Hybrid Theranostic Platforms for Cancer Nanomedical Treatment

Dissertation by

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In Partial Fulfillment of the Requirements

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ABSTRACT

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Cancer is a leading cause of mortality worldwide. Governments spent multibillion expenses on treatment and palliative care of diseased people. Despite these generous funding and intensive research with aim to find a cure or efficient treatment for cancer, until now there is a lack in selective cancer management strategies. Conventional treatment strategies for cancer, such as surgery, cytotoxic chemotherapy, radiation therapy, hormone therapy don’t have selectivity toward cancer – the property of discrimination of healthy organs and tissues from the diseased site. Chemotherapy is very challenging as the difference between effective and lethal doses is very minuscule in most cases. Moreover, devastating side effects dramatically changes the quality of life for cancer patients. To address these issues two main strategies are intensively utilized in chemistry: (I) the design and synthesis of novel anticancer organic compounds with higher selectivity and low toxicity profiles and the second, design and preparation of biocompatible nanocarriers for imaging and anticancer compound selective delivery nanomedicine. The following dissertation combines the above two strategies as bellows: First project is related to the design and synthetic route development toward novel nature-inspired group of heterocyclic compounds – iso-
Phidianidines. The second project focused on design, preparation and evaluation of hybrid theranostics (therapeutic and diagnostic in a single entity).

Chapter 1 is a general background review of the major topics that will be discussed in this dissertation.

The first efficient and high-yielding synthetic route toward iso-phidianidines, containing regioisomeric form of 1,2,4-oxadiazole linked to the indole via methylene bridge is reported in Chapter 2. In vitro test of the synthesized library of iso-phidianidines revealed micromolar range of cytotoxicity toward human cervical cancer cell line. Structure activity relationship revealed the importance of presence of monosubsituted amine in 3 position of oxadiazole to maintain activity. Moreover, gradual increase of activity was detected in increasing of the length of the diamine. Polyamine (spermidine) side chain demonstrated strongest anticancer activity, identified as lead compound and may be studied further as a good candidate for cervical cancer treatment. Finally, the remaining high activity of amino-terminated iso-phidianidines demonstrated that presence of guanidine group in termini is not necessary for high cytotoxicity.

The second part of this dissertation (Chapter 3) discusses the rational design, wet protocol synthesis and complete characterization of the novel hybrid material – polydopamine coated iron-cobalt nanocubes (PDFCs). This material was loaded with anticancer model drug doxorubicin in one step procedure (PDFC-DOX) and the
resulting drug-delivery vehicle was found to be successfully internalized by cervical cancer cells. The cytotoxicity test demonstrated inhibition of 50% of the cells at the concentration of 30µg/ml for PDFC-DOX. Moreover, the release was highly attenuated and pH-sensitive in acidic range. PDFC was also modified with fluorescein leading to green fluorescent nanoparticles PDFC-FITC, which demonstrated excellent intracellular molecular imaging property. PDFCs with one of the highest magnetic saturation among the materials used in biomedicine (226 emu/g based on core) showed the absence of any cytotoxicity in vitro and excellent MRI contrasting property ($r_2 = 186.44$ mMs$^{-1}$; higher than commercial contrast agents Ferridex® and Clio®), both in vitro and in vivo on mice. They were cleared out from the mice bodies in month without affecting their health. Due to the high density of core (8.3 g/cm$^3$) they demonstrated ability to be contrast materials also for X-Ray CT diagnostic modality, increasing the tumor detection and visualization probability in combination with MRI.

In addition to its diagnostic and drug-delivery modalities, PDFC was evaluated also for microwave-induced cytotoxicity as a novel concept in cancer treatment. As low as 10 µg/ml concentration of PDFCs in human cervical cancer cells caused extensive death above 73% upon exposure to 2.45 GHz of microwaves for one minute. Laser irradiation (808 nm, 15 minutes) of cancer cells with internalized PDFCs caused cell death above 60%. The specific absorption rate of PDFCs at 470 MHz frequency and 20 mT of the alternating magnetic field power was 180 W/g, which is nearly 100 W higher than for commercial nanoparticles (Ferridex®).
Thus, PDFCs are a material with unprecedented seven theranostic modalities - “heptapeutics” and represents a typical example of “One-for-All” approach in cancer nanomedicine.

