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35-959 Rzeszów, ul. prof. S. Pigoń 6,
tel. 17 872 14 26, tel./fax 17 872 14 26
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Łukasz Ożóg ^{1(ABGF)}, Jacek Tabarkiewicz^{2,3(ABGF)}, David Aebisher ^{2(ABGF)}

Chemiluminescence-driven Dye Excitation for Dark Photodynamic Therapy

¹ Department of Biophysics, Faculty of Mathematics and Natural Sciences, University of Rzeszów, Poland

² Department of Human Immunology, Faculty of Medicine, University of Rzeszów, Poland

³ Center for Innovative Research in Medical and Natural Sciences,
University of Rzeszów, Poland

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 ($^1\text{O}_2$), 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 $^1\text{O}_2$ which reacts with cell molecules ultimately resulting in tissue damage and cell death. The mechanism of $^1\text{O}_2$ formation in these system is energy transfer from excited PS to ground state oxy-

Corresponding author: David Aebisher, e-mail: daebisher@ur.edu.pl

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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 ($< 1 \mu\text{s}$) in biological tissue thus for photodynamic therapy it is desirable to generate $^1\text{O}_2$ 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 apoptosis.¹ Photosensitizers cannot generate ROS in the absence of light or other excitation energy source and ideally PS is nontoxic to healthy 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 (^1PS) to the first excited state singlet ($^1\text{PS}^*$) can be accomplished by excitation with external visible light or by chemiluminescence. Intersystem crossing (ISC) to the first excited state triplet ($^3\text{PS}^*$) followed by collisional energy transfer to ground state oxygen ($^3\text{O}_2$) generates cytotoxic singlet oxygen ($^1\text{O}_2$) 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 OPV with the negatively charged oxidized luminol dianion placed the donor and acceptor in close proximity for chemiluminescence resonance energy transfer (CRET). Excitation of OPV was achieved as luminol chemiluminescence has a maximum at 425 nm and OPV has an

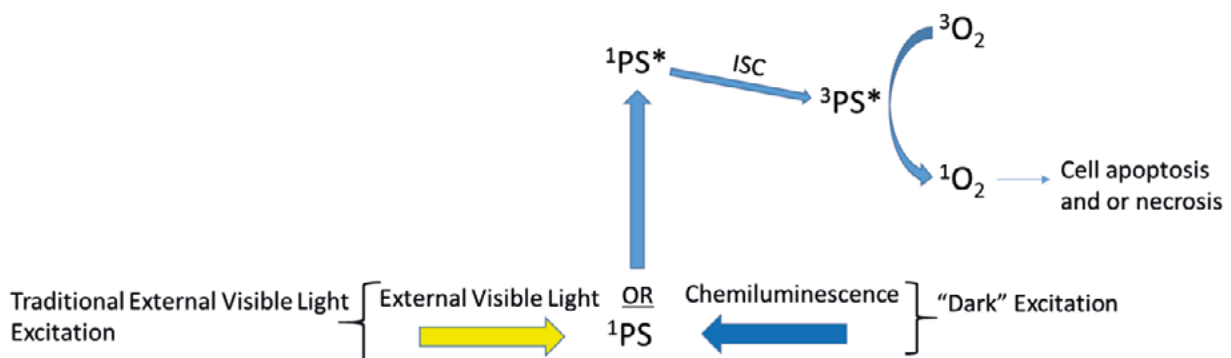


Figure 1. Photosensitizer (PS) excitation from the ground state (^1PS) to the 1^{st} excited state singlet ($^1\text{PS}^*$) by excitation with external visible light or, alternatively, by chemiluminescence. Intersystem crossing (ISC) to the first excited state triplet ($^3\text{PS}^*$) followed by energy transfer to ground state oxygen ($^3\text{O}_2$) generates cytotoxic singlet oxygen ($^1\text{O}_2$)

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 $^1\text{O}_2$.

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 chemiluminescence-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 a 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 $\mu\text{g mL}^{-1}$.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 individually showed minimal apoptosis 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 $^1\text{O}_2$ 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/acceptor

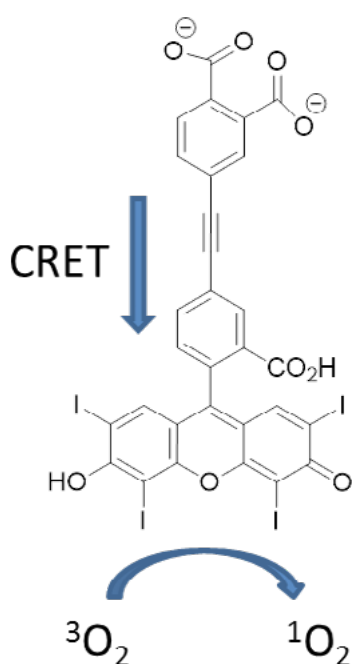


Figure 2. A system developed by Akkaya et al. for excitation of pendant tetraiodoxanthene and subsequent generation of $^1\text{O}_2$ via through-bond CRET

tor conjugate with hydrogen peroxide is advantageous since this may also occur readily in cancer cells.¹¹ Generation of $^1\text{O}_2$ 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.

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REVIEW PAPER

Zuzanna Bober ^{1(ABGF)}, David Aebisher  ^{2(ABF)}, Jacek Tabarkiewicz ^{1,2(F)}, Wiesław Guz  ^{1,3(F)},
Piotr Tutka ^{1,4(F)}, Dorota Bartusik-Aebisher  ^{4(ABF)}

Investigation of pharmaceuticals by nuclear magnetic resonance imaging and spectroscopy

¹ Center for Innovative Research in Medical and Natural Sciences, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

² Department of Human Immunology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

³ Department of Electroradiology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

⁴ Department of Experimental and Clinical Pharmacology, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

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

Corresponding author: Dorota Bartusik-Aebisher, e-mail: dbartusik-aebisher@ur.edu.pl

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

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 process 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 $\gamma=42,58 \text{ s}^{-1}\text{T}^{-1}$. 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 ^1H 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 (Stimulated Echo Acquisition 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 (Stimulated Echo Acquisition 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 development²²⁻²³ and drug release²⁴⁻²⁸ 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

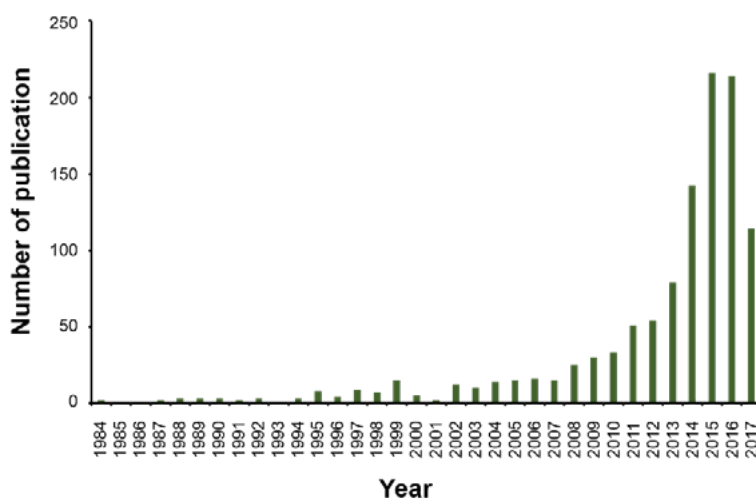


Figure 1. Publications on MRI Applications in Pharmacy

understanding the processes that occur in drug metabolism. This may have a significant impact on the development of a new generation of pharmaceuticals²⁹. 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

