian of the genome".[29–31] Fetus exposed to hyperthermia can be born with spina bifida, encephalocele, microphthalmia, microthynthesis, external ear anomalies, heart defects, hypospadias, gastrointestinal defects, cleft lip and / or cleft palate, abdominal wall defects, diaphragmatic hernia, Hirschsprung disease, Moebius syndrome, and can also lead to spontaneous miscarriage.[32]

Taking of paracetamol in the first trimester of pregnancy for illnesses with fever does not increase the risk of serious congenital abnormalities and may reduce the risk of several developmental defects.[33]

Drugs

Various drugs interfering with metabolism of folic acid or disturbing its absorption like antiepileptic drugs (such as valproate and carbamazepine), sulfamethoxazole, trimethoprim (antimicrobials), methotrexate, azathioprine (immunosuppressant), acetylsalicylic acid (anticoagulant), sulfadoxin-pyrimethamine (anti-malaria agent), sulfasalazine (anti-ulcerative colitis), antacids, rifampicin (anti-tuberculosis) and androgenic hormones, etc. may increase NTD risk. These medicines should be avoided or used with caution especially in women of childbearing age.[34,35] Kondo et al. in 2013 performed a case-control study that revealed the use of antiepileptic drugs (AEDs) without folic acid supplementation resulted in a 20.2-fold higher risk for an affected pregnancy, compared to using no AEDs or using AEDs with folic acid supplementation.[36]

Cigarettes smoking during pregnancy

Pregnant women who smoked had significantly lower concentrations of serum folate and lower concentrations of red blood cell folate, than pregnant women who did not smoke. Lower levels of serum folate may account for the higher rate of miscarriage, stillbirth and fetal anomalies like NTD, that are observed in pregnant women who smoke.[37]

Obesity

Obesity is also a risk factor for NTD, and the effect of extreme obesity is independent of the effect of folate intake. Data shows that NTD risk increased from 1.9%, for women weighing 80 to 89 kg, to 4.0% for women weighing 110 kg or more compared to women from 50 to 59 kg. NTD risk decreases by 40% with folic acid 400 µg or more / day in women weighing less than 70 kg, but folic acid supplementation has no benefit in women with higher body weight.[38,39]

Diabetes mellitus

Diabetes mellitus in pregnant mothers is a risk factor for NTD. The relative risks for major central nervous system (NTDs) and cardiovascular system defects among infants of mothers with insulin-dependent diabetes mellitus increase. Strict metabolic control well before conception and knowledge about the risk of diabetes mellitus can significantly reduce the incidence of birth malformations among their infants. Hyperinsulinemia is also a strong risk factor for NTD.[40,41]

Epidemiological and experimental studies on NTD provide some evidence that a host of physical agents (e.g. X-irradiation, hyperthermia, stress), drugs (e.g. thalidomide, folate antagonists, and hypervitaminosis A), substance abuse (e.g. alcohol), chemical agents (e.g. organic mercury, lead), maternal infections (e.g. rubella, cytomegalovirus, *Toxoplasmo gondii*, syphilis), maternal metabolic conditions (e.g. phenylketonuria, diabetes mellitus, endemic cretinism), etc. are capable of causing congenital malformations of central nervous system structures.[42]

Vitamin A

It has been shown during experiments on animals that retinoic acid reveals teratogenic activity. Studies performed by Rothman et al. (1995) have shown that pregnant women who took daily ≥ 15 000 IU of vitamin A or less than 5000 IU, prevalence of NTD was 3% and 1.3% respectively. High vitamin A consumption during pregnancy is obviously harmful and must be avoided.[43]

Genetic Factors

Genetic abnormalities undoubtedly have an important impact in inducing neural tube defects in children. Animal studies have shown that there are as many as 100 mutant genes affecting neurulation and almost all of them have their homologs in humans. This suggests that NTD has a multifactorial genetic etiology. However, we know of no single gene which is solely responsible for NTD in humans. NTD are related to genes encoding proteins that are directly or indirectly connected with folic acid and methionine metabolism. NTD are more common in females than in males, and occurs more frequently in families where previously born children were also affected with NTD, although they do not follow a strict Mendelian pattern of inheritance. This risk is 3-5 fold higher than in the general population. One of the most common mutations associated with NTD has been identified in the 5, 10-methylenetetrahydrohydrofolate reductase (MTHFR) gene. The folate pathway enzyme, MTHFR is one of the most important factors that enables cell regulation of the intracellular concentration of methionine and homocysteine. This is associated with NTD by preventing the conversion of the homocysteine to metionine. A thermolabile variant of MTHFR 677TT, reduces enzyme activity and causes enzyme deficiency in 4-16% of the population, depending on ethnic group (the biggest proportion of MTHFR exhibited in the Japanese male, the lowest in the non-white Brazilian).[44] Polymorphism of C677T (C for cytosine, T for thymidine) in the MTHFR gene has been identified as a mutation that renders the enzyme thermolabile and makes it prone to higher temperature, decreases its activity leading to an increased serum homocysteine concentration.[45]

Prevention of neural tube defects and a summary

Undeniable evidence for the effectiveness of periconceptional folic acid supplementation in preventing the majority of NTD, as reported by the Medical Research Council (MRC) of the United Kingdom, has been available since 1991.[46] Periconceptional consumption of folic acid supplements is strongly recommended for women who have affected pregnancy or have a family history of NTD. Furthermore in 2015, the North American Teratology Society published officially recommendations for women of child-bearing age or women planning to conceive to take folic acid supplements prior to conception and the global strategic plan for the total prevention of folic acid-preventable spina bifida and folic acid-preventable anencephaly by 2024. Unfortunately, many studies show that there is still insufficient knowledge of women who are planning a pregnancy, about the role of folic acid in NTD prevention and the absolute need for supplementation.[47] A good solution is provided by governments that institute mandatory folic acid fortification of a centrally produced food (such as, but not limited to, wheat flour, corn flour or meal, rice, and maize flour or meal) to provide almost all adults with at least an additional 150 micrograms of folic acid per day. Presently, 80 countries have registration (Food fortification Initiative 2016) to mandate fortification of wheat flour with folic acid, and six countries implemented the fortification of rice with folic acid. Approximately 180,000 spina bifida and anencephaly pregnancies occur in 120 countries each year that may be preventable through mandatory folic acid fortification.[48] Data shows that folic acid fortification is highly cost-effective, saving approximately $5 billion dollars in direct costs in the United States alone over a 10-year span (1996–2006).[49]

References

1. Khoshnood B, Loane M, de Walle H, et al. Long term trends in prevalence of neural tube defects in Europe: population based study. The BMJ. 2015;351.
2. Yamaguchi Y, Miyazawa H, Miura M. Neural tube closure and embryonic metabolism: Neural tube closure and metabolism. Congenit Anom. 2017. doi:10.1111/cga.12219.
3. Sadler TW. Mechanisms of neural tube closure and defects. Ment Retard Dev Disabil Res Rev. 1998;4:247–53.
4. Sasai Y, De Robertis EM. Ectodermal Patterning in Vertebrate Embryos. Dev Biol. 1997;182:5-20.
5. Araya C, Carmona-Fontaine C, Clarke JDW. Extracellular matrix couples the convergence movements of mesoderm and neural plate during the early stages of neurulation. Dev Dyn Off Publ Am Assoc Anat. 2016;245:580-9.
6. Greene NDE, Stanier P, Copp AJ. Genetics of human neural tube defects. Hum Mol Genet. 2009;18(R2):R113-129.
7. Kamen B. Folate and antifolate pharmacology. Semin Oncol. 1997;24,5:18-39.
8. Figueiredo JC, Grau MV, Haile RW, et al. Folic Acid and Risk of Prostate Cancer: Results From a Randomized Clinical Trial. JNCI J Natl Cancer Inst. 2009;101:432-5.
9. Berry RJ, Li Z, Erickson JD, et al. Prevention of Neural--Tube Defects with Folic Acid in China. N Engl J Med. 1999;341:1485-90.
10. Czeizel AE, Dud?s I. Prevention of the First Occurrence of Neural-Tube Defects by Periconceptional Vitamin Supplementation. N Engl J Med. 1992;327:1832-5.
11. Hibbard BM. The role of folic acid in pregnancy, with particular reference to anaemia, abruption and abortion. J Obstet Gynaecol Br Commonw. 1964;71:529-42.
12. van Straaten HW, Copp AJ. Curly tail: a 50-year history of the mouse spina bifida model. Anat Embryol (Berl). 2001;203:225-37.
13. Greene NDE, Leung K-Y, Gay V, et al. Inositol for the prevention of neural tube defects: a pilot randomised controlled trial. Br J Nutr. 2016;115:974-83.
14. Cavalli P, Tonni G, Grosso E, Poggiani C. Effects of inositol supplementation in a cohort of mothers at risk of pro-

ducing an NTD pregnancy. Birt Defects Res A Clin Mol Teratol. 2011;91:962-5.
15. Pai YJ, Leung K-Y, Savery D, et al. Glycine decarboxylase deficiency causes neural tube defects and features of non-ketotic hyperglycinemia in mice. Nat Commun. 2015;6:6388.
16. Vieira AR, Castillo Taucher S. Influence of maternal age on the risk for neural tube defects, a meta analysis. Rev Médica Chile. 2005;133:62-70.
17. Brough L, Rees GA, Crawford MA, Dorman EK. Social and ethnic differences in folic acid use preconception and during early pregnancy in the UK: effect on maternal folate status. J Hum Nutr Diet. 2009;22:100-7.
18. Smith K, Freeman KA, Neville-Jan A, Mizokawa S, Adams E. Cultural Considerations in the Care of Children with Spina Bifida. Pediatr Clin North Am. 2010;57:1027-40.
19. Brender JD, Felkner M, Suarez L, Canfield MA, Henry JP. Maternal Pesticide Exposure and Neural Tube Defects in Mexican Americans. Ann Epidemiol. 2010;20:16-22.
20. Gonzalez-Herrera L, Martin Cerda-Flores R, Luna-Rivero M, et al. Paraoxonase 1 polymorphisms and haplotypes and the risk for having offspring affected with spina bifida in Southeast Mexico. Birt Defects Res A Clin Mol Teratol. 2010;88:987-94.
21. Herdt-Losavio ML, Lin S, Chapman BR, et al. Maternal occupation and the risk of birth defects: an overview from the National Birth Defects Prevention Study. Occup Environ Med. 2010;67:58-66.
22. Suarez L, Felkner M, Hendricks K. The effect of fever, febrile illnesses, and heat exposures on the risk of neural tube defects in a Texas-Mexico border population. Birt Defects Res A Clin Mol Teratol. 2004;70:815-9.
23. Muñoz JB, Lacasaña M, Aburto VHB, Sánchez LET, García AMG, Carrillo LL. Socioeconomic Factors and the Risk of Anencephaly in a Mexican Population: A Case-Control Study. Public Health Rep. 2005;120:39-45.
24. Hosako H, Francisco LE, Martin GS, Mirkes PE. The roles of p53 and p21 in normal development and hyperthermia--induced malformations. Birth Defects Res B Dev Reprod Toxicol. 2009;86:40-7.
25. MJ E. Prenatal loss of foetuses and abortion in guinea--pigs. - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/?term=Edwards+MJ.+1966.+Prenatal+loss+of+foetuses+and+abortion+in+guinea-pigs.+Nature+210%3A223%E2%80%932. Accessed June 18, 2017.
26. Moretti ME, Bar-Oz B, Fried S, Koren G. Maternal Hyperthermia and the Risk for Neural Tube Defects in Offspring: Systematic Review and Meta-Analysis. Epidemiology. 2005;16:216-9.
27. Edwards MJ, Saunders RD, Shiota K. Effects of heat on embryos and foetuses. Int J Hyperthermia. 2003;19:295-324.
28. Kline J, Stein Z, Susser M, Warburton D. Fever during pregnancy and spontaneous abortion. Am J Epidemiol. 1985;121:832-42.
29. Little SA, Kim WK, Mirkes PE. Teratogen-induced activation of caspase-6 and caspase-7 in early postimplantation mouse embryos. Cell Biol Toxicol. 2003;19:215-26.
30. Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? Cell Death Differ. 2006;13:1027-36.
31. Hofseth LJ, Hussain SP, Harris CC. p53: 25 years after its discovery. Trends Pharmacol Sci. 2004;25:177-81.
32. Martínez-Frías ML, García Mazario MJ, Caldas CF, Conejero Gallego MP, Bermejo E, Rodríguez-Pinilla E. High maternal fever during gestation and severe congenital limb disruptions. Am J Med Genet. 2001;98:201-3.
33. Feldkamp ML, Meyer RE, Krikov S, Botto LD. Acetaminophen use in pregnancy and risk of birth defects: findings from the National Birth Defects Prevention Study. Obstet Gynecol. 2010;115:109-15.
34. Russell RM, Golner BB, Krasinski SD, Sadowski JA, Suter PM, Braun CL. Effect of antacid and H2 receptor antagonists on the intestinal absorption of folic acid. J Lab Clin Med. 1988;112:458-63.
35. Hernández-Díaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. N Engl J Med. 2000;343:1608-14.
36. Kondo A, Morota N, Ihara S, et al. Risk factors for the occurrence of spina bifida (a case-control study) and the prevalence rate of spina bifida in Japan: Risk Factors and Prevalence of Spina Bifida. Birt Defects Res A Clin Mol Teratol. 2013;97:610-5.
37. McDonald SD, Perkins SL, Jodouin CA, Walker MC. Folate levels in pregnant women who smoke: an important gene/environment interaction. Am J Obstet Gynecol. 2002;187:620-5.
38. Gao L-J, Wang Z-P, Lu Q-B, Gong R, Sun X-H, Zhao Z-T. Maternal overweight and obesity and the risk of neural tube defects: a case-control study in China. Birt Defects Res A Clin Mol Teratol. 2013;97:161-5.
39. Dietl J. Maternal obesity and complications during pregnancy. J Perinat Med. 2005;33.
40. Becerra JE, Khoury MJ, Cordero JF, Erickson JD. Diabetes mellitus during pregnancy and the risks for specific birth defects: a population-based case-control study. Pediatrics. 1990;85:1-9.
41. Hendricks KA, Nuno OM, Suarez L, Larsen R. Effects of hyperinsulinemia and obesity on risk of neural tube defects among Mexican Americans. Epidemiol Camb Mass. 2001;12:630-5.
42. Padmanabhan R. Etiology, pathogenesis and prevention of neural tube defects. Congenit Anom. 2006;46:55-67.
43. Rothman KJ, Moore LL, Singer MR, Nguyen U-SDT, Mannino S, Milunsky A. Teratogenicity of High Vitamin A Intake. N Engl J Med. 1995;333:1369-73.
44. Perez ABA, D'Almeida V, Vergani N, de Oliveira AC, de Lima FT, Brunoni D. Methylenetetrahydrofolate reductase (MTHFR): incidence of mutations C677T and A1298C in Brazilian population and its correlation with plasma homocysteine levels in spina bifida. Am J Med Genet. 2003;119A:20-5.