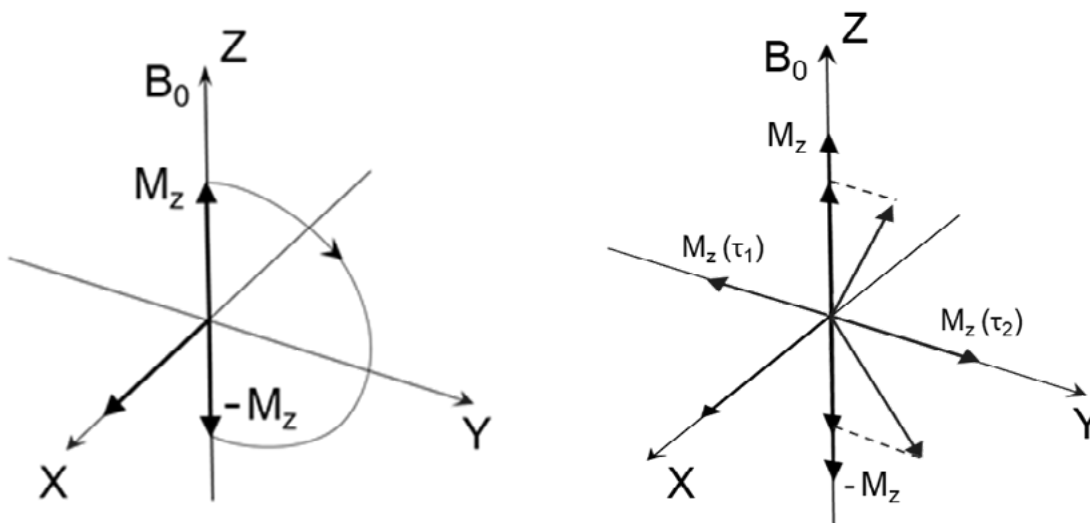


Figure 2. The MRI phenomenon

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 $MZ = -M_0$. The T_1 relaxation makes the magnetization increase during time interval from $-M_0$

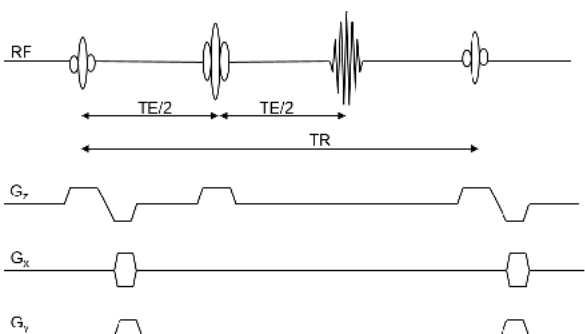


Figure 3. Partial or progressive saturation in a 2D Fourier Transform spin-echo pulse sequence

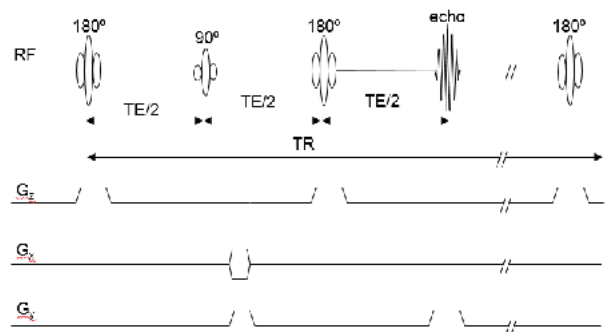


Figure 4. Inversion Recovery

throughout zero until it is back to original value $MZ = M_0$. 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 θ 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.

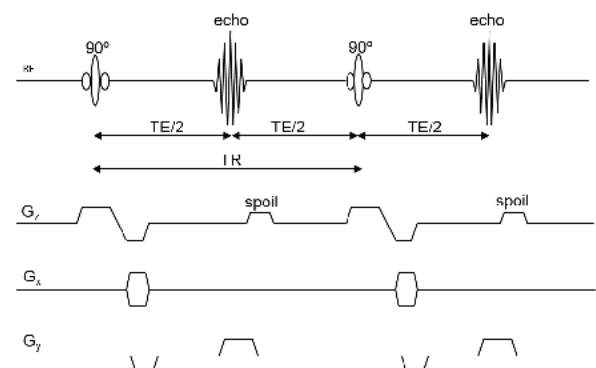


Figure 5. Variable tip angle, 2D Fourier transform gradient recalled echo pulse sequence

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

This assumes perfect 90° pulses. Also to eliminate diffusion effects, a plot of $1/T_{1\text{eff}}$ against TE2 yields a straight line whose intercept at TE = 0 is $1/T_1 - T_{1\text{eff}}$ 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 $\text{SI} = S_0 \exp(\text{TM}/T_1)$. 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 improved by the SNR by changing the flip angle of the second pulse to 180 degree. It 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, measurements of the variation between subjects and assessment of the extent of brain temperature variation.⁵⁵ MRSI also provides 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 acqui-

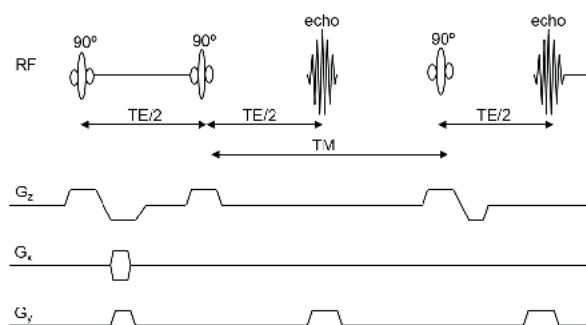


Figure 6. STE sequence

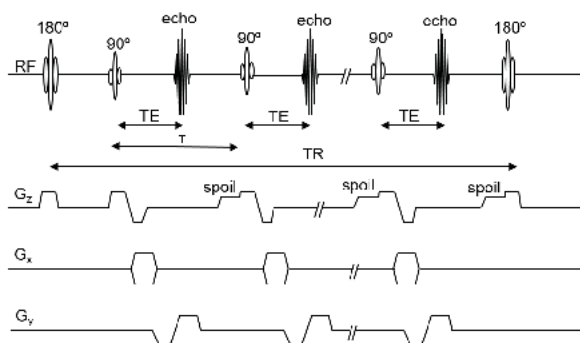


Figure 7. Multiple gradient echo

sition 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

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 <i>in vitro</i>		
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. <i>et al.</i> / 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. <i>et al.</i> / 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. <i>et al.</i> / ⁷¹	phantom: GdCl ₃ concentration was varied from 0–140 μ mol/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 <i>et al.</i> / 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 μ mol/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. <i>et al.</i> / 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. <i>et al.</i> / 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 poly-lactide-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.

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REVIEW PAPER

Zuzanna Bober ^{1(ABGF)}, David Aebisher  ^{2(ABF)}, Łukasz Ożóg  ^{3(F)},
Jacek Tabarkiewicz ^{1,2(F)}, Piotr Tutka ^{1,4(F)}, Dorota Bartusik-Aebisher  ^{4(ABF)}

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

¹ Center for Innovative Research in Medical and Natural Sciences, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

² Department of Human Immunology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

³ Department of Biophysics, Faculty of Mathematics and Natural Sciences, University of Rzeszow, Rzeszow, Poland

⁴ Department of Experimental and Clinical Pharmacology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

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 ¹⁹F 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 ¹⁹F MRI biomarkers. Molecules with ¹⁹F substituents are particularly attractive for use in drug tracking by ¹⁹F MRI due to 100% ¹⁹F abundance, high ¹⁹F MRI sensitivity (0.83 relative to ¹H MRI) and an impressively large chemical shift range (400 ppm). Another benefit in the use of ¹⁹F MRI is a zero background signal in biological samples due to lack of endogenous fluorine. Therefore, drugs containing fluorine atom have potential for ¹⁹F MRI imaging drug delivery to tissue. This article will review recent developments in the use of ¹⁹F 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 (¹⁹F) Magnetic Resonance Imaging and Spectroscopy, ¹⁹F relaxation time, contrast agents, drug delivery, oxygen concentration in tumor tissue and individual cells. Our interest is in review of ¹⁹F 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 ¹⁹F MRI techniques, so that an anti-tumor drug containing ¹⁹F can not only be delivered to the cell, but also released inside the cell itself and constantly monitored. Current drug targeting research shows possibility

Corresponding author: Dorota Bartusik-Aebisher, e-mail: dbartusik-aebisher@ur.edu.pl

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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 ^1H 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 a quantitative way of *in vitro* imaging high ^{19}F MRI sensitivity (0.83 relative to ^1H 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 a 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.

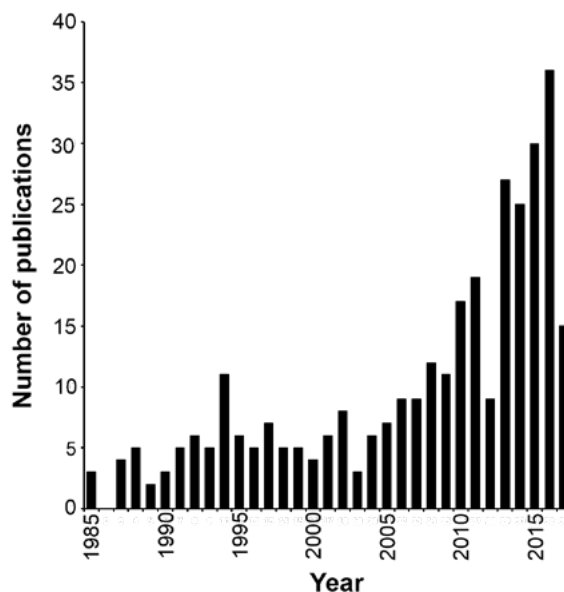


Figure 1. Number of publications on ^{19}F MRI collected from Library of National Center for Biotechnology Information (NCBI) PubMed Data Base

Among various types of imaging, ^{19}F MRI can provide 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, $\text{C}_8\text{F}_{17}\text{SO}_3\text{N}(\text{C}_2\text{H}_5)_4$), aqueous solutions of $\text{C}_9\text{F}_{19}\text{COON}(\text{CH}_3)_4$, fluoro- and hydrocarbon surfactants with different tail lengths and counterions ($^+\text{N}(\text{CH}_3)_4$, $^+\text{N}(\text{C}_3\text{H}_7)_4$, 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

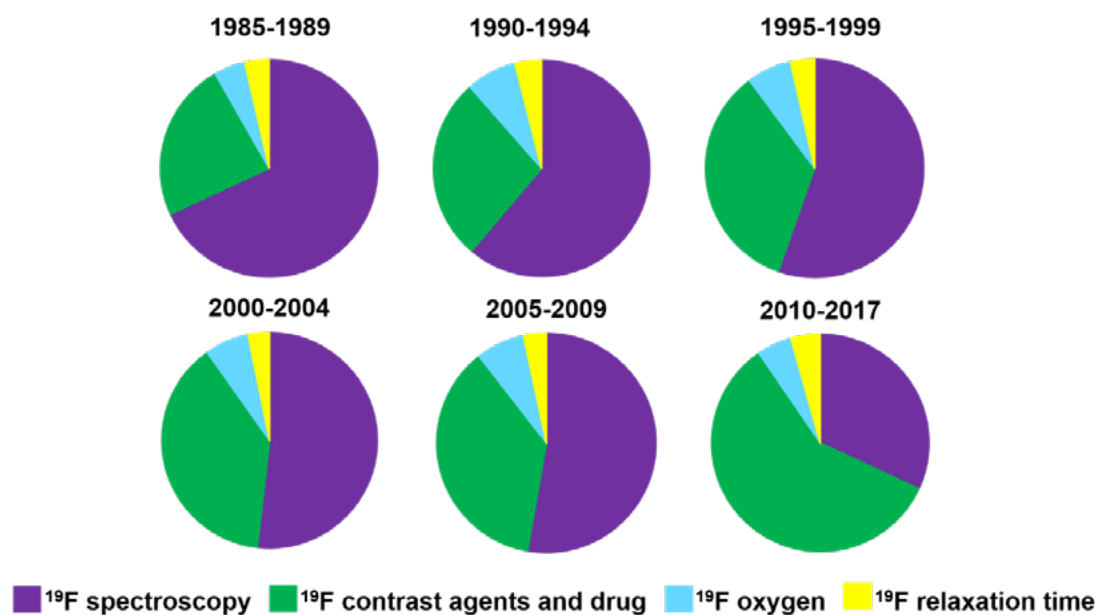


Figure 2. The statistic of ¹⁹F research collected from Library of National Center for Biotechnology Information (NCBI) PubMed Data Base

have been demonstrated that ¹⁹F 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 from 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, ¹⁹F spectroscopy, ¹⁹F contrast agents and drug ¹⁹F oxygen and ¹⁹F relaxation time were grouped. Figure 2 below shows the percentage distribution of topics in each year during 1985-2017.

¹⁹F contrast agents and drugs

Fluoroorganic and fluoroinorganic compounds are very rare in nature and the few that do occur in nature are highly toxic. The reaction to introduce ¹⁹F to compounds is called fluorination. Examples of popular organofluorine reagents are acetyl hypofluorite which was used to fluorinate aromatic rings¹¹ and acetyl hypofluorite which has been intensively studied to use for addition to double bonds¹², fluorination of lithium enolates¹³, 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, ¹⁹F MRS has contributed to the development of agents modulating tumor drug metabolism. To date, the review of literature showed

a research done with 5-FU¹⁶, NaF¹⁷⁻¹⁸, lescol¹⁹, flunarizinium²⁰, mirenin²¹, fevarin²², sevofluran²³, ciprofloxacin²⁴, haloperidol²⁵, prozac²⁶, ciprinol flumetasone²⁷, perfluorodecalin²⁸ and trastuzumab²⁹. To obtain a quantitative estimate of the drug, ¹⁹F MRS is necessary to study dynamic abundance of ¹⁹F. In addition to the ¹⁹F drugs, ongoing research is underway to find effective marker cells in ¹⁹F MRI. Therapeutic cells are usually marked with MRI contrast agents, including gadolinium ions (Gd³⁺), 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, ¹⁹F is known as a creator of a strong contrast signal *in vitro* and *in vivo* in background-free samples or tissue. *In vivo* ¹⁹F 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 ¹⁹F 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 ¹⁹F compounds together with specifically adapted hardware and acquisition methods.³⁵ In general, the ¹⁹F 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 $54\pm 2\%$, $50\pm 3\%$ and $45\pm 1\%$ 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 emissions. 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 synthesize 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. Perfluoropolyethers (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. Fluorescent (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--CF₂ (CF₂)^x CF₂] 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.

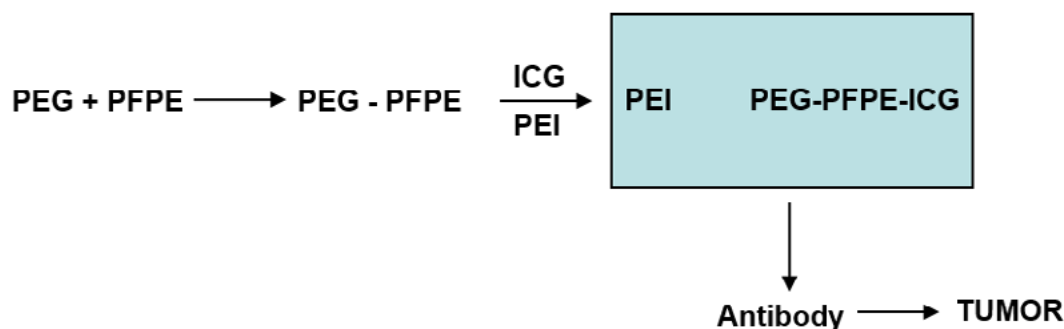


Figure 3. The synthetic scheme of polyethylene glycols (PEG) + Linear PFPE + polyethyleneimine (PEI)