45. Mohd-Zin SW, Marwan AI, Abou Chaar MK, Ahmad-Annuar A, Abdul-Aziz NM. Spina Bifida: Pathogenesis, Mechanisms, and Genes in Mice and Humans. Scientifica. 2017;2017:1-29.
46. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. The Lancet. 1991;338:131-7.
47. Smith MA, Lau C. A resolution on folic acid fortification. Birt Defects Res A Clin Mol Teratol. 2015;103:1-2.
48. Odewole OA, Williamson RS, Zakai NA, et al. Near-elimination of folate-deficiency anemia by mandatory folic acid fortification in older US adults: Reasons for Geographic and Racial Differences in Stroke study 2003-2007. Am J Clin Nutr. 2013;98:1042-7.
49. Centers for Disease Control and Prevention (CDC). Spina bifida and anencephaly before and after folic acid mandate--United States, 1995-1996 and 1999-2000. MMWR Morb Mortal Wkly Rep. 2004;53:362-5.

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REVIEW PAPER

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Combined effect of flavonoid compounds and cytostatics in cancer treatment

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ABSTRACT

Aim. The aim of the study was to review the literature on the combination of cytostatics with flavonoids as a promising way to improve the cancer therapy.

Material and methods. A review of Polish and foreign literature was performed. The following databases were searched: PubMed, Scopus, Science Direct, and Polish Medical Bibliography.

Literature analysis. Effective strategies to inhibit the progression of cancer are needed. Compounds of natural origin, including plant polyphenols, are a part of our diet. Due to their availability, and antioxidant properties, they may serve as efficacious adjuvants in cancer therapy, enhancing the effectiveness of chemotherapeutics. Epidemiological studies have shown an inverse relationship between diets rich in fruits, vegetables, and supplements, and the risk of all causes of death from cancer. Based on their diverse biological activity, flavonoids may be potential adjuvant therapeutic agents that act synergistically with cytostatics for treatment of many types of cancer. This review of the results is a summary the research on anticancer activity of flavonoids and may also raise consciousness of consumers, who will be able to compose their diet armed with the knowledge of preventive and therapeutic anticancer properties of food ingredients. There is need for further research on polyphenols of plant origin, including interactions among food components that coexist. Another important aspect is to understand how the activity of phytocompounds depends on concentration and the presence of additional factors (e.g. microflore, metal ions), which could possibly make a compound harmful, instead of having positive theraputics effect. Elucidation of the mechanisms involved in biological activity of the described phytocompounds is essential for a better understanding of their influence on an organism.

Keywords. flavonoids, anticancer drugs, co-delivery system, cytostatics

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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Introduction

At the present time, a considerable portion of cancer diseases are attributed to lifestyle choices such as smoking. Unfortunately, in many cases prognosis for cancer patients is not optimistic and the survival period is usually no longer than a couple of years after diagnosis. The majority of tumors develop very slowly and worrying symptoms appear late. An additional difficulty in cancer therapy is the fact that cancer cells are capable of recurrence and dispersion among healthy cells, so that complete removal of a tumor by surgery is not possible. A large number of cancers are also highly resistant to pharmacotherapy. Using aggressive chemotherapy usually worsens the condition of patients, who suffer many devastating side effects. Many oncological drugs destroy cancer cells along with normal cells. For this reason there is a search for new effective therapies that would eliminate cancer cells by the process of programmed cell death and prevention of migration. Currently, there is a tendency to look for natural ingredients with interesting biological properties that would support pharmacological therapy. We still do not exactly know what role diet ingredients that are delivered with food play during therapy, especially those with confirmed antioxidant activity. Hence, there is a necessity to broaden knowledge of interactions between drugs and specific antioxidants inside cancer cells. Research indicates that a properly composed diet that contains natural antioxidants may reduce toxic effects of chemotherapy.[1]

Biologically active flavonoid compounds

Flavonoids belong to one of the most widespread groups of natural compounds in nature composed of over 7000 different chemical compounds with highly diverse chemical structures and biological properties. They are found in the leaves, flowers, fruits, seeds, roots and bark of plants. Flavonoids have been tested for inhibition of the key enzymes involved in the mitochondrial respiratory chain among other properties. Some flavonoids show inhibitory activity towards particular groups of enzymes such as: hydroxylases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases and oxygenases. It was also shown that they have anti-inflammatory properties and may act as hormones. Flavonoid compounds are known to be scavengers of free radicals of various kinds, such as peroxide anion, peroxide radical or hydroxyl radical. They may also serve as singlet oxygen quenchers. Their capability to inhibit free radicals arises from their chemical structure. The presence of hydroxyl groups in the flavonoid ring is responsible for the ability to inhibit free radicals and prevent oxidative stress. Results of a recent study have shown that dietary flavonoids have a significant effect on complicated regulatory processes taking place in cancer cells. They may improve conditions of patients in various stages of cancer disease considerably. Many studies have been performed in order to find cytotoxic anticancer compounds in plants, especially in those that have been long-known in traditional medicine. Literature data indicates that plant-derived polyphenol compounds are promising "nutraceuticals" which combine nutritional value and pharmaceutical properties and may contribute to fighting various diseases such as diseases of the circulatory system, obesity, neurological diseases and cancers.

There is evidence that consumption of flavonoids considerably reduces the risk of certain types of cancers. A diet rich in isoflavones may lead to reduction in breast cancer occurrence in women and prostate cancer in men. The antitumor activity of flavonoids is considered to be related to interactions with the enzymes involved in neoplasia. A mechanism of flavonoid-induced blocking of DNA replication by inhibiting activity of enzymes such as DNA polymerase II and topoisomerases I and II are known. Flavonoid compounds also take part in inhibition of the cell cycle, which results in blocking proliferation and inducing apoptosis of cancer cells. Flavonoids are also capable of preventing oncogene activation by interactions with metabolic enzymes, for example, by inhibition of cytochrome P450s such as CYP1A1 and CYP1A2.[2]

Due to their pro-health properties, hop flavonoids and their synthetic derivatives have also been studied. Xanthohumol, the most important chalcone which constitutes 1% of hop-cone dry weight, has many biological properties. Apart from strong antioxidant activity, it also has antiviral, antimicrobial and anti-inflammatory properties.[3-5] Moreover, an *in vitro* study demonstrated that xanthohumol inhibits formation of new blood vessels during carcinogenesis and has antiproliferative properties against the human cancer cell lines: breast (MCF-6, MCF-7, T47-D), colon (HT-29), ovarian (A-2780) and prostate.[6-10]

Research over the last few years revealed that naringenin, which is a precursor of most flavonoids, may control fat tissue accumulation by induction of apoptosis in fat cells (adipocytes), inhibition of their formation (adipogenesis) and by increasing lipolysis.[11] There is also great interest in isoxanthohumol and 8-prenylnaringenin, compounds in hop cones 10 to 100 times lower in concentration than xanthohumol, as evidenced by a large number of scientific papers.[12] 8-Prenylnaringenin, a potential anticancer drug, demonstrates strong *in vitro* affinity for the estrogen receptor ERα found mainly in the mammary gland, the endometrium and the ovary. The binding affinity of 8-Prenylnaringenin is stronger than for coumestrol and genistein which are considered the most active flavonoids known.[13]

In addition to being promising agents in cancer therapy, flavonoid compounds may be used for prevention and treatment of anemia and circulatory disorders, in dermatology for treatment of atopic dermatitis, and as

anti-inflammatory agents. They may also prevent infections and the skin ageing process.[14] Moreover, recent research showed that naringenin may be used as an analgesic agent.[15] In lipopolysaccharide-activated mouse macrophages it efficiently inhibited expression of TNF-α gene, nitric oxide synthase, cyclooxygenase (COX-2) inhibiting release of inflammatory mediators (TNF-α, nitric oxide and prostaglandins).[16]

Additionally, flavonoids have a positive effect on the peripheral and central nervous systems by improving blood flow to the brain. This helps in formation of new blood vessels and growth of hippocampal neurons, which improves memory. Such properties help to maintain brain cognitive skills which may be important, for example, in Alzheimer's disease therapy. In research on hippocampus cells, naringenin (present in citrus fruits and tomatoes) was found to promote neurogenesis and to stimulate growth of damaged neurons.[17] Although plants have been used for a long time for therapy of various diseases, it is important to know the activity of individual compounds that they contain. According to research by Kuete et al., naringenin isolated from *Aframomum arundinaceum* was more toxic to drug resistant cancer cells than the plant extract itself.[18]

Bonina et al. provided evidence that quercetin, hesperetin and naringenin protect *in vitro* skin cells against UV radiation.[19] The mechanism proposed involved inhibition of peroxidation of phosphatidylcholine in liposome membranes and decreased production of malondialdehyde (MDA). The flavonoids decreased the amount of MDA, in direct proportion to concentrations used. Their activity can be ranked as follows: quercetin > hesperetin > naringenin. However, due to better absorption and the ability to penetrate into deeper skin layers, naringenin and hesperetin were the most profitable as active ingredients of protective preparations and cosmetics.[20] Plant polyphenols, due to their immunomodulatory properties and the ability to scavenge oxygen free radicals, may contribute to acceleration of wound healing. Recently, research has indicated that isoflavones and their derivatives may also be used in prevention of thyroid and lung cancers.[21] Isoprenylated flavonoid compounds may act as inhibitors of protein kinases, taking part in initiation of inflammation or cancer diseases. Studies carried out by Nishimura et al. demonstrated that prenylated flavonoids from hop (*Humulus lupulus* L.), namely xanthohumol and its derivatives, induced cancer cell apoptosis in the neuroblastoma cells IMR-32 and NB-39.[22] Prenyl, geranyl, furan and pyran derivatives of baicalein and 3,7-dihydroxyflavone obtained by chemical synthesis were tested for pro-apoptotic activity towards breast and lung cancer cell lines.[23] A remarkable inhibition of tumor cell growth was observed for derivatives containing a single geranyl group and also for the compounds with furan and pyran fused rings. Hisanaga et al. demonstrated that 8-prenylquercetin has stronger anti-inflammatory activity than its derivatives that lack a prenyl chain.[24] Substitution of the prenyl group increases the hydrophobicity of flavonoids and may modulate their absorption and excretion from the body.[25] This finding suggests that 8-prenylnaringenin reduces the rate of excretion of naringenin from blood, allowing for circulation in the blood stream for much longer periods than non-prenylated naringenin, so higher accumulation to target tissue may be achieved.