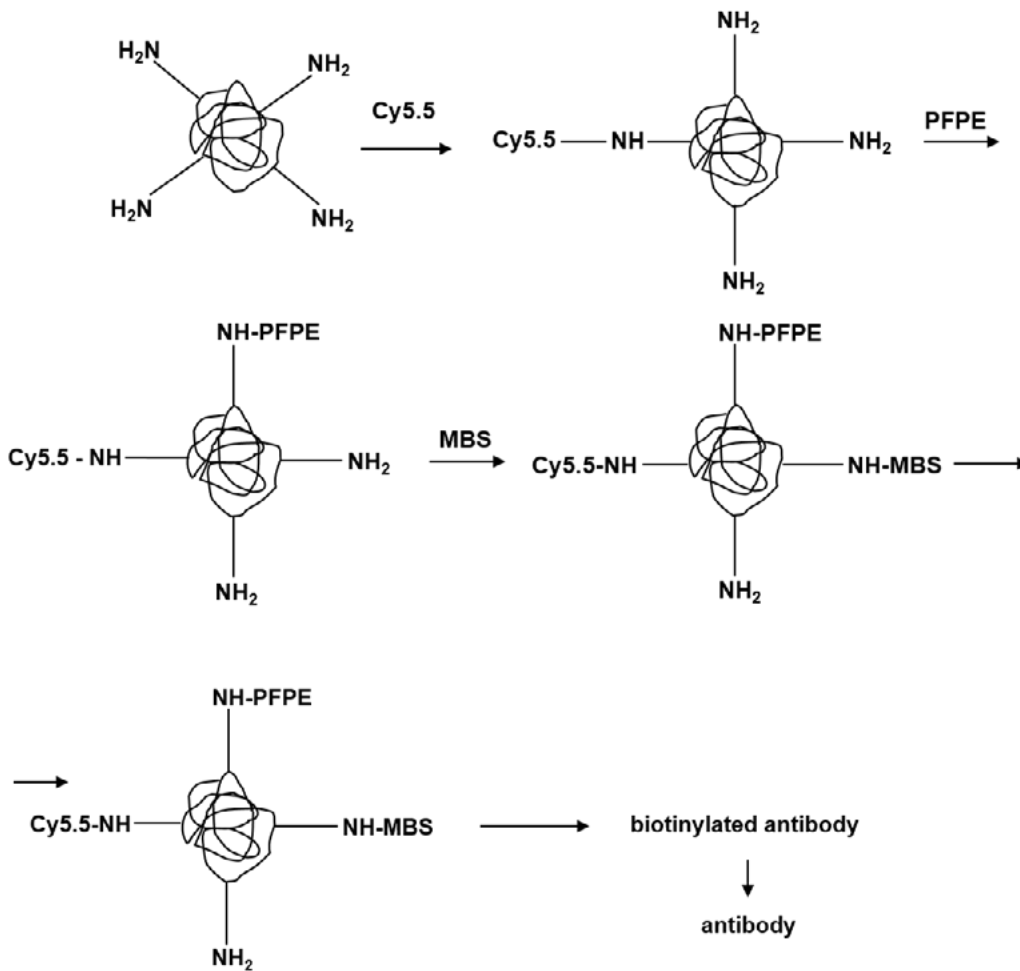


Figure 4. The synthetic scheme of linear PFPE+dextran+Cy5.5+Ab

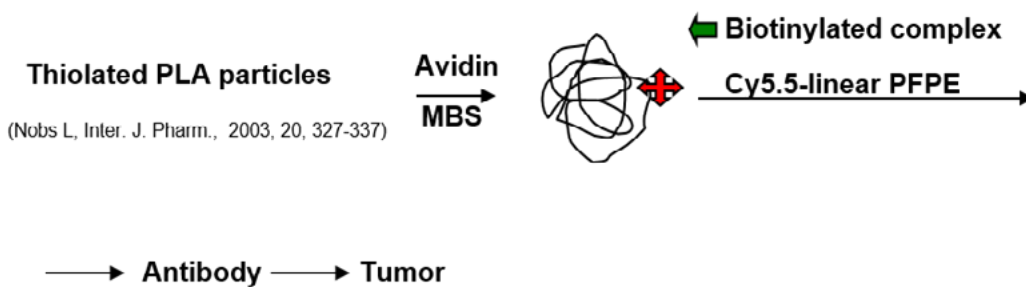


Figure 5. The synthetic scheme of linear PFPE + aminated dextran + Cy5.5+Ab

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-Hydroxyssucinimide 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 method⁴³ followed by the coupling of cystamine dihydrochloride. Next, Avidin can be covalently bounded to the functional PLA particles via the sulfo-

MBS. Next, fluorescent (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--CF₂(CF₂)_xCF₂]-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)OCH₂CF₃), 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-

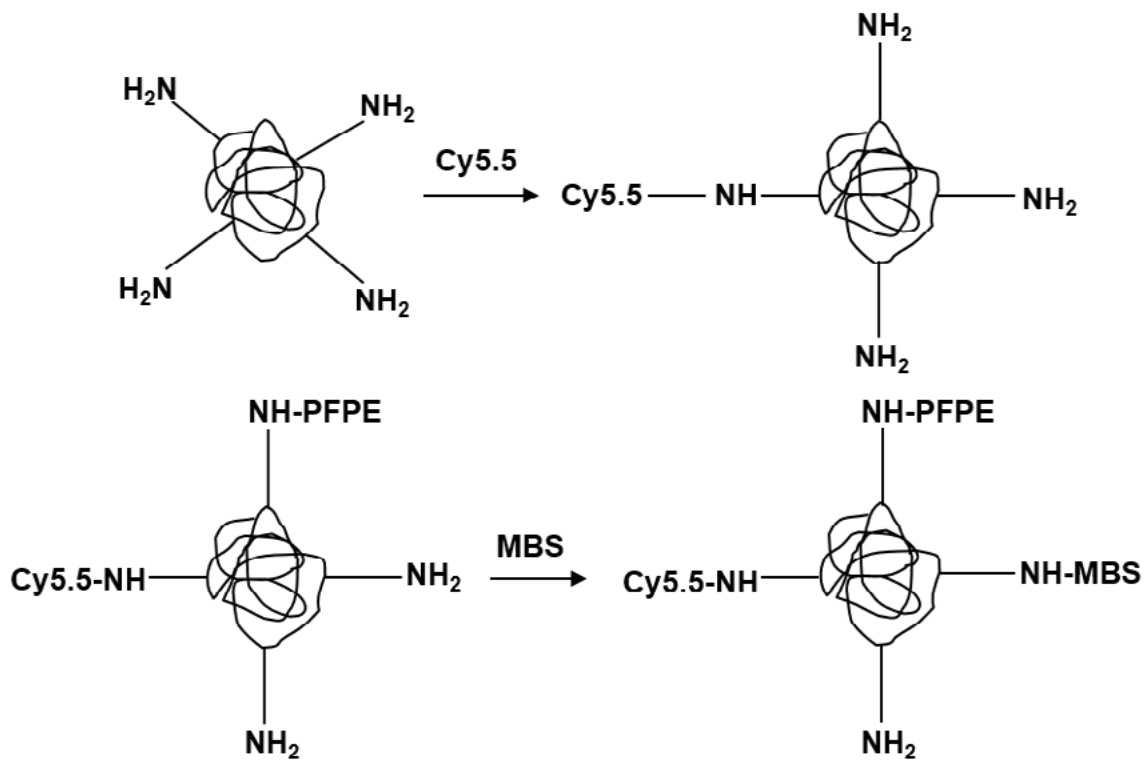


Figure 6. The synthetic scheme of fluorescent (Cy5.5) – linear PFPE

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

¹⁹F oxygen

Oxygen content in tumor tissue can be monitored with ¹⁹F MRI. We hypothesize that the advantage of intracellular O₂ 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 cancer⁵⁰ 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,

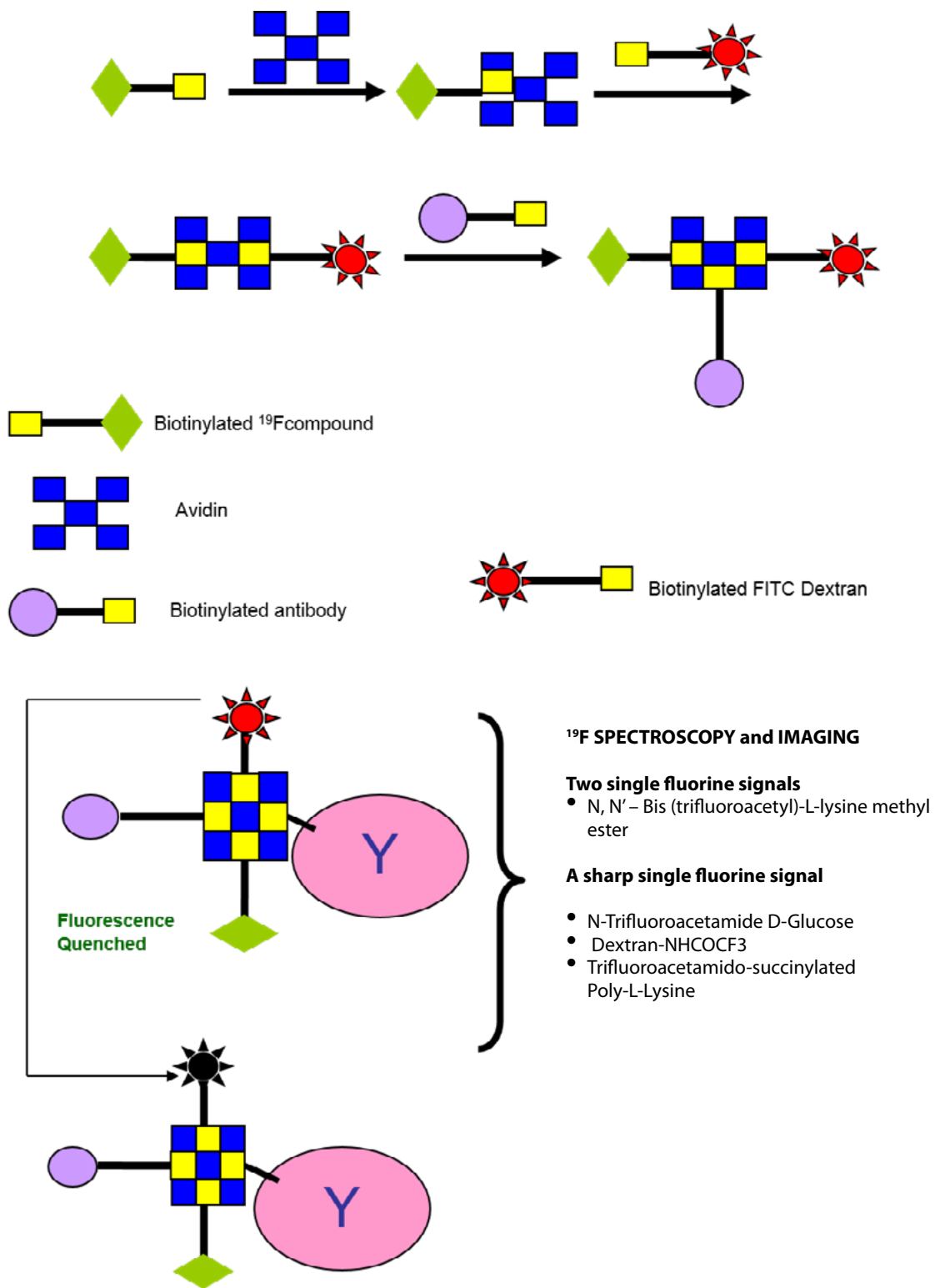


Figure 7. Biotinylated ¹⁹F compound constructed from FITC dextran, antibody and avidin

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 \geq 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 the success of radiotherapy in animal tumor. Hypoxic cells impair the effectiveness 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 clonogenic cells within solid tumors before and during treatment would be extremely valuable. The spin-lattice relaxation rate ($1/T_1$) of PFOB will increase 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}F MRI.⁵⁷

A fluorinated cobalt(II) complex serves as a turn-on ^{19}F MRI tracer for reactive oxygen species includ-

ing 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 $\text{Na}_2\text{S}_2\text{O}_4$. ^{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 ($\sim 4 \times 10^4$) 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 nor-fluoxetine) *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 non-toxic 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 ¹⁹F atoms per molecule for enhancing sensitivity. The center CF₂ groups can generate a strong peak (>0.9 at -92 ppm) that dominates 90% of its ¹⁹F signal, whereas the end CF₂ 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 $T_1\rho$ of fluorine atoms in solid dispersions were determined at various temperatures (-20 to 150 °C). Correlation time (τ_{auc}), which is a measure of rotational molecular mobility, was calculated from the observed T_1 or $T_1\rho$ value and that of the T_1 or $T_1\rho$ minimum, assuming that the relaxation mechanism of spin-lattice relaxation of FLF fluorine atoms does not change with temperature.⁷² MRI of the ¹⁹F 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 SF₆ 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

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

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REVIEW PAPER

Adrian Truskiewicz ^{1(ABGF)}, David Aebisher ^{2(ABF)}, Przypek Aneta ^{1(F)},
Wiesław Guz ^{1,3 (F)}, Dorota Bartusik-Aebisher ^{3(ABF)}

Functional MRI – how does it work?

¹ Department of Electroradiology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

² Department of Human Immunology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

³ Center for Innovative Research in Medical and Natural Sciences, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

⁴ Department of Experimental and Clinical Pharmacology, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

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 (BOLD) 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

Corresponding author: Adrian Truskiewicz, e-mail: atruszkiewicz@gmail.com

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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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 electroencephalography¹, 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, ¹H MRI is most often used, but is increasingly implemented with ¹³C, ¹⁵N, ¹⁹F, and ³¹P.

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

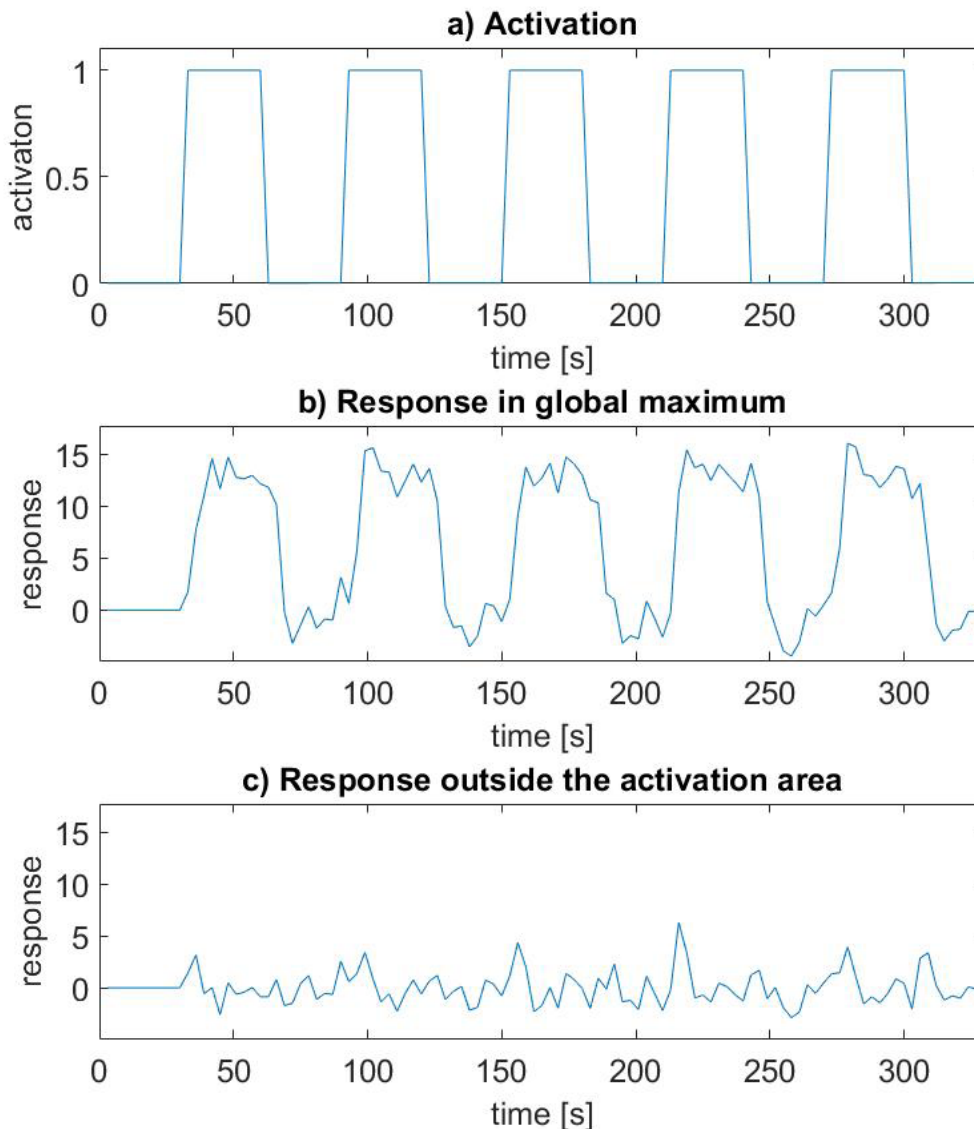


Figure 1. a) temporary activation, b) response at the global maximum, c) response from the place where activation did not take place

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.