Combined action of flavonoids and cytostatics

Chemotherapy is a systemic method of cancer treatment with the use of cytostatics. Combination therapy (or polytherapy) involves using two or more anticancer drugs, which administered together are more effective.[26-27] In this form of treatment, flavonoids, which are contained in food, seem to be very promising. Numerous studies have confirmed the synergistic effect of natural polyphenols and cytostatics on the programmed cell death induction in cancer cells. They may increase susceptibility of cancer cells to subsequent lines of attack in chemotherapy.

Polyphenols may selectively enhance the activity of some cytostatics against tumor cells, and at the same time, exert a cytoprotective effect on normal tissues. The most often described mechanism of flavonoid anticancer activity is their ability to inhibit proliferation and induce programmed cell death in cancer cells. At the molecular level, this activity is related to inhibition of intramolecular signal transduction pathways necessary for cell survival such as the Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, Ras/Ras protein, Raf/Raf kinase, MEK/mitogen activated protein kinase, ERK/extracellular signal regulated kinase, PI3K/phosphoinositide 3-kinase, Akt/PKB/protein kinase B, and mTOR/mammalian target of rapamycin kinase.

Paclitaxel, known also as Taxol, isolated from the bark of the Pacific yew (*Taxus brevifolia*) is widely used in treatment of breast, ovarian and lung cancers. Moreover, the combination of paclitaxel and cisplatin is an effective second-line therapy for patients with metastatic breast cancer. Paclitaxel analogues, such as docetaxel and cabazitaxel, are also used as anticancer drugs (for treatment of aggressive breast and prostate cancers). A recent study confirmed that prenylated compounds derived from hop (*Humulus lupulus L.*), such as isoxanthohumol, enhance *in vivo* activity of paclitaxel.[28] According to the literature, naringenin also enhances the sensitivities of cancer cells to doxorubicin both *in vitro* and *in vivo*.[29]

In another study, central nervous system cancer cells were used as an experimental model. This group of cancers is difficult to treat. The complex anatomical and histological structure of the central nervous system is the reason why complete removal of the cancer-affected tissue is often impossible. These types of cancer are also highly resistant to pharmacotherapy. Additional difficulty in the therapy is the necessity to protect neu-

rons from damage. It is a known fact that nerve cells are very sensitive to oxidative stress. Having high antioxidant activity, flavonoids may play an important role in preventing neuronal death during anticancer therapy. Therefore, the study on employment of flavonoids in combination therapy of the central nervous system cancers were preceded by the assessment of their impact on normal nerve cell survival.

Glioblastomas are brain cancers that arise from astrocytes in the glial tissue. One of the most malignant is an anaplastic astrocytoma (AA) (lat. astrocytoma anaplasticum, WHO grade III) and glioblastoma multiforme (GBM) (lat. glioblastoma multiforme, WHO grade IV). They represent about 50% of all brain tumors. Unfortunately, prognoses for patients with these diseases are not optimistic and the life expectancy from the time of diagnosis is about a year for GBM and 3-5 years for AA. Gliomas develop slowly and the symptoms appear late. An additional difficulty in therapy of gliomas is the ability of cancer cells to migrate and disperse through the normal brain cells, so it is impossible to completely remove cancer tissue. They are also resistant to pharmacotherapy. The drug frequently used for treatment of glioblastomas is temozolomide (Temodal®, TMZ). This is an alkylating agent and its anticancer activity is based on formation of O^6-methylguanine in the DNA strand, which mispairs with thymine instead of cytosine during the next DNA replication cycle. This leads to prolonged G2-M arrest in glioma cells and ultimately cell death. An *in vitro* study revealed that using quercetin in combination with temozolomide enhances the therapeutic effect of this drug. Among others, stronger inhibition of cancer cells growth was observed, along with higher level of caspase-3, an important marker of apoptosis, compared to temozolomide alone.

Another promising example of using quercetin in combination chemotherapy was a study carried out with MOGGCCM astrocytoma cells. Preincubation of the glioblastoma cell line with this flavonoid increased sensitivity of the cells to induction of programmed cell death by means of Temodal. Interestingly, the type of programmed cell death induced by these two compounds was dependent on quercetin concentration. Incubation of the astrocytoma cells with Temodal and this flavonoid at the concentration of 1-5 μM effectively inhibited autophagy, whereas higher concentrations of the natural compound (about 30 μM) induced apoptosis.[30]

Another compound used in anticancer therapy, which is acts synergistically with quercetin in apoptosis induction, is doxorubicin, belonging to anthracyclines. In doxorubicin-resistant human pancreatic carcinoma cell lines EPP85-181 this flavonoid inhibited expression of P-glycoproteins, which are responsible for multi-drug resistance. In consequence, the tested cell line became more sensitive to the antibiotic-induced apoptosis. An additional advantage of this therapy is the ability of quercetin to protect normal cells from death, which was often observed in the case of treatment with daunorubicin alone.[31] Moreover, this flavonoid acted synergistically with doxorubicin, another anthracycline antibiotic, as inhibitor of breast cancer cells proliferation, and with tamoxifen as angiogenesis inhibitor in this cancer.[32] Similar results were obtained in treatment of a drug-resistant breast cancer with the help of quercetin or luteolin combined with doxorubicin (cytostatic) and tamoxifen (anti-estrogen), which led to inhibition of both proliferation and angiogenesis of the cancer cells.[33-34]

The most recent literature reports indicate that also other flavonoids are used as adjuvants in cancer therapies.[35-44] Chrysin in combination with celecoxib may help in treatment of diseases associated with COX-2 cyclooxygenases inhibition.[45-46] Whereas, naringenin administered with ABT-737, a drug being Bcl-2 protein inhibitor, enhanced its cytotoxic effect to gastric cancer cells.[35]

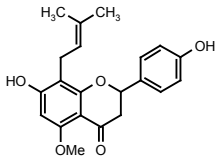
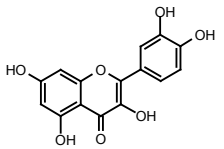
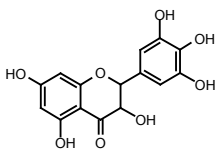
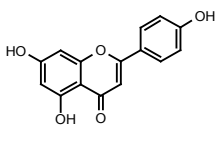
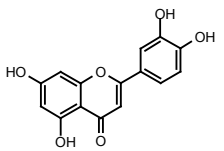
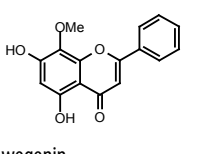
Similar research showed that quercetin also enhanced proapoptotic and pro-autophagic properties of the anticancer agent sorafenib (Nexavar) used in treatment of kidney cancer.[47] The molecular mechanism of this drug is based on inhibition of Raf serine/threonine kinase which plays a key role in the intracellular Ras/Raf/MEK/ERK signal transduction pathway, which in consequence leads to inhibition of cell proliferation. Combination of sorafenib and quercetin significantly increased sensitivity of MOGGCCM cells to induced apoptosis mediated by the mitochondrial pathway.

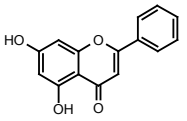
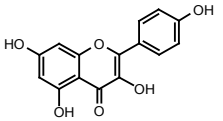
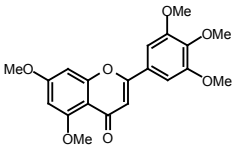
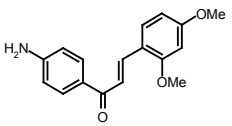
The combination of chrysin with cytostatics is more effective in induction of programmed cell death than using single chemotherapeutics. For the majority of tested cancer cells, changes in the mechanisms regulating cell cycle progression were observed. Mutual action of anticancer drugs and other flavonoids, such as combination of temozolomide and quercetin, significantly increased apoptosis of human glioblastoma cells induced by temozolomide, the anticancer drug used in treatment of brain cancers. Moreover, quercetin administered at the proper concentration considerably increased the chemosensitivity of breast and liver cancer cells to doxorubicin, and therefore enhanced the response of tumors to chemotherapy.[48] Luteolin and silibinin in combination of 20 μM and 50 μM, respectively were more effective than temozolomide (100 μM), the commonly used chemotherapy for glioblastoma.[49] Flavonoid compounds that have been used in combination therapy with cytostatics are listed in Table 1.

Summary

Clinical use of anticancer drugs is limited due to their dose-dependent side effects. Food intervention with the use of plants containing a proper composition of bioactive compounds may be a safe and effective way to pre-

Table 1. Combined effect of flavonoids and cytostatics in cancer therapy

Flavonoid compound	Anticancer drug	Biological model	Type of experiment	Ref.
		Flavanones		
 isoxanthohumol	paclitaxel	rats	in vivo	Krajnović et al. 2016
 quercetin	tamoxifen	rats breast cancer therapy	in vivo	Silva et al. 2017
	adriamycin	mice after P388 cell inoculation	in vivo	Han et al. 2014
			in vitro	Han et al. 2015
	doxorubicin	SMMMC7721 liver cancer cell	in vitro	Wang et al. 2012
		MCF-7 MDA-MB-231 MCF-10A	in vitro	Staedler et al. 2011
	daunorubicin	EPP85-181P EPP85-181DB human pancreatic carcinoma	in vitro	Borska et al. 210
	cisplatin	HeLa human cervix carcinoma	in vitro	Jakubowicz-Gil et al. 2005
	sorafenib	T98G MOGGCCM Human anaplastic astrocytoma and glioblastoma multiforme	in vitro	Jakubowicz-Gil et al. 2014
 dihydromyricitein	paclitaxel doxorubicin	A2780 SKOV3 IOSE80 ovarian carcinoma	in vitro	Xu et al. 2017
	nedaplatin	SMMC7721 QGY7701 HL7702 human hepatocellular	in vitro	Jiang et al. 2015
		Flavones		
 apigenin	cisplatin	PC3 prostate cancer	in vitro	Erdogan et al. 2017
	paclitaxel	HeLa cells	in vitro	Xu et al. 2011
 luteolin	tamoxifen	MCF-7, BT-483, BT-474; ER– cells: MDA-MB-231 human breast cancer	in vitro	Tu et al. 2013
	cisplatin	Mice (male C57BL)	in vivo	Kang et al. 2011
	doxorubicin	MCF-7 MDA-MB-453	in vitro	Sato et al. 2015
 wogonin	cisplatin	AMCHN2, -HN3, -HN4, -HN5, and -HN9, and SNU-1041, -1066, and -1076 human head and neck cancer	in vitro	Kim et al. 2016
		A549 lung cancer		He et al. 2012

 chrysin	doxorubicin	BEL-7402 hepatocellular carcinoma	in vitro	Gao et al. 2013
 kamferol	cisplatin	OVCAR-3 ovarian cancer	in vitro	Luo et al. 2010
 3',4',5',5,7-pentamethoxyflavone	cisplatin	A549 lung cancer	in vitro	Hou et al. 2015
Chalcones				
	cisplatin	ES-2 Hey-A8 human ovarian cancer	in vitro	Su et al. 2017

vent life-style diseases, including cancers. *In vitro* studies may contribute to a better understanding the role of antioxidants in chemotherapy, because at the moment, the effects of antioxidant supplementation are still unclear. Not only do antioxidants directly participate in free-radical reactions, but also have influence on the activity of many enzymes and expression of genes participating in apoptosis and DNA repair. Due to synergistic action, it could be possible to decrease drug dose, while providing the same therapeutic effect. Additionally, there is a possibility of reducing harmful side effects of chemotherapeutics on normal cells without loss of effectiveness of the treatment, because antioxidants can stabilize DNA and contribute to strengthening the antioxidant barrier, which is highly beneficial to chemotherapy. Preclinical and clinical studies with cancer patients is a serious challenge in this area. There is a need to perform more detailed studies that would lead to the development of new, innovative molecularly targeted therapeutic approaches for cancer treatments.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest.