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REVIEW PAPER

Łukasz Ożóg ^{1(ABGF)}, David Aebisher ^{2(ABF)}, Zuzanna Bober ^{3(ABF)},
Dorota Bartusik-Aebisher ^{4(GF)}, Wiesław Guz ^{3(F)}, Marian Cholewa ^{1(AF)}

Magnetic Resonance Elastography – noninvasive method to assess liver disease

¹ Department of Biophysics, Faculty of Mathematics and Natural Sciences, University of Rzeszów,

² Department of Human Immunology, Faculty of Medicine, University of Rzeszów

³ Center of Innovative Research in Medical and Natural Sciences, Faculty of Medicine, University of Rzeszów

⁴ Department of Clinical and Experimental Pharmacology, Faculty of Medicine, University of Rzeszów

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-

Corresponding author: Łukasz Ożóg; E-mail: luozog@ur.edu.pl

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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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 be 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

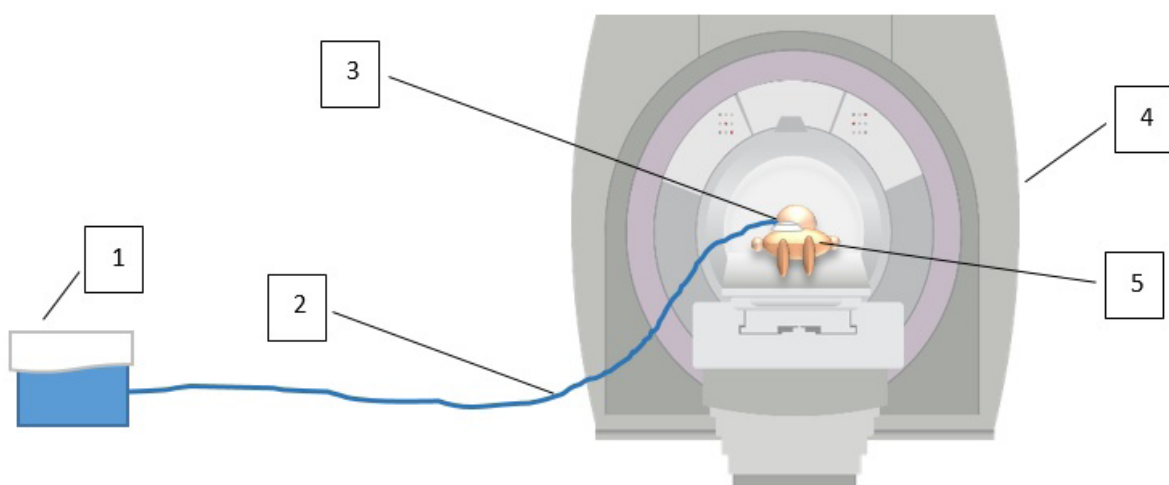


Figure 1. Scheme of a liver MRE system. 1 – Active Driver (source of mechanical waves placed outside the MR room); 2 – Connecting Tube (plastic tube transmitting acoustic vibration); 3 – Passive Driver (non-metallic element with flexible membrane, located on the liver); 4 – magnet; 5 – patient

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

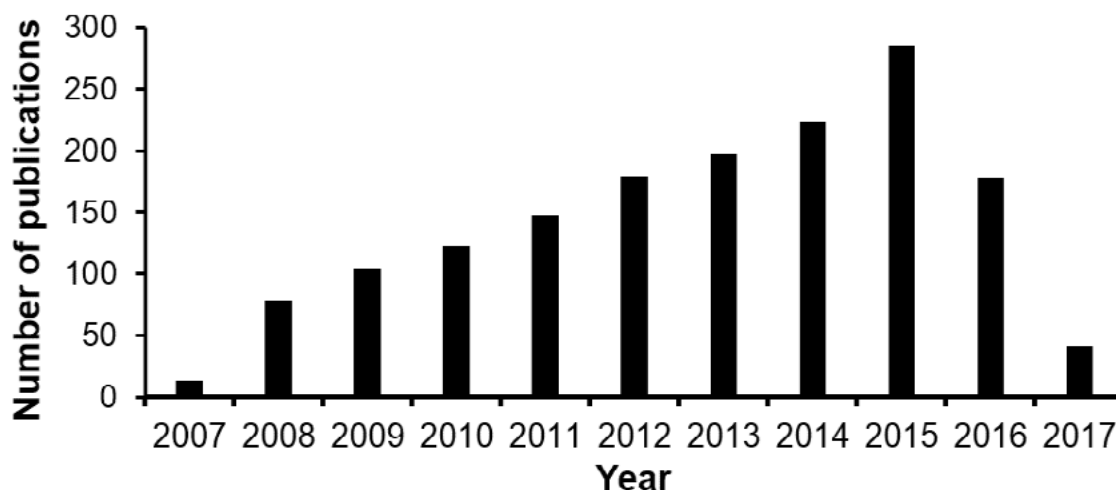


Figure 2. Number of publications in the PubMed National Center for Biotechnology Information (NCBI) for "magnetic resonance elastography liver fibrosis" from 2007 to 2017

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.

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REVIEW PAPER

Wiesław Guz ^{1,2(ABDF)}, Zuzanna Bober ^{1(BG)}, Łukasz Ożóg ^{3(BG)}, Adrian Truskiewicz ^{1(BG)},
Aneta Przypek ^{2(BG)}, David Aebisher ^{4(BGF)}, Dorota Bartusik-Aebisher ^{5(A)},
Andrzej Urbanik ^{2,6(A)}

Clinical application of advanced neuroimaging techniques – Magnetic Resonance Spectroscopy

¹ Center for Innovative Research in Medical and Natural Sciences, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

² Department of Elektroradiology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

³ Department of Biophysics, Faculty of Mathematics and Natural Sciences,
University of Rzeszow, Rzeszow, Poland

⁴ Department of Human Immunology, Faculty of Medicine, University of Rzeszow,
Rzeszow, Poland

⁵ Department of Experimental and Clinical Pharmacology, Faculty of Medicine,
University of Rzeszow, Rzeszow, Poland

⁶ Department of Radiology, Collegium Medicum, Jagiellonian University, Krakow, Poland

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

Corresponding author: Wiesław Guz, e-mail: wguz@op.pl

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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 (^1H) 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 ^1H nuclei, but also other nuclides such as ^{13}C , ^{15}N , ^{19}F , ^{23}Na and ^{31}P . Compared to ^1H 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. ^1H 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 ^1H 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 $2 \times 2 \times 2$ cm i.e. 8 cm³. 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 ^1H 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 flat 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 (^1H 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)

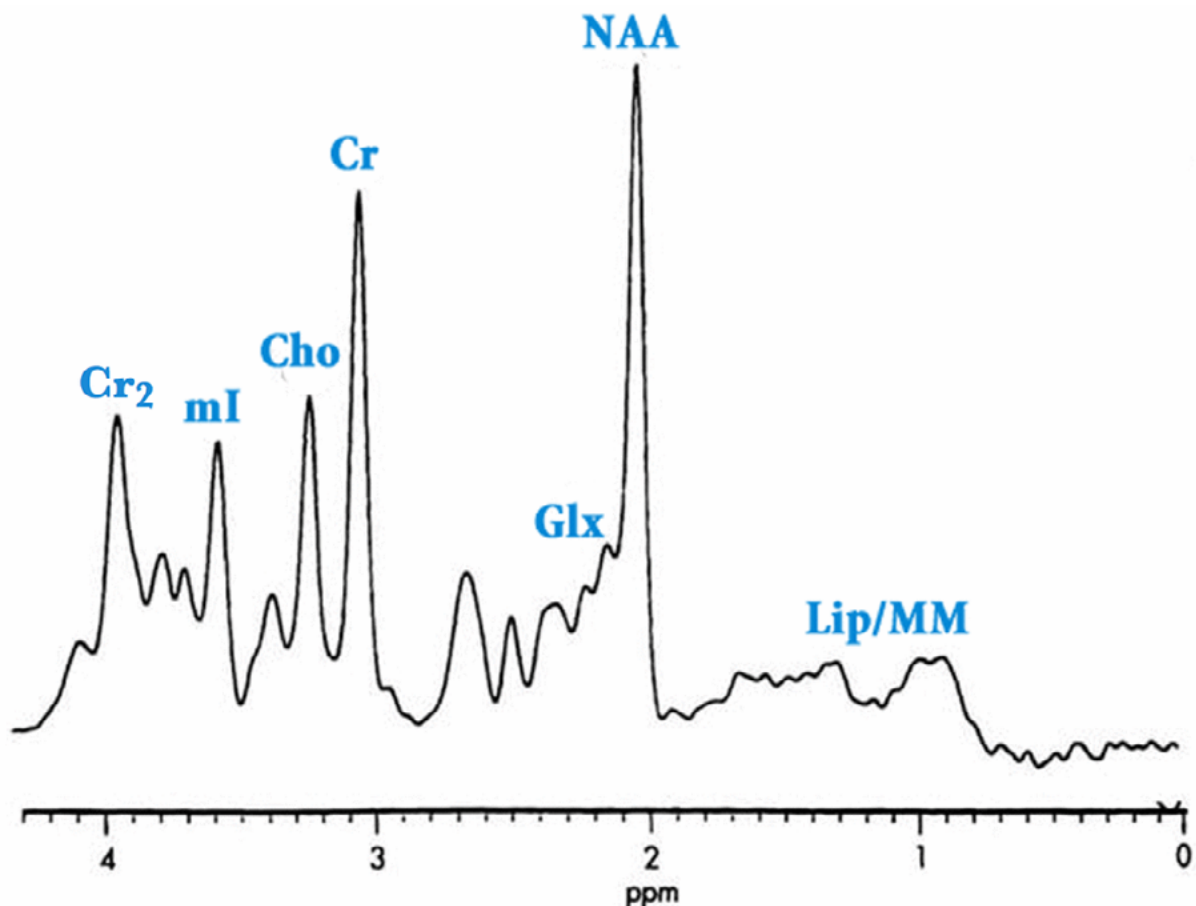


Figure 1. MRS spectrum sample

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-electrocardiomas 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 ¹H 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 - ¹H MRS:

1. In brain cancer⁹⁻¹⁴

- 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 ^1H 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 (mI) - 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 ^1H MRS-CSI).