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References

1. Kang KP, Park SK, Kim DH, et al. Luteolin ameliorates cisplatin-induces acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis. Nephrol Dial Transplant. 2011;26:814-22.
2. Yuan Y, Qui C, Nicoli D, et al. Inhibition of human cytochrome P450 enzymes by hops (*Humulus lupulus*) and hop prenylphenols. Eur J Pharm Sci. 2014;53: 55-61.
3. Wang Q, Ding Z, Liu J, Zheng Y. Xanthohumol, a novel anti--HIV-1 agent purified from hops *Humulus lupulus*. Antivir Res. 2004;64:189–94.
4. Stompor M, Żarowska B. Antimicrobial activity of xanthohumol and its selected structural analogues. Molecules. 2016;21:608.
5. Cho Y-C, You S-K, Kim HJ, Cho C-W, Lee I-S, Kang BY. Xanthohumol inhibits IL-12 production and reduces chronic allergic contact dermatitis. Inter Immunol. 2010;10: 556-61.
6. Miranda CL, Stevens JF, Helmrich A, et al. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. Food Chem Toxicol. 1999;37:271-85.
7. Gerhauser C, Alt A, Heiss E, et al. Cancer chemopreventive activity of xanthohumol, a natural product derived from hop. Mol Cancer Ther. 2002;1:959-69.
8. Ho Y-C, Liu C-H, Chen C-N, Duan K-J, Lin M-T. Inhibitory effects of xanthohumol from hops (*Humulus lupulus* L.) on human hepatocellular carcinoma cell lines. Phytother Res. 2008;22:1465-8.
9. Vanhoecke B, Derycke L, Marck VV, Depypere H, Keukeleire DD, Bracke M. Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer. Int J Cancer. 2005;117:889-95.

10. Monteiro R, Faria A, Azevedo I, Calhau C. Modulation of breast cancer cell survival by aromatase inhibiting hop (*Humulus lupulus* L.) flavonoids. J Steroid Biochem Mol Biol. 2007;105:124–130.
11. Morikawa K, Nonaka M, Mochizuki H, Hanada K, Hanada H, Hirota K. Naringenin and hesperetin induce growth agrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. J Agric Food Chem. 2008;26:11030-7.
12. Stevens JF, Taylor AW, Clawson JE, Deinzer ML. Fate of xanthohumol and related prenylflavonoids from hops to beer. J Agric Food Chem. 1999;47:2421-28.
13. Brunelli E, Minassi A, Appendino G, Moro L. 8-Prenylnaringenin, inhibits estrogen receptor-α mediated cell growth and induces apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol. 2007;107:140-8.
14. Chen W, Becker T, Qian F, Ring J. Beer and beer compounds: physiological effects on skin health. J Eur Acad Dermatol Venerol. 2014;28:142-150.
15. Pinho-Ribeiro FA, Zarpelon AC, Fattori V, et al. Naringenin reduces inflammatory pain in mice. Neuropharmacol. 2016;105:508-19.
16. Paoletti T, Fallarini S, Gugliesti F, Minassi A, Appendino G, Lombardi G. Anti-inflammatory and vascularprotective properties of 8-prenylapigenin. Eur J Pharmacol. 2009;620:120-130.
17. Xu X-H, Ma C-M, Han Y-Z, et al. Protective effect of naringenin on glutamate-induced neurotoxicity in cultured hippocampal cells. Arch Biol Sci. 2015;67:639-46.
18. Kuete V, Ango PY, Yeboah SO, et al. Cytotoxicity of four *Aframomum* species (*A. arundinace*um, *A. alboviolaceum*, *A. kayserianum* and *A. polyanthum*) towards multi-factorial drug resistant cancer cell lines. BMC Complement Altern Med. 2014;14:340-7.
19. Bonina F, Lanza M, Montenegro L, et. al. Flavonoids as potential protective agents against photo-oxidative skin damage. Int J Pharm. 1996;145:87-94.
20. Skorek M, Jurczyk K, Sajewicz M, Kowalska T. Thin-layer chromatographic identification of flavonoids and phenolic acids contained in cosmetic raw materials. J Liq Chromatograph Rel Tech. 2016;39:286-91.
21. Jiang L, Zhang Q, Ren H, et al. Dihydromyricetin enhances the chemo-sensivity of nedaplatin via regulation of the p53/Bcl-2 pathway in hepatocellular carcinoma cells. Plos One. 2015;10:e0124994.
22. Nishimura R, Tabata K, Arakawa M, et al. Isobavachalcone, a chalcone constituent of *Angelica keiskei*, induces apoptosis in neuroblastoma. Biol Pharm Bull. 2007;30:1878-83.
23. Neves MP, Cidade H, Pinto M, et al. Prenylated derivatives of baicalein and 3,7-dihydroxyflavone: synthesis and study of their effects on tumor cell lines growth, cell cycle and apoptosis. Eur J Med Chem. 2011;46:2562-74.
24. Hisanaga A, Mukai R, Sakao K, Terao J, Hou D-X. Anti-inflammatory effects and molecular mechanisms of 8-prenyl quercetin. Mol Nutr Food Res. 2016;60:1020-32.
25. Mukai M, Fujikura Y, Murota K, et al. Prenylation exhances quercetin uptake and reduces efflux in Caco-2 cells and enhances tissue accumulation in mice fed long-term. J Nutr. 2013;143:1558-64.
26. Gao A-M, Ke Z-P, Shi F, Sun G-C, Chen H. Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway. Chem Biol Interact. 2013;206:100-8.
27. Jakubowicz-Gil J, Paduch R, Piersiak T, Głowniak K, Gawron A, Kandefer-Szerszeń M. The effect of quercetin on proapoptotic activity of cisplatin in HeLa cells. Biochem Pharmacol. 2005;69:1343-50.
28. Krajnović T, Kalucrossed D, Signerović GN, Wessjohann LA, Mijatović S, Masimović-Ivanić D. Versatile antitumor potential of isoxanthohumol: enhancement of paclitaxel activity in vivo. Pharmacol Res. 2016;105:62-73.
29. Zhang FY, Du GJ, Zhang CL, Lu WL, Liang W. Naringenin enhances the anti-tumor effect of doxorubicin through selectively inhibiting the activity of multidrug resistance-associated proteins but not P-glycoprotein. Pharm Res. 2009;26:914-25.
30. Jakubowicz-Gil J, Langner E, Rzeski W. Kinetic studies of the effects of Temodal and quercetin on astrocytoma cells. Pharmacol Rep. 2011;63:403-16.
31. Borska S, Sopel M, Chmielewska M, Zabel, Dzegiel P. Quercetin as a potential modulator of P-glycoprotein expression and function in cells of human pancreatic carcinoma line resistant to daunorubicin. Molecules. 2015;15:857-70.
32. Staedler D, Idrizi E, Kenzaoui BH, Juillerat-Jeanneret L. Drug combinations with quercetin: doxorubicin plus quercetin in human breast cancer cells. Cancer Chemother Pharmacol. 2011;68:1161-72.
33. Silva FC, Bramatti, Toledo AG, Salles FM, Itnose AM, Marek CB. Antihyperglycemic effect of quercetin in ovariectomized rats treated with tamoxifen. J Med Food. 2017; 20:235-42.
34. Tu S-H, Ho C-T, Liu M-F, et al. Luteolin sensitises drug-resistant human breast cancer cells to tamoxifen via the inhibition of cyclin E2 expression. Food Chem. 2013;14: 1553-61.
35. Zhang H, Zhong X, Zhang X, Shang D, Zhou Y, Zhang C. Enhanced anticancer effect of ABT-737 in combination with naringenin on gastric cancer cells. Exp Ther Med. 2016;11:669-73.
36. Su Y-K, Huang W-C, Lee W-H, et al. Methoxyphenyl chalcone sensitizes aggressive epithelial cancer to cisplatin through apoptosis induction and cancer stem cell eradication. Tumor Biol. 2017;39:1010428317691689.
37. Erdogan S, Turkekul K, Serttas R, Erdogan Z. The natural flavonoid apigenin sensitizes human CD44+ prostate cancer stem cells to cisplatin therapy. Biomed Pharmacol. 2017;88:210-7.
38. Kim EH, Jang HJ, Shin D, Baek SH, Roh J-L. Targeting Nrf2 with wogonin overcomes cisplatin resistance in head and neck cancer. Apoptosis. 2016;21:1265-78.

39. Hou X, Bai X, Gou X, et al. 3',4',5',5,7-Pentamethoxyflavone sensitizes cisplatin-resistant A549 cells to cisplatin by inhibition of Nrf2 pathway. Mol Cells. 2015;38: 396-401.
40. Luo H, Daddysman MK, Rankin GO, Jiang BH, Chen YC. Kaempferol enhances cisplatin's effect on ovarian cancer cells through promoting apoptosis caused by down regulation of cMyc. Cancer Cell Int. 2010;10:16.
41. Sato Y, Sasaki N, Saito M, Endo N, Kugawa F, Ueno A. Luteolin attenuates doxorubicin-induced cytotoxicity to MCF-7 human breast cancer cells. Biol Pharm Bull. 2015;38: 703-9.
42. Xu Y, Xin Y, Diao Y, et al. Synergistic effects of apigenin and paclitaxel on apoptosis of cancer cells. PLoS ONE. 2011;6:e29169.
43. Hao T, Ling Y, Wu M, et al. Enhanced oral bioavailability of docetaxel in rats combined with myricetin: in situ and in vivo evidences. Eur J Pharm Sci. 2017;101:71-79.
44. He F, Wang Q, Zheng XL, et al. Wogonin potentiates cisplatin-induced cancer cel apoptosis through accumulation of intracellular reactive oxygen species. Oncol Rep. 2012;28(2):601-605.
45. Darwish HA, Arab HH, Abdelsalam RM. Chrisin alleviates testicular dysfunction in adjuvant arthritic rats via suppression of inflammation and apoptosis; comparison with celecoxib. Toxicol Appl Pharmacol. 2014;279:129-40.
46. Han Y, Yu H, Wang J, Ren Y, Su X, Shi Y. Quercetin alleviates myocyte toxic and sensitizes anti-leukemic effect of adriamycin. Hamatolohy. 2015;20:276-83.
47. Jakubowicz-Gil J, Langner E, Bądziul D, Wertel I, Rzeski W. Quercetin and sorafenib as a novel and effective couple in programmed cell death induction in human gliomas. Neurotox Res. 2014;26:64-77.
48. Wang G, Zhang J, Liu L, Sharma S, Dong Q. Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl. PLoS ONE. 2012; 7:e51764.
49. Chakrabarti M, Ray SK. Anti-tumor activities of luteolin and silibinin in glioblastoma cells: overexpression of miR-7-1-3p augmented luteolin and silibinin to inhibit autophagy and induce apoptosis in glioblastoma in vivo. Apoptosis. 2016;21:312-28.

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REVIEW PAPER

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Cytotoxic and anti-cancer activity of the *Cistus* species of herbal plants

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ABSTRACT

Aim. The aim of this paper is to provide an overview of the cytotoxic and anti-cancer properties of the major species of the genus *Cistus*.

Materials and methods. Thirty four papers that discuss the medicinal history and current research of *Cistus* species as phytotherapeutics were used for this discussion.

Literature analysis.The growing popularity of the *Cistus* species of herbs is mainly due to its anti-inflammatory, antimicrobial, antifungal and antioxidant properties. The results of in vitro studies indicate that the presence of pear extract significantly affects leukemia, leukemia, breast, colon, ovarian, pancreatic, and melanoma carcinomas. The significant growth inhibition of these cells, underlines its valuable anti-tumor properties and allows for the possibility of use as a therapeutic aid.

Keywords. anti-tumor activity, *Cistus species*, cytotoxic activity, phytotherapeutics

Introduction

Herbs are a natural source of compounds that demonstrate bioactivity in humans.[1,2] *Cistus* species *(Cistaceae)* are of particular interest in the area of herbal plants because of the valuable aspects of pharmacological and antioxidant activity.[3-6]

The Cistus species of the family *Cistaceacea* are perennial shrubs naturally occurring in the Mediterranean, Europe and western Africa, and Asia.[7,8,9] *Cistus species* have been used since antiquity in Mediterranean cultures for their medicinal properties. Scientific literature confirms their valuable phytotherapeutic properties as anti-inflammatory, antibacterial, antifungal, antiviral, anti-allergic, antimicrobial, and analgesic agents.[10-18] Their biological activity is mainly due to antioxidant polyphenolic compounds that are present which are considered as potential therapeutic agents in a wide range of diseases such as hypertension, diabetes, and Alzheimer's disease among others.[4,19-21] Representative *Cistus species* incanus and creticus are shown in Figures 1 and 2.

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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Figure 1. Herb Cistus incanus 22

The absence of antioxidant compounds such as phytotherapeutics in the human diet which provide the ability to deactivate free radicals can lead to dysfunction in the body, causing many diseases such as cancer, premature aging, and heart attacks.[23-26]

The aim of this paper is to characterize the cytotoxic and anti-cancer properties of the major species of the genus *Cistus*, with particular reference to the *Cistus creticus* subspecies *cretenicus L., Cistus incanus L. and Cistus monspeliensis L., C. creticus ssp. Creticus, Cistus libanotis, C. villosus and C. monspeliensis, C. ladanifer* and *C. populifolius, C. salviifolius* and their role as phytotherapeutic compounds.

Dimas et al. isolated 9 labdane diterpenes from the *Cistus creticus* subspecies *cretenicus (L.)* plant and ladano resin (extracted to the surface of leaves and stems of the plant). In vitro studies of the effects of the above diterpenes on 14 lines of human leukemic cells (CCRF-CEM, MOLT-3 H33AJ-JA13, HUT78, H9 (T cell lines) KM3, NAMALWA, DAUDI SDK, JIYOYE, CCRF- HL 60 (promyelocyte cell line) K562 (proerythrocytes) and U937 (monocytes) indicated cytotoxic activity. (13E) -labd-13-ene-8 alpha 15-diol showed cytotoxic activity against 13 cell lines tested, while (13E) -labd-7,13-dienol showed activity only against the HL60 cell line.[28]

In studies by Vitali et al., a significant effect of polyphenol compounds present in *Cistus incanus L.* and *Cistus monspeliensis L.* was indicated. These polyphenolic compounds showed cytotoxic effects on the human prostate cells (PZ-HPV-7 and PNT1A) and the lung fibroblast cell line (V79-4) in a reduction of cell viability.[29] These substances present in the extracts of *Cistus incanus L.* and *Cistus monspeliensis L.* may be beneficial in the treatment of benign prostatic hypertrophy (BPH).[29]

Studies of *C. ladanifer* and *C. populifolius* subspecies analyzed in vitro have confirmed their valuable antioxidant properties as well as cytotoxicity to human tumor cells. *C. populifolius* and *C. ladanifer* extract showed the ability to inhibit the proliferation of M220 pancreatic cancer cells and the breast cancer cells MCF7 / HER2 and JIMT-1.[30] The leaves of these plants are a source of water-soluble polyphenol extracts enriched with ellagitannins with antioxidant activity, and their cytotoxic effect on neoplastic cells deserves further attention.

Figure 2. Herb Cistus creticus L. 27

Another subspecies of *C. creticus ssp.* has also been characterized by cytotoxic activity against tumor cells. Ethanol extracts of *C. creticus ssp*. present in culture inhibit the development of cervical cancer cell lines (HeLa), breast cancer (MDA-MB-453) and melanoma (FemX). It was determined that the agents responsible for this inhibition are present in the diterpenes type labdan purified extract.[31]

The phytochemical studies of extracts from *Cistus libanotis, C. villosus* and *C. monspeliensis,* highlight their antiproliferative activity. When introduced to the culture, extracts from these species show great antiproliferative activity against human melanoma cell lines (A-375) than human breast cancer cells (MCF-7).[32]

El Euch et al. attempted to evaluate the difference in cytotoxicity between leaf extracts and flower buds (FB) of the *Cistus salviifolius* strain. They determined that the FB extract exhibited higher cytotoxic activity against OVCAR and MCF-7 ovarian cancer cells compared to leaves that were inactive at a concentration of 50 mg/L. The extract location was found to significantly affect the composition and biological activity of C. salviifolius.[33]

Studies have reported that extracts from *Cistus incanus L.* and pomegranate (*Punica granatum L.)* which are rich in polyphenolic compounds showed significant antioxidant activity. The addition of *Cistus* to breast cancer cell lines (MCF-7) and colon (LOVO) and addition of pomegranate extracts to both drug-sensitive and drug-resistant (doxorubicin-resistant) tumor cells resulted in apoptosis.[34] A higher proapoptotic effect of extract was observed in drug-sensitive cell lines than in drug-resistant cells. The authors suggest that the extracted could be used by persons exposed to oxidative stress.[34]

Conclusion

The results of scientific research literature presented in this paper characterize *Cistus* species as a medicinal plant with biological activity with emphasis on their antitumor properties. This is due to the presence of polyphenolic compounds such as labdan type diterpens and ellagitannins that may be considered potential therapeutic agents in the treatment of many cancers. However, the use of *Cistus* extracts as a complement to the treatment of human cancers requires further research to thoroughly understand the effects and interactions with recommended medicines.

References

1. Burt S. Essential oils: their antibacterial properties and potential applications in foods- a review. Int J Food Microbiol. 2004;94:223-53.
2. Kazimierczak R, Hallmann E, Sokołowska O, Rembiałkowska E. Zawartość związków bioaktywnych w roślinach zielarskich z uprawy ekologicznej i konwencjonalnej. J Res Appl Agric Engng. 2011;55:200-5.
3. Jeszka M, Faczyk E, Kobus-Cisowska J, Dziedzic K. Związki fenolowe– charakterystyka i znaczenie w technologii żywności. Nauka Przyroda Technologie. 2010;4:1-13.
4. Loizzo MR, Jemia MB, Senatore F, Bruno M, Menichini F, Tundis R. Chimistry and functional properties in prevention of neurodegenerative disorders of five Cistus species essential oils. Food Chem Toxic. 2013;59:586-94.
5. Riehle P, Vollmer M, Rohn S. Phenolic compounds in Cistus incanus herbal infusions - Antioxidant capacity and thermal stability during the brewing process. Food Res Inter. 2013;53:891-9.
6. Morales-Soto A, Oruna-Concha MJ, Elmore JS, et al. Volaile profile of Spanish Cistus plants as sources of antimicrobials for industrial applications. Industrial Crops and Products. 2015;74:425–33.
7. Comandini O, Contu M, Rinaldi AC. An overview of Cistus ectomycorrhizal fungi. Mycorrhiza. 2006;16:381–95.
8. Guzmán B, Vargas P. Systematics, character evolution, and biogeography of Cistus L. (Cistaceae) based on ITS, trnL-trnF, and matK sequences. Molecular Phylogenetics and Evolution. 2005;37:644-60.
9. Catoni R, Gratani L, Varone L. Physiological, morphological and anatomical trait variations between winter and summer leaves of Cistus species. Flora –Morphology, Distribution, Functional Ecology of Plants. 2012;207:442-9.
10. Küpeli E, Yesilada E. Flavonoids with anti-inflammatory and antinociceptive activity from Cistus laurifolius L. leaves through bioassay-guided procedures. J Ethnopharmacol. 2007;112:524-30.
11. Tomás-Menor L, Morales-Soto A, Barrajón-Catalán E, Roldán-Segura C, Segura-Carretero A, Micol V. Correlation between the antibacterial activity and the composition of extracts derived from various Spanish Cistus species. Food Chem Toxic. 2013;55:313-22.
12. Demetzos C, Dimas K, Hatziantoniou S, Anastasaki T, Angelopoulou D. Cytotoxic and anti-inflammatory activity of labdane and cis-clerodane typediterpenes. Planta Medica. 2001;67:614–8.
13. Hannig C, Spitzmüller B, Al-Ahmad A, Hannig M. Effects of Cistus-tea on bacterial colonization and enzyme activities of the in situ pellicle. J Dent. 2008;36:540-5.
14. Hannig C, Sorg J, Spitzmüller B, Al-Ahmad A, Hannig M. Polyphenolic beverages reduce initial bacterial adherence to enamel in situ. J Denti. 2009;37:560-6.
15. Haouat AC, Sqalli H, Farah A, Haggoud A, Iraqui M. Activité antimycobactérienne des extraits de deux espèces marocaines du genre Cistus. Phytotherapie. 2013;11:365–72.
16. Barros L, Dueñas M, Aloes CT, Silva S, Henriques M, Santos-Buelga C, Ferreira ICFR. Antifungal activity and detailed chemical characterization of Cistus ladanifer phenolic extracts. Industrial Crops and Products. 2013;41:41–5.
17. Pomponio R, Gotti R, Santagati NA, Cavrini V. Analysis of catechins in extracts of Cistus species by microemulsion electrokinetic chromatography. J Chromatogr A. 2003; 990:215-23.
18. Toniolo C, Nicoletti M. HPTLC Analyses on Different Populations of Cistus salvifolius L. Austin Chromatography. 2014;1:1-4.
19. Barrajón-Catalán E, Fernández-Arroyo S, Roldán C, et al. A systematic study of the polyphenolic composition of aqueous extracts deriving from several Cistus genus species. Evolutionary relationship. Phytochem Anal. 2011;22:303-12.