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

^1H 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 ($G1-2=2.05\pm0.18$, $G3=2.58\pm0.11$, $G4=5.1\pm0.89$). 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 ^1H MRS in evaluation of malignant malignant tumors¹⁰⁻¹²

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 ^1H 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 ^1H MRS in differentiation of other tumors¹³

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 ^1H 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 ^1H 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 ^1H MRS results.

Clinical applications of ^1H 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 ^1H 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. ^1H MRS seems to be helpful in assessing axonal lesions and demy-

elination, and together with DTIs, it can better monitor the evolution of MS lesions.

Clinical applications of ^1H MRS in cerebral infectious diseases¹⁹

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 ^1H MRS in metabolic diseases¹⁴

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 ^1H 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 ^1H MRS by N-acetyl aspartate (NAA) and glutamate (Glx) and choline (Cho) growth. ^1H 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 ^1H 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 ^1H MRS in brain injury¹⁴

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 ¹H MRS in ischemic diseases¹⁴

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 myosinosin (mI) is evident. Phosphoric spectroscopy (³¹P MRS) in ischemia decreases the ratio of phosphocreatine to inorganic phosphorus (PCr/Pi).

Clinical applications of ¹H MRS in epilepsy²⁰

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 ¹H MRS in psychiatry²¹⁻²⁴

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 ³¹P 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.

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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

¹ Department of Medical Biology, Institute of Experimental and Clinical Medicine,
Medical Faculty of University of Rzeszow, Poland

² Centre for Innovative Research in Medical and Natural Sciences,
Medical Faculty of University of Rzeszow, Poland

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 these 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. Furthermore, 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 inten-

sive 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-

Corresponding author: Dorota Bądziul, email: dbadziul@ur.edu.pl

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 particularly 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 submembranous 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 blebbing 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 pre-clinical 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 act as an antimetabolic 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 efficacy 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 physician's 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 therapy³⁰, 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 glioblastoma patients and provides more evidence supporting the use of TTFs as an efficacious, antimetabolic 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.






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REVIEW PAPER

Wiesław Guz ^{1,2(ABDF)}, Zuzanna Bober ^{1(BG)}, Łukasz Ozóg ^{3(BG)}, Adrian Truszkiewicz ^{1(BG)},
Aneta Przypek ^{2(BG)}, David Aebisher ^{4(BG)}, Dorota Bartusik-Aebisher ^{5(A)},
Andrzej Urbanik ^{2,6(A)}

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

¹ Center for Innovative Research in Medical and Natural Sciences, Faculty of Medicine,
University of Rzeszow, Poland

² Department of Elektoradiology, Faculty of Medicine, University of Rzeszow, Poland

³ Department of Biophysics, Faculty of Mathematics and Natural Sciences,
University of Rzeszow, Poland

⁴ Department of Human Immunology, Faculty of Medicine, University of Rzeszow, Poland

⁵ Department of Experimental and Clinical Pharmacology, Faculty of Medicine,
University of Rzeszow, Poland

⁶ Department of Radiology, Collegium Medicum, Jagiellonian University, Krakow, Poland

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

Corresponding author: Wiesław Guz, e-mail: wguz@op.pl

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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 devi-

ces 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 developed¹⁻³:

- 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 Arterial Spin Labeling)

The first PASL (Pulsed Arterial 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 application⁶:

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. Subtraction, 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 gradient⁸ 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 multi-layer 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 matter 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 hemorrhage 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.

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REVIEW PAPER

Rafał Podgórski ^{1,2(ABDFG)}, Monika Stompor ^{1,2(BFG)}, Tomasz Kubrak ^{1,2(BFG)},
Dominika Podgórska ^{3(ABFG)}

Neural tube defects: risk factors and prevention

¹ Centre for Innovative Research in Medical and Natural Sciences, University of Rzeszów

² Department of Biochemistry, Faculty of Medicine, University of Rzeszów

³ Department of Rheumatology, Regional Hospital No. 2 in Rzeszów

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-

Corresponding author: Rafał Podgórski, e-mail: rpodgorski@ur.edu.pl

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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 begins 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 cause 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 exposed 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, microthyntesis, 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, *Toxoplasma 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 $\geq 15\,000$ 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-methylenetetrahydrofolate 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 methionine. A thermolabile variant of MTHFR 677T, 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 official 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

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REVIEW PAPER

Monika Stompor ^{1,2(ABFGH)}, Rafał Podgórski ^{1,2(FG)}, Tomasz Kubrak ^{1,2(FG)}

Combined effect of flavonoid compounds and cytostatics in cancer treatment

¹Centre for Innovative Research in Medical and Natural Sciences,
University of Rzeszów, Poland

²Department of Biochemistry, Faculty of Medicine,
University of Rzeszów, Poland

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 phytochemicals depends on concentration and the presence of additional factors (e.g. microflora, metal ions), which could possibly make a compound harmful, instead of having positive therapeutics effect. Elucidation of the mechanisms involved in biological activity of the described phytochemicals is essential for a better understanding of their influence on an organism.

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

Corresponding author: Monika Stompor, e-mail: monika.stompor@gmail.com

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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 ER α 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-prenyl naringenin 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 O⁶-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 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 querce-

tin 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

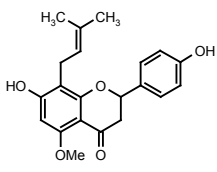
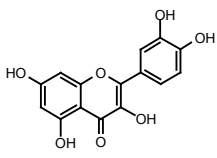
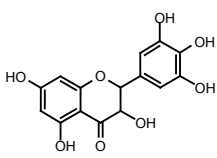
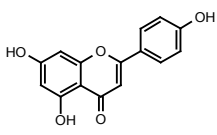
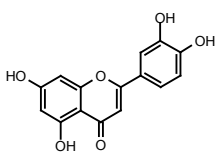
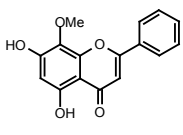
Similar research showed that quercetin also enhanced proapoptotic and pro-autophagic properties of the anticancer agent sorafenib (Nexavar) used in treatment of kidney cancer.⁴⁷ The molecular mechanism of this drug is based on inhibition of Raf serine/threonine kinase which plays a key role in the intracellular 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.

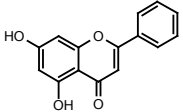
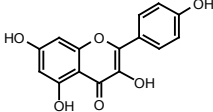
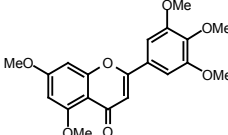
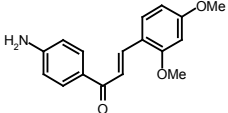
The combination of chrysin with cytostatics is more effective in induction of programmed cell death than using single chemotherapeutics. For the majority of tested cancer cells, changes in the mechanisms regulating cell cycle progression were observed. Mutual action of anticancer drugs and other flavonoids, 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				
 <p>isoxanthohumol</p>	paclitaxel	rats	in vivo	Krajnović et al. 2016
	tamoxifen	rats breast cancer therapy	in vivo	Silva et al. 2017
 <p>quercetin</p>	adriamycin	mice after P388 cell inoculation	in vivo	Han et al. 2014
			in vitro	Han et al. 2015
	doxorubicin	SMMM7721 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
	T98G MOGGCCM		in vitro	Jakubowicz-Gil et al. 2014
	sorafenib	Human anaplastic astrocytoma and glioblastoma multiforme	in vitro	
 <p>dihydromyricetin</p>	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				
 <p>apigenin</p>	cisplatin	PC3 prostate cancer	in vitro	Erdogan et al. 2017
	paclitaxel	HeLa cells	in vitro	Xu et al. 2011
 <p>luteolin</p>		MCF-7, BT-483, BT-474; ER- cells: MDA-MB-231 human breast cancer	in vitro	Tu et al. 2013
	tamoxifen			
	cisplatin	Mice (male C57BL)	in vivo	Kang et al. 2011
	doxorubicin	MCF-7 MDA-MB-453	in vitro	Sato et al. 2015
 <p>wogonin</p>		AMCHN2, -HN3, -HN4, -HN5, and -HN9, and SNU-1041, -1066, and -1076 human head and neck cancer		Kim et al. 2016
	cisplatin	A549 lung cancer	in vitro	He et al. 2012

	doxorubicin	BEL-7402 hepatocellular carcinoma	in vitro	Gao et al. 2013
chrysin				
	chrysin	OVCA-3 ovarian cancer	in vitro	Luo et al. 2010
kamferol				
	3',4',5',5',7-pentamethoxyflavone	A549 lung cancer	in vitro	Hou et al. 2015
Chalcones				
	chalcone	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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REVIEW PAPER

Agnieszka Ewa Stępień  ^{1,2}(AFG)

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

¹ Centre for Innovative Research in Medical and Natural Sciences, Medical Faculty, University of Rzeszow, Poland

² Department of Dietetics, Institute of Nursing and Health Sciences, Medical Faculty, University of Rzeszow, Poland

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, 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 *Cistaceae* 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.

Corresponding author: Agnieszka Ewa Stępień, Department of Dietetics, Institute of Nursing and Health Sciences, Medical Faculty, University of Rzeszow, Al. mjr. W. Kopisto 2 a, 35-310 Rzeszów, Poland, tel.: 48 178516846

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 ellagittannins 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.

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REVIEW PAPER

Tomasz Kubrak ^{1,2} (ABCDEF GH), Rafał Podgórski ^{1,2} (ABCDEF GH), Monika Stompor ^{1,2} (ABCDEF GH)

Natural and Synthetic Coumarins and their Pharmacological Activity

¹ Centre for Innovative Research in Medical and Natural Sciences, University of Rzeszów

² Department of Biochemistry, Faculty of Medicine, University of Rzeszów

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 coumarin 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 species of plants belonging to almost 30 different families. The families containing the highest content of coumarins are: *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 a coumarin 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 -pyrone. Substituents are often hydroxyl, methoxy and aliphatic groups, at the C7, C6 and C3 positions of benzopyrone (Fig. 1).

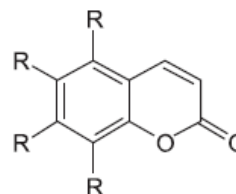


Figure 1. Chemical structure of coumarin

Corresponding author: Tomasz Kubrak, e-mail: tkubrak@ur.edu.pl

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*, *Aspergillus*, *Fusarium*, *Penicillium*, *Stremtomyces* and the few plants belonging to families: *Compositae*, *Leguminosae* and *Myriaceae*.⁴

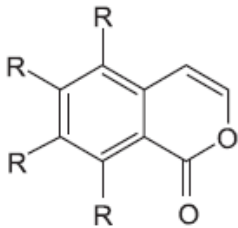


Figure 2. Chemical structure of isocoumarin

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).

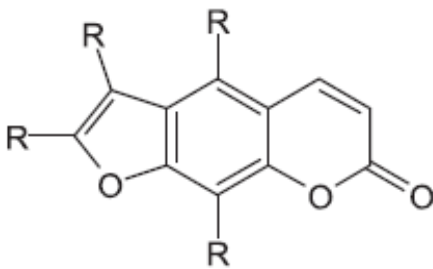


Figure 3. Chemical structure of furanocoumarin

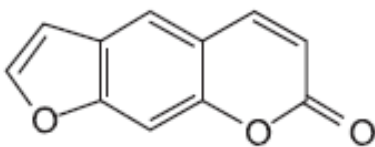


Figure 3A. Psoralen type

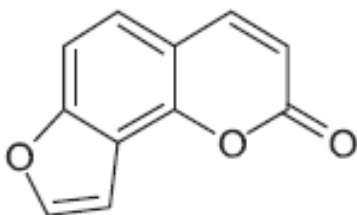


Figure 3B. Angelicin type

4. **pyrancoumarin derivatives**, coumarin 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).

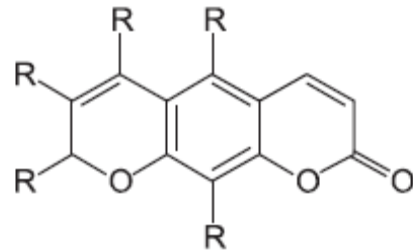


Figure 4. Chemical structure of pyrancoumarin

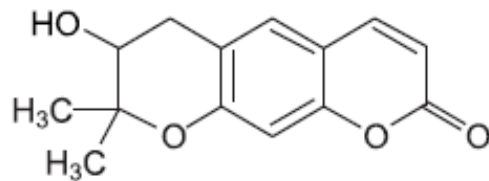


Figure 4A. Xanthyletin-type

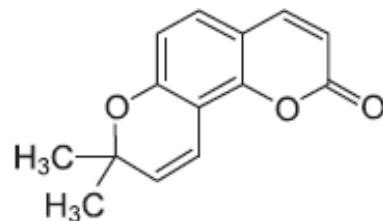


Figure 4B. Seselin-type

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) have anti-inflammatory properties and have been used to treat oedema, helping wound healing. This removes protein and oedema fluid from injured tissue by stimulating phagocytosis and proteolytic enzyme production.⁵ Esculetin exhibited anti-inflammatory activity in 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 ammosesinol and ostruthin have a high activity towards gram(+) bacteria and show 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 Imperatorin shows high activity towards *Shigella dysenteriae*.¹⁰

Pyranocoumarins such as grandivittin, agasyllin, aegelinol benzoate and osthole isolated from the root *Ferula gocompestris* (Besser) Grecescu (*Apiaceae*) show activity towards both gram(+) and gram(-) 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* and gram(-) organisms such as *Haemophilus influenzae*, *Neisseria meningitides* and *Pasteurella*. Coumermycin, structural similar to novobiocin, exhibits almost 50 times more potency against bacteria belonging to *Escherichia coli* strains and *Staphylococcus aureus* strains. Also chartreusin, 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 in scopoletin, umbelliferone, phellodenol A, marmezin and xanthyletin.¹²

Antifungal activity of coumarins

The broad spectrum of antifungal activity is shown by osthole, a derivative of coumarin isolated from celery plants. This derivative demonstrates activity against *Rhizoctonia solani* [Kühn], *Phytophthora capsici* [Leonian], *Botry-*

tis cinerea [Pers.], *Sclerotinia sclerotiorum* [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 effect and two isomers (+)-calanolide A and (-)-calanolide B isolated from *Calophyllum lanigerum* (*Clusiaceae*), belong to pyranocoumarin, inhibit RT activity and completely deactivate the replication process of HIV-1.^{15,16} Others coumarin derivatives – inophyllum B i P obtained from a giant African snail *Achatina fulica* [Férussac] significantly inhibit RT activity in HIV-1 cell cultures.¹⁷

Aminomethyltrimethyl psoralen (AMT) is used as 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 on blood vessels resulting in a drop in blood pressure. A similar action was shown by dihydromammea; a coumarin isolated from the seed of the tree *Mammea africana* [L.] (*Guttiferae*).¹

Visnadine – pyranocoumarin, 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 within the molecule.²¹

Coumarin compounds can directly affect the properties of antioxidant enzymes. Luczaj et 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 glutathione were 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. Orhan et al. have demonstrated significant inhibition of acetyl- and butyrylcholinesterase levels after application with bergapten, xanthotoxin, scopoletin, umbelliferone, and 4-methylumbelliferone.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

Osthole present among others in *Cnidium monnieri* (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-acetyltransferase inhibitor and 8-MOP (8-methoxypsoralen) in phototherapy for psoriasis and vitiligo.

In treatment of the skin disorders vitiligo, psoriasis and atopic inflammation, bergapten has also been successful.^{30,31} Human keratinocytes of the NCTC-2544 line were exposed to bergapten and xanthotoxin and 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 for autoimmune 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 repigmentation. 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 GLC₄ (small cell lung carcinoma) and COLO 320 (colorectal cancer) cell lines, it has been shown that the cytotoxicity 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 methoxylated coumarin 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. by QSAR 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 nifcoumar) 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 Attempts have been made to synthesize a coumarin-like compound with selective and targeted action on tumor cells. Extremely cytotoxic heterocyclic coumarin derivatives which 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 felamidine, four natural coumarins isolated from *Ferulagocampes-tris* (*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 (ER α). ER α 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 ER α 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

Conclulsion

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

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