20. Rauha JP, Remes S, Heinonen M, et al. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Inter J Food Microbiol. 2000;56:3–12.
21. Middleton EJR, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacological Review. 2000;52:673-751.
22. Swett R. Cistineae: the Natural oreder of cissus, or Rockrose. London, James Ridgeway.1652-1830;1-487.
23. Dietrych-Szóstek D, Burda S. Występowanie i rola przeciwutleniaczy w żywności. Biuletyn Informacyjny, Instytut Uprawy, Nawożenia i Gleboznastwa. 1999;11:18-22.
24. Heinonen IM, Meyer AS. Antioxidant in fruits, berries, vegetables. Friut and vegetable processing– Improving quality. Ed. W. Jongen. Cambridge, Woodhesd Publishing Ltd and CRC Press, LLC;2002.
25. Madsen HL, Andersen CM, Jorgensen LV, Skibsed LH. Radical scavenging by dietary flavonoids. A kinetic study of antioxidant efficiencies. EUR Food Res Technol. 2000;211: 240-6.
26. Szajdek A, Borowska J. Właściwości przeciwutleniające żywności pochodzenia roślinnego. Żywność Nauka Technologia Jakość. 2004;4:5-28.
27. Sarapan Pagi Biblika site. http://www.sarapanpagi.org/tanaman-indah-dalam-alkitab-vt6580-20.html. Accessed March 7, 2016.
28. Dimas K, Demetzos C, Marsellos M, Sotiriadou R, Malamas M, Kokkinopoulos D. Cytotoxic activity of labdane type diterpenes against human leukemic cell lines in vi-tro. Planta Medica. 1998;64:208-11.
29. Vitali F, Pennisi G, Attaguile G, Savoca F, Tita B. Antiproliferative and cytotoxic activity of extracts from Cistus incanus L. and Cistus monspeliensis L. on human prostate cell lines. J Natural Product Research. Formerly Natural Product Letters. 2011;5:188-202.
30. Barrajón-Catalán E, Fernández-Arroyo S, Saura D,et al. Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells. Food Chem Toxicol. 2010;48:2273–82.
31. Skorić M, Todorović S, Gligorijević N, Radulovic S. Cytotoxic activity of ethanol extracts of in vitro grown Cistus creticus ssp. creticus L. on human cancer cell lines. Ind Crop Prod. 2012;38:153-9.
32. Jemia MB, Kchouk ME, Senatore F, et al. Antiproliferative activity of hexane extract from Tunisian Cistus libanotis, Cistus monspeliensis and Cistus villosus. Chem Cent J. 2013;7:47-54.
33. El Euch SK, Bouajila J, Bouzouita N. Chemical composition, biological and cytotoxic activities of Cistus salviifolius flower buds and leaves extracts. Industrial Crops and Products. 2015;76:1100-5.
34. Moreira H, Ślęzak A, Szyjka A, Oszmiański J, Gąsiorowski K. Antioxidant and cancer chemopreventive activities of cistus and pomegranate polyphenols. Acta Poloniae Pharmaceutica - Drug Research. 2017;74:688-98.

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REVIEW PAPER

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Natural and Synthetic Coumarins and their Pharmacological Activity

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ABSTRACT
Coumarins are a structurally diverse group of natural substances derives from plants that display a host of bioactivities. In this paper, we will introduce the reader to coumarins and their applications as medicinal substances. The great diversity in courmarin structure will be discussed along with their extensive use as pharmaceutical agents. Coumarins display a wide range of antimicrobial activity and applications of coumarins as antifungal and antiviral agents will be addressed. Other properties of coumarins such as their role in neuroprotection, anticancer, and as antioxidants will also be reviewed.
Keywords. coumarins, antimicrobial agents, neuroprotection, natural products in medicine

Introduction

Coumarins form an extensive group of natural substances known as secondary metabolites. They are found in over 150 different speciesof plantsbelonging to almost 30 different families.The families containingthe highest content of coumarinsare: *Rutaceae, Clusiaceae, Guttiferae, Caprifoliaceae, Oleaceae, Nyctaginaceae*and*Apiaceae*.[1] Coumarin compounds accumulate in large quantities in fruits (such as citrus fruits), vegetables (eg. celery), roots, flowers and leaves. In smaller quantities they are isolated from bark and stems.[2]

Coumarins, in addition to occurring in vascular plants, are also found in bacteria and fungi such as novobiocin and coumermicin which are known antibiotics synthesized by bacteria. In contrast, *Aspergillus flavus* is a source of aflatoxin, a highly carcinogenic substance with acoumarin ring in its structure.[3]

Structural diversity of coumarins

The structural diversity of natural coumarins is the basis for classifying them into four groups:

1. **coumarin derivatives, e.g.simple coumarin**, compounds formed by two rings: benzene and α-piron. Substituents are often hydroxyl, methoxy and aliphatic groups, at the C7, C6 and C3 positions of benzopyrone (Fig. 1).

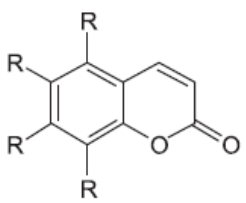

Figure 1. Chemical structure of coumarin

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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2. **isocoumarin derivatives**,formed by benzene rings and α-isopirone. They have substituents in positions C3, C6, C7 and C8 (Fig. 2). They are isolated mainly from fungi: *Artemisia, Aspergilus, Fusarium, Penicillinium, Stremtomyces*and the few plants belonging to families:*Compositae, Leguminoseae*and *Myriaceae*.[4]

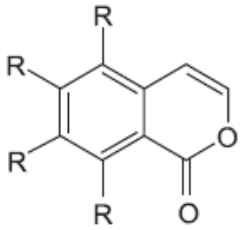

Figure 2. Chemical structure of isocoumarin

3. **furanocoumarin derivatives**, (Fig. 3) formed by the coupling of the coumarin ring with the furan ring at the C6-C7 position (psoralen type, Fig. 3A) or in the C7-C8 position (angelicin type, Fig. 3B).

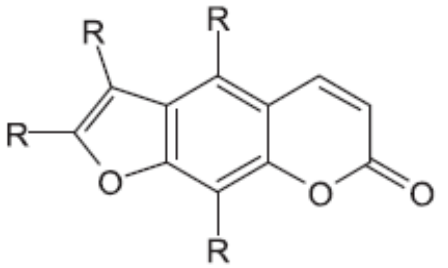

Figure 3. Chemical structure of furanocoumarin

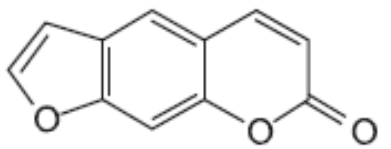

Figure 3A. Psoralentype

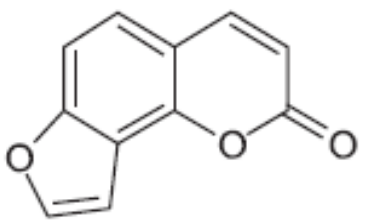

Figure 3B. Angelicintype

4. **pyrancoumarin derivatives**,coumarine ring is condensed with pyran ring (Fig. 4). Ring condensation at the C6-C7 position is defined by the xanthyletin-type (Fig. 4A), or in position C7-C8 a seselin-type (Fig. 4B).

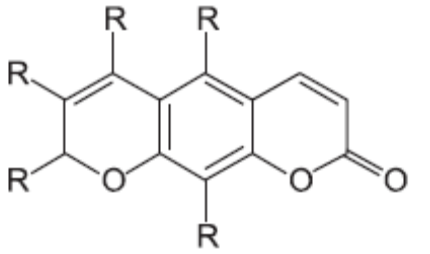

Figure 4. Chemical structure ofpyrancoumarin

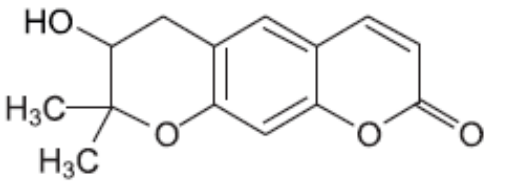

Figure 4A. Xanthyletin-type

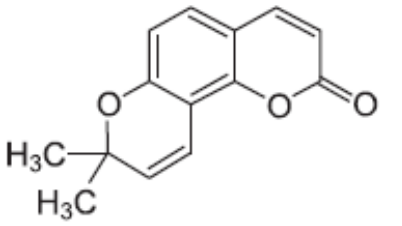

Figure 4B. Seselin-type

Pharmacological Activity of Coumarins

Coumarins are a group of biologically active compounds. They are produced by living organisms (plants, fungi and bacteria) as secondary metabolites. Their activities are, among others, anti-inflammatory, antithrombotic, antimicrobial, antifungal, antiviral (including anti-HIV), anticonvulsant, antioxidant, and antitumor.[1]

Anti-inflammatory activity of coumarins

Coumarins (1,2-benzopyrone) haveanti-inflammatory properties andhavebeen used to treat oedema, helping wound healing. This removes protein and oedemafluid from injured tissue by stimulating phagocytosis and proteolytic enzyme production.[5] Esculetin exhibited anti-inflammatory activityin rat colitis.[6] Also, esculetin inhibits the cyclooxygenase and lipoxygenase enzymes, which results in an anti-inflammatory effect.[7]

Anticoagulant activity of coumarins

Vitamin K is a co-catalyst for the carboxylation reaction of the glutamic acid residue with γ-carboxyglutamic acid. The carboxylation process affects the further normal activity of coagulation factors II, VII, IX and X. Warfarin interferes with the cycle of vitamin K metabolism, resulting in liver deposition of partially carboxylated and decarboxylated proteins. These proteins are characterized

by decreased procoagulant activity.[8] Coumarins interfere with the carboxylation process of C and S protein, causing a procoagulant effect.[1]

It has been shown that the warfarin - coumarin derivative, used as an oral anticoagulant, negatively affects the γ-carboxylation of glutamate residues of bone proteins. As a result of its action in pregnant mothers and those taking warfarin preparation, the fetal skeleton develops abnormally.[8]

Warfarin has shown particularly promising results in the treatment of SCCL (Small Cell Carcinoma Lung) a tumour cell type that is characterised by a coagulation-associated pathway.[9]

Antimicrobial activity of coumarins

Most coumarins have very low antimicrobial activity, but compounds having long chain hydrocarbon substitutions such as ammoresinol and ostruthin have a high activity towards gram(+) bacteria andshow antimicrobial activity on *Bacillus megaterium, Micrococcus luteus, Micrococcus lysodeikticus*and *Staphylococcus auerus.*

Anthogenol, a coumarin derivative isolated from green fruits *Aegle marmelos* (L.),exhibits antimicrobial activity against bacteria of the genus *Enterococcus*.[1] Imperatorinshows high activity towards *Shigella dysenteriae*.[10]

Pyranocoumarins such as grandivittin, agasyllin, aegelinol benzoate and osthole isolated from the root *Ferulagocampestris*(Besser) Grecescu(*Apiaceae*) show activity towards both gram(+) andgram(-)bacteria, for example on *Staphylococcus aureus, Salmonella typhi, Enterobacter cloacae, Enterobacter aerogenes*and *Helicobacter pylori.*[11]

Coumarins are mainly isolated from higher plants, but some of them have been discovered in microorganisms. Examples include novobiocin, coumermycin, and chartreusin. Novobiocin, a secondary metabolite of *Streptomyces niveus*and *Streptomyces spheroides*exhibit very high activity towards gram(+) organisms such as *Corinebacterium diphtheria, Staphylococcus aureus, Streptomyces pneumoniae, Streptomyces pyogenes*andgram(-) organisms such as *Haemophillusinfluenzae, Neisseria meningitides* and*Pasteurella*. Coumermycin, structural similar to novobiocin, exhibits almost 50 times more potency againstbacteria belonging to *Escherichia coli* strains and *Staphylococcus aureus*strains. Alsochartreusin, isolated from *Streptomyces chartreusis*,shows activity towards gram(+) bacteria, but due to its toxicity, chartreusin has not been tried for treatment.[1] Antituberculous activity against *Mycobacterium tuberculosis*are found inskopoletin, umbelliferone, phellodenol A, marmezin and xanthyletin.[12]

Antifungal activity of coumarins

The broad spectrum of antifungal activity is shown byosthol, a derivative of coumarin isolated from celery plants. This derivative demonstrates activity against *Rhizoctoniasolani* [Kühn], *Phytophthoracapsici* [Leonian], *Botrytis cinerea* [Pers.], *Sclerotiniasclerotiorum* [de Bary] and *Fusarium graminearum* [Patch].[13] A number of coumarins have been tested for antifungal activity, and the three most effective ones are psoralen, imperatorin, and ostruthin.[14]

Antiviral activity of coumarins

Coumarin derivatives can also have a reverse transcriptase (RT) inhibitory effectand two isomers (+)-calanolide A and (-)-calanolide B isolated from*Calophyllumlanigerum*(*Clusiaceae*), belong to pyranocoumarin, inhibit RT activityandcompletely deactivate the replication process of HIV-1.[15,16] Others coumarin derivatives – inophyllum B i P obtained from an giant African snail *Achatinafulica*[Férussac]significantly inhibit RT activity in HIV-1 cell cultures.[17]

Aminomethyltrimethyl psoralen (AMT) is usedas a photo-sterilizing agent and is added to blood products followed by exposure to UVA radiation. AMT has inactivated DNA and RNA viruses.[18] Sancho et al. showed that imperatorin also inhibits either vesicular stomatitis virus or gp-160-enveloped recombinant HIV-1 infection in several T-cell lines and in HeLa cells.[19]

Antihypertensive activity of coumarins

Scopoletin, a coumarin isolated from the fruits of *Tetrapleuratetraptera* (*Mimosaceae*), in laboratory animals shows a smooth muscle relaxant effect onblood vessels resulting in a drop in blood pressure. A similar action wasshown by dihydromammea; a coumarin isolated from the seed of the tree *Mammeaafricana* [L.] (*Guttiferae*).[1]

Visnadine – pyranokumarin, an active ingredient extracted from the fruit of *Ammi visnaga*, exhibited peripheral and coronary vasodilator activities. It is used adjunctively to treat angina pectoris.[20]

Antioxidant activity of coumarins

Coumarin antioxidant activity is manifested by the ability to inhibit reactive oxygen species(ROS) and to capture them. Studies in rats have shown that the inhibition of xanthine oxidase - the enzyme responsible for xanthine biosynthesis, is directly proportional to the amount of hydroxyl groups that are contained withinthe molecule.[21]

Coumarin compounds can directly affect the properties of antioxidant enzymes.Łuczajet al. have demonstrated the effect of coumarin on superoxide dismutase, catalase, and glutathione peroxidase activity in plasma, liver, kidney and brain of rats.[22,23] Following administration of esculetin and 7-hydroxycoumarin in mice, increased levels of vitamin E, vitamin C and glutathionewere found.[24] It has been shown that fraxin in 0.5 mM concentration protects human umbilical vein endothelial cells (HUVEC) against oxidative stress caused by hydrogen peroxide.[25]

The antioxidative and cytopreparative character of fraxetin has been demonstrated. It effectively prevents

oxidative stress-induced apoptosis of neuroblastoma cells-which can be used in the treatment of Parkinson's disease and other neurodegenerative diseases.[26]

Neuroprotective activity of coumarins

Alzheimer's disease (AD) is a degenerative and progressive neurological disorder. It is characterized by variable levels of cholinergic enzymes and the formation of senile plaques containing β-amyloid proteins in cerebral tissue. In patients with Alzheimer's disease is observed decreased or unchanging levels of acetylcholinesterase (AChE), level of second enzyme butyrylcholinesterase (BChE) increase. Therefore, the levels of AChE and BChE enzymes are considered to be crucial in the treatment of this disease. Orhanet al.have demonstrated significant inhibition of acetyl- and butyrylcholinesterase levels after application with bergapten, xanthotoxin, scopoletin, umbelliferone, and 4-methyloumbelliferone.[27]

Recent computer techniques have allowed the design of an amine-substituted coumarin derivative.The synthesized compound 3-(4-{[benzyl(ethyl)amino]methyl} phenyl)-7-[4-(diethylamino)butoxy]-2H-chromen-2-one exhibits neuroprotective activity, expressed in AChE inhibition, and is a potential candidate for Alzheimer's disease treatment.[28]

Ostholepresent among others in *Cnidiummonnieri* (L.) fruits is a commonly used substance in traditional Chinese medicine. Chen et al. investigated the effect of osthole on the demyelination process in the central nervous system of mice in an experimental model of multiple sclerosis.[29] The results showed that osthole delayed disease progression and could find use in the treatment of multiple sclerosis.[29]

The use of coumarins in the treatment of skin diseases and of the hematopoietic system diseases

Therapeutic use has been found for two furanocoumarin derivatives 5-MOP (5-methoxypsoralen) as an N-acetyltransferaseinhibitor and 8-MOP (8-methoxypsoralen)in phototherapy for psoriasis and vitiligo.

In treatment of the skin disordersvitiligo, psoriasis and atopic inflammation,bergapten has also been successful.[30,31] Human keratinocytes of the NCTC-2544 line were exposed to bergaptene and xanthotoxinand exposed to UVA light, which resulted in cell cycle inhibition in G1 phase and increase in cellular apoptosis level.[32]

Very effective psoriasis treatment was achieved using xanthotoxin and the PUVA method which involves administering xanthotoxin gel directly onto the patient's skin and then irradiating with UVB light.[33,34]

The Jurkat cell line (T-cell leukemia line) and normal lymphocytes were exposed to 8-MOP and then exposed to UVA light. There was a marked induction of apoptosis and a significant increase in caspases: 8 and 9 (initiator caspases) and 3 and 7 (effector caspases).[35] This method, called photophoresis, which uses extracorporeal irradiation of blood cells previously exposed to 8-MOP, has been implicated in therapy forautoimmune diseases, such as T-cell lymphoma.[36] Photophoresis increases apoptosis in lymphocytes, causing them to die and induce the formation of postapoptotic vesicles with anti-inflammatory properties.[37] Another feature of xanthotoxin used in vitiligo treatment is the ability to induce skin repigment. Coumarin increases the intracellular concentration of calcium ions and affects the organization of actin fibers in the cytoskeleton of melanocytes, which in turn leads to their migration.[38]

Anticancer activity of coumarins

In research on GLC_4 (small cell lung carcinoma) and COLO 320 (colorectal cancer) cell lines, it has been shown that the cytotoxity of coumarin is due to the presence of at least two phenolic groups at the 6,7- or 6,8-position in the ring of the molecule.[39] The proliferation of the 786-O and A-498 (kidney cancer) and DU145 and LNCaP (prostate cancer) cells line were inhibited by coumarin and its hydroxyl derivative, umbelliferone.[40,41]

Several hydroxylated and methoxylatedcoumarin derivatives were tested for their relative cytotoxicity on four human HSC-2 tumor cell lines, HSC-3 (oral squamous cell carcinoma), A-375 (melanoma) and HL-60 (promyelocytic leukemia). It has been shown that the cytotoxicity of 6,7-dihydroxycoumarin towards HL-60 tumor cells can be further enhanced by substituting the -OH in 3 and/or position 4.[42] Similar conclusions were made by Budzisz et al.byQSAR regression analysis of the relationship between biological activity and physicochemical properties of test compounds. The cytotoxic effect increases with increasing hydrophobic substituents in 2, 3 and 4 positions of the benzopyrene ring.[43]

The cytotoxic activity of organometallic coumarin complexes (umbelliferone, mendiaxon, warfarin, coumachlor, and niffcoumar) towards P3HR1, K-562 and THP-1 leukemia cells lines were confirmed.[44]Nitrocoumarin derivatives of 7-hydroxy-6-nitrocoumarin and 7-hydroxy-3,6,8-trinitrocoumarin exhibited cytotoxic activity against tumor cells of the melanocytic line (SK-MEL-31).[41] On the other hand, 8-nitro-7-hydroxycoumarin induced apoptosis of leukemic cell lines K562 and HL-60.[45]

Coumarin derivatives exhibit specific cytotoxicity, which is closely related to the chemical structure of their molecules.[43] Attemptshave been made to synthesize a coumarin-like compound with selective and targeted action on tumor cells. Extremely cytotoxic heterocyclic coumarin derivativeswhich have 1,2,4-triazole, 4,5-dicyanoimidazole or purine groups have been obtained. In addition, the 1,2,4-triazole-3-carboxamide derivative exhibited particular selectivity to HeLa human epithelial

cells (cervical cancer).[44] In contrast, the presence of the 2-amino-6-chloropurine group conditioned the cytostatic effects on HepG2 (hepatoma) and SW620 (colon) cells line, leading to mutations in the p53 gene.[46]

Osthole stops proliferation of human breast cancer cells MCF-7 and MDA-MB231 by inhibiting metalloproteinases in the outer cell matrix, slowing down the migration and further invasion of tumor cells.[47,48]

Grandivittin, agasyllin, aegelinol benzoate and felamidin, four natural coumarins isolated from *Ferulagocampestris* (*Apiaceae*), and several synthetic ester derivatives of aegelinol were tested against four tumor cell lines. Some of them were shown to be marginally cytotoxic against the A549 lung cancer cell line.[49] From*Canophyllumdispar* (*Clusiaceae*), eight 4-phenylfuranocoumarin derivatives were isolated that showed significant cytotoxicity to cervical cancer (KB).[50]

Panno et al. stimulated bergapten on breast cancer cells MCF-7 (human adenocarcinoma cell line) and SKBR-3 (malignant breast cancer cell line). Bergapten, independently of photoactivation, caused cell cycle arrest in G0 / G1 phase, inserting breast cancer cells into the pathway of apoptosis, and counteracting the stimulating effect of IGF-I/E2 on MCF-7 cell line growth.[51] Further study of the team, conducted on human breast cancer cells MCF-7, ZR-75 and SKBR-3, confirmed the antiproliferative and apoptotic effects of bergapten and its UV-activated derivative.[52] Molecular studies of mammary gland cells have determined the function of the membrane estrogen receptor α (ERα). ERα is involved in the normal development of the mammary gland as well as in the tumor-resistant MCF-7 breast cancer resistant to tamoxifen. Stimulation with bergapten causes ERα to decrease with anti-tumor and mitogenic effects.[53] Recent studies show that bergapten induces the metabolic reprogramming of breast cancer cells MCF-7 and ZR75. Therapy with bergapten causes changes in metabolic pathways, inducing cell death.[54]

Xanthoxylethin isolated from *Erythrinvariegata* [L.], stimulated gastric cancer cells of the SGC-7901 line, induced apoptosis and cell cycle arrest. It was noted that this action was associated with DNA damage. The process of apoptosis in cells was caused by mitochondrial damage and the cell cycle was stopped in phase S.[55]

In cancer therapy, a very important issues are angiogenesis, i.e. the formation of blood vessels within the tumor and metastasis.The 7-diethylaminocoumarin derivatives exhibited activity as angiogenesis inhibitors towards to tumor cells and were highly selective to normal HUVEC (human umbilical vein endothelial cells).[56] In other studies, the synthetic brominated coumarin derivative showed cytotoxic and anti-proliferative effects on EAC (Ascitic Carcinoma) and DLA (lymphoma) carcinoma cell lines. Inhibition of blood vessel formation and stimulation of apoptosis has also been observed.[57]

It has been observed that combination therapy with dicumarol, coupled with a chemotherapeutic agent, can improve efficacy and reduce toxicity compared to coumarin alone. The use of the dicumarol with taxol complex has antiproliferative effects on the hedgehog larvae (*Strongylocentrotuspurpuratus*) [Stimpson]. The positive result was explained by the synergism of the cytostatic and dicumarol. The authors suggest that the future of the development of combined pharmacotherapy may be the basis of modern chemotherapy.[58]

It has been found that coumarins eaten in human diet can positively affect the body. Observations indicate that even if present at low levels in apiaceous vegetables, imperatorin, trioxsalen and isopimpinellin may contribute significantly to CYP1A2 inhibition and potentially decreased procarcinogen activation.[59]

Conclusion

Coumarins are a large group of biologically active compounds commonly used in natural medicine.

References

1. Venugopala KN, Rashmi V, Odhav B. Review on Natural Coumarin Lead Compounds for Their Pharmacological Activity. Bio Med Res Int. 2013;963248:1-14.
2. Kohlmünzer S. Farmakognozja. Podręcznik dla studentów farmacji. Wydawnictwo Lekarskie PZWL;2000.
3. Borges F, Roleira F, Milhazes N, Santana L, Uriarte E. Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity. CurrMed Chem. 2005;12:887-916.
4. Superchi S, Phi D, Salvadori P, et al. Synthesis and Toxicity to Mammalian Cells of the Carrot Dihydroisocoumarins. Chem Res Toxicol. 1993;6:46-9.
5. Piller NB. A comparison of the effectiveness of some anti inflammatory drugs on thermal oedema. Brit J ExperPath. 1975;56:554–60.
6. Witaicenis A, Seito LN, Di Stasi LC. Intestinal anti-inflammatory activity of esculetin and 4-methylesculetin in the trinitrobenzenesulphonic acid model of rat colitis. ChemBiol Interact. 2010;186:211–8.
7. Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nicolaides DN. Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities. Curr Pharm Design. 2004;10:3813–33.
8. Hirsh J, Dalen JE, Anderson DR, et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest. 1998;119:8–21.
9. Lacy A, O'Kennedy R. Studies on Coumarins and Coumarin-Related Compounds to Determine their Therapeutic Role in the Treatment of Cancer. Curr. Pharm. Design. 2004;10:3797-811.
10. Raja SB, Murali MR, Roopa K, Devaraj SN. Imperatorin a furocoumarin inhibits periplasmic Cu-Zn SOD of *Shigelladysenteriae*their by modulates its resistance towards

phagocytosis during host pathogen interaction. Biomed Pharmacother. 2011;5:560-8.
11. Basile A, Sorbo S, Spadaro V, et al. Antimicrobial and Antioxidant Activities of Coumarins from the Roots of *Ferulagocampestris* (Apiaceae). Molecules 2009;14:939-52.
12. Chiang CC, Cheng MJ, Peng CF, Huang HY, Chen IS. A novel dimeric coumarin analog and antimycobacterial constituents from *Fatouapilosa*. ChemBiodivers. 2010;7:1728-36.
13. Wang CM, Zhou W, Li CX, Chen H, Shi ZQ, Fan YJ. Efficacy of osthol, a potent coumarin compound, in controlling powdery mildew caused by *Sphaerothecafuliginea*. J Asian Nat Prod Res. 2009;11:783-91.
14. Bourgaud F, Hehn A, Larbat R, et al. Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzymes. PhytochemRev. 2006;5:293–308.
15. McKee TC, Fuller RW, Covington CD, et al. New pyranocoumarins isolated from *Calophyllumlanigerum* and *Calophyllumteysmannii*. J Nat Prod. 1996;59:754-8.
16. Spino C, Dodier M, Sotheeswaran S. Anti-HIV coumarins from calophyllum seed oil. Bioorg Med Chem Lett. 1998;8:3475–8.
17. Patil AD, Freyer AJ, Eggleston DS, et al. The inophyllums, novel inhibitors of HIV-1 reverse transcriptase isolated from the Malaysian tree, *Calophylluminophyllum* Linn. J Med Chem. 1993;36:4131–8.
18. Margolis-Nunno H, Robinson R, Ben-Hur E, Chin S, Orme T, Horowitz B. Elimination of potential mutagenicity in platelet concentrates that are virally inactivated with psoralens and ultraviolet A light. Transfusion 1995;35:855-62.
19. Sancho R, Marquez N, Gomez-Gonzalo M, et al. Imperatorin inhibits HIV-1 replication through an Sp1-dependent pathway. J Biol Chem. 2004;279:37349–59.
20. Iranshahi M, Askari M, Sahebkar A, Hadjipavlou-Litina D. Evaluation of antioxidant, anti-inflammatory and lipoxygenase inhibitory activities of the prenylatedcoumarinumbelliprenin. DARU J Pharm Sci. 2009;17:99–103.
21. Lee BC, Lee SY, Lee HJ, et al. Anti-oxidative and photo-protective effects of coumarins isolated from *Fraxinuschinensis*. Arch Pharm Res. 2007;30:1293-301.
22. Łuczaj W, Jarocka-Karpowicz I, Bielawska K, Skrzydlewska E. Sweet grass protection against oxidative stress formation in the rat brain. Metab Brain Dis. 2015;30:183–90.
23. Łuczaj W, Stankiewicz-Kranc A, Milewska E, Roszkowska-Jakimiec W, Skrzydlewska E. Effect of sweet grass extract against oxidative stress in rat liver and serum. Food ChemToxicol. 2012;50:135-40.
24. Martin-Aragòn S, Benedi JM, Villar AM. Effects of the antioxidant (6,7-dihydroxycoumarin) esculetin on the glutathione system and lipid peroxidation in mice. Gerontology. 1998;44:21-5.
25. Whang WK, Park HS, Ham I, et al. Natural compounds, fraxin and chemicals structurally related to fraxin protect cells from oxidative stress. ExpMol Med. 2005;37:436–46.
26. Molina-Jiménez MF, Sánchez-Reus MI, Andres D, Cascales M, Benedi J. Neuroprotective effect of fraxetin and myricetin against rotenone-induced apoptosis in neuroblastoma cells. Brain Res. 2004;1009:9-16.
27. Orhan I, Tosun F, Sener B. Coumarin, anthroquinone and stilbene derivatives with anticholinesterase activity. Z Naturforsch C 2008;63:366-70.
28. Montanari S, Bartolini M, Neviani P, et al. Multitarget strategy to address Alzheimer's Disease: design, synthesis, biological evaluation, and computational studies of coumarin-based derivatives. Chem Med Chem. 2016;11:1296-308.
29. Chen X, Pi R, Zou Y, et al. Attenuation of experimental autoimmune encephalomyelitis in C57 BL/6 mice by osthole, a natural coumarin. Eur J Pharmacol. 2010;629:40–6.
30. da Silva VB, Kawano DF, Carvalho I, da Conceição EC, de Freitas O, da Silva CH. Psoralen and bergapten: in silico metabolism and toxicophoric analysis of drugs used to treat vitiligo. J Pharm Pharm Sci. 2009;12:378-87.
31. Lohr C, Raquet N, Schrenk D. Application of the concept of relative photomutagenic potencies to selected furocoumarins in V79 cells. Toxicol In Vitro 2010;24:558-66.
32. Viola G, Fortunato E, Cecconet L, Del Giudice L, Dall'Acqua F, Basso G. Central role of mitochondria and p53 in PUVA-induced apoptosis in human keratinocytes cell line NCTC-2544. ToxicolApplPharmacol. 2008;227:84-96.
33. Asawanonda P, Amornpinyokeit N, Nimnuan C. Topical 8-methoxypsoralen enhances the therapeutic results of targeted narrowband ultraviolet B phototherapy for plaque-type psoriasis. J EurAcadDermatolVenereol. 2008;22:50-5.
34. SeckinD,UstaI,Yazici Z, Senol A. Topical 8-methoxypsoralen increases the efficacy of narrowband ultraviolet B in psoriasis. PhotodermatolPhotoimmunolPhotomed. 2009;25:237-41.
35. Miyazaki M, Yamazaki H, Takeuchi H, Kamataki T. Mechanisms of chemopreventive effects of 8-methoxypsoralen against 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone- induced mouse lung adenomas. Carcinogenesis. 2005;11:1947-55.
36. Bissaccia E, Vonderheid EC, Geskin L. Safety of a new, single, integrated, closed photopheresis system in patients with cutaneous T-cell lymphoma. Br J Dermatol. 2009;161:167-9.
37. Stadler K, Frey B, Munoz LE, et al. Photopheresis with UV-A light and 8-methoxypsoralen leads to cell death and to release of blebs with anti-inflammatory phenotype in activated and non-activated lymphocytes. BiochemBiophys Res Commun. 2009;386:71-6.
38. Zhang XQ, Zheng M, Mou KH, Feng J. Effects of 8-Methoxypsoralen on intracellular Ca(2+)i and cytoskeleton actin organization in human melanocytes in vitro. J Zhejiang Univ Med Sci. 2009;38:348-51.
39. Kolodziej H, Kayser O, Woerdenbag HJ, van Uden W, Pras N. Structure-cytotoxicity relationships of a series of natural and semi-synthetic simple coumarins as assessed in two human tumour cell lines. Z NaturforschC. 1997;52:240-4.

40. Myers RB, Parker M, Grizzle WE. The effects of coumarin and suramin on the growth of malignant renal and prostatic cell lines. J Cancer Res ClinOncol. 1994;120:S11-3.
41. Bielawska K, Malinowska M, Cyuńczyk M. Wpływ kumaryn na organizm człowieka. BromatChemToksykol. 2014;2: 213–21.
42. Kawase M, Sakagami H, Hashimoto K, Tani S, Hauer H, Chatterjee SS. Structure-cytotoxic activity relationships of simple hydroxylated coumarins. Anticancer Res. 2003;23:3243-6.
43. Budzisz E, Brzezinska E, Krajewska U, Rozalski M. Cytotoxic effects, alkylating properties and molecular modelling of coumarin derivatives and their phosphonic analogues. Eur J Med Chem. 2003;38:597-603.
44. Manolov I, Kostova I, Netzeva T, Konstantinov S, Karaivanova M. Cytotoxic activity of cerium complexes with coumarin derivatives. Molecular modeling of the ligands. Arch Pharm. 2000;333:93-8.
45. Egan D, James P, Cooke D, O'Kennedy R. Studies on the cytostatic and cytotoxic effects and mode of action of 8-nitro-7-hydroxycoumarin. Cancer Lett. 1997;118:201-11.
46. Benci K, Mandić L, Suhina T, et al. Novel coumarin derivatives containing 1,2,4-triazole, 4,5-dicyanoimidazole and purine moieties: synthesis and evaluation of their cytostatic activity. Molecules 2012;17:11010-25.
47. Yang D, Gu T, Wang T, Tang Q, Ma C. Effects of osthole on migration and invasion in breast cancer cells. BiosciBiotechBiochem.2010;74:1430-4.
48. Zhang ZR, Leung WN, Cheung HY, Chan CW. Osthole: A review on its bioactivities, pharmacological properties, and potential as alternative medicine. Evid Based Complement Alternat Med. 2015;2015:919616.
49. Rosselli S, Maggio AM, Faraone N, et al. The cytotoxic properties of natural coumarins isolated from roots of *Ferulagocampestris* (Apiaceae) and of synthetic ester derivatives of aegelinol. Nat Prod Commun. 2009;4:1701-6.
50. Guilet D, Helesbeux JJ, Seraphin D, Sevenet T, Richomme P, Bruneton J. Novel cytotoxic 4-phenylfuranocoumarins from *Calophyllumdispar.* J Nat Prod. 2001;64:563-68.
51. Panno ML, Giordano F, Palma MG, et al. Evidence that bergapten, independently of its photoactivation, enhances p53 gene expression and induces apoptosis in human breast cancer cells. Curr Cancer Drug Targets 2009;9:469-81.
52. Panno ML, Giordano F, Mastroianni F, et al. Breast cancer cell survival signal is affected by bergapten combined with an ultraviolet irradiation. FEBS Lett. 2010;584: 2321-6.
53. Panno ML, Giordano F, Rizza P, et al. Bergapten induces proteasome-dependent degradation of ER in breast cancer cells: Involvement of SMAD4 in the ubiquitination process. Breast Cancer Res Treat. 2012;136:443–55.
54. Santoro M, Guido C, De Amicis F, et al. Bergapten induces metabolic reprogramming in breast cancer cells. Oncol Rep. 2016;35:568-76.
55. Rasul A, Khan M, Yu B, Ma T, Yang H. Xanthoxyletin, a coumarin induces S phase arrest and apoptosis in human gastric adenocarcinoma SGC-7901 cells. Asian Pac J Cancer Prev. 2011;12:1219-23.
56. Lee S, Sivakumar K, Shin WS, Xie F, Wang Q. Synthesis and anti-angiogenesis activity of coumarin derivatives. Bioorg Med Chem Lett. 2006;16:4596-9.
57. Vijay Avin BR, Thirusangu P, Lakshmi Ranganatha V, Firdouse A, Prabhakar BT, Khanum SA. Synthesis and tumor inhibitory activity of novel coumarin analogs targeting angiogenesis and apoptosis. Eur J Med Chem. 2014;75: 211-21.
58. Madari H, Panda D, Wilson L, Jacobs RS. Dicoumarol: a unique microtubule stabilizing natural product that is synergistic with Taxol. Cancer Res. 2003;63:1214-20.
59. Kang AY, Young LR, Dingfelder C, Peterson S. Effects of furanocoumarins from apiaceous vegetables on the catalytic activity of recombinant human cytochrome P-450 1A2. Protein J. 2011;30:447-56.

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