In Conclusion (Chapter 4, designing of new small molecule motifs for enhanced chemotherapy can only work together with fabricating novel delivery platforms. We believe that combining both strategies will have a better chance in succeeding to provide a consumer effective product for cancer treatment in the near future.
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My appreciation also goes to my friends, laboratory colleagues and Core Lab staff for making my time at King Abdullah University of Science and Technology a great experience. I also want to extend my thanks all people who built and run the King Abdullah University of Science and Technology, providing us excellent environment for productive work and comfortable life. Finally, my heartfelt gratitude is extended to my parents for their encouragement and to my wife and son for their patience and support.
DEDICATION

This work is dedicated to my mother, Aghavni Araqelyan MD, who infected me with her passion towards the science of medicinal chemistry and who fell in the unequal fight with cancer.
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<td>AFM</td>
<td>alternating magnetic field hyperthermia</td>
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<tr>
<td>AMPA</td>
<td>R-amino-3-hydroxy-5 methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>CCK</td>
<td>cell count kit</td>
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<tr>
<td>CVC</td>
<td>consonant-vowel-consonant</td>
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<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNT</td>
<td>carbon nanotube</td>
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<tr>
<td>CTAB</td>
<td>N,N,N,-cetyltrimethylammonium bromide</td>
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<tr>
<td>DAT</td>
<td>dopamine transporter</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<td>DDW</td>
<td>degassed and deionized water</td>
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<tr>
<td>DHI</td>
<td>dehydroxyindole</td>
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<tr>
<td>DIPEA</td>
<td>diisopropyl ethyl amine</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
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<td>DOPA</td>
<td>3,4-dyhydroxy-L-phenyl indole</td>
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DOX  doxorubicin

DSC  differential scanning calorimetry

EtOAc  ethyl acetate

EDAX  energy-dispersive x-Ray spectroscopy

FACS  fluorescence-activated cells sorting

FBS  fetal bovine serum

FITC  fluorescein isothiocianate

HATU  1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate

IC  inhibition concentration

MRI  magneto-resonance imaging

MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NET  norepinephrine transporter

NIR  near-infrared

NMR  nuclear magnetic resonance
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<td>polydopamine</td>
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<td>PDFC</td>
<td>polydopamine coated iron cobalt nanocubes</td>
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<td>PET</td>
<td>positron emission tomography</td>
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<td>PLGA</td>
<td>polylactic-\textit{co}-glycolic acid</td>
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<td>PTT</td>
<td>photothermal therapy</td>
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<td>SAR</td>
<td>specific absorption rate</td>
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<td>SERT</td>
<td>serotonin transporter</td>
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<tr>
<td>SLP</td>
<td>specific loss power</td>
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<td>SPION</td>
<td>superparamagnetic iron oxide nanoparticles</td>
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<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<td>SQUID</td>
<td>superconducting quantum interference device</td>
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<tr>
<td>PTT</td>
<td>photothermal therapy</td>
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<tr>
<td>RPM</td>
<td>rounds per minute</td>
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<td>ROI</td>
<td>region of interest</td>
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<td>TEM</td>
<td>transition electron microscopy</td>
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<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>TGA</td>
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Figure 3.13.1 Dead HeLa cells count on FACS with propidium iodide staining. The dark green represents the cells which has internalized PDFC’s and was irradiated with 808 nm 0.5 W/cm² laser for 15 min.

Table 3.11. Quantitative results of death by the HeLa cells count on FACS with propidium iodide staining. 808 nm 0.5 W/cm² laser for 15 min kills 60.44% of HeLa cell after incubation with 30 µg/ml PDFC’s

Figure 3.12.1. Specific absorption rate of PDFC's at 1mg/ml concentration and 370 KHz of working frequency.

Figure 3.12.2. Specific absorption rates for PDFC's at different concentrations and 470 KHz of working frequency.
Chapter 1
Introduction

1.1 Significance and Importance

Cancer is the leading cause of global mortality and morbidity. According to the latest World Cancer Report 2014\(^1\), which have been composed by the International Agency for Research on Cancer at World Health Organization, nearly 14 million new cases of cancer and 8 million cancer-related deaths have been registered worldwide only in the year 2012 (GLOBOCAN 2012)\(^2\). The age-standardized incidence and mortality rates

![Figure 1.1.1 Estimated number of cancer deaths for all ages and both sexes worldwide – World Cancer Report 2014 (WHO)](image-url)
estimated as 182 and 102 per 100000 of the population, respectively.

According to the American Cancer Society at least one in three people will face a cancer diagnosis in their lifetime and the lifetime risk of developing cancer is higher in men (slightly less than one in two) than for women (a little more than one in three)³. The same source reports that the direct medical costs for cancer in the US in 2011 were $88.7 billion. Thus, there is a high demand for continuous research to find new cancer treatments.

Classical treatments of cancer – surgical removal of tumors, radiation therapy, chemotherapy, hormonal therapy, immune therapy, and targeted therapy don’t always

Figure 1.1.2 Estimated age-standardized cancer incidence and mortality worldwide rates per 100 000 of population, by major organs, in men and women, 2012
lead to successful long term remission and cure. Moreover, many of the classical treatment methods such as radiotherapy and chemotherapy are leading to the devastating side effects and high systemic toxicity. Another major issue is the low selectivity of current chemotherapy agents. To address these issues two main strategies were pursued: (1) the synthesis of novel small molecule drug candidates with increased cellular selectivity profile and (2) the development of nanocarriers for selective drug delivery, locally targeted treatment and the better resolution imaging necessary for early diagnosis and detection of cancer. Accurate and sensitive ways of diagnosis are prerequisite for early cancer detection, which has proven potential to save lives$^4$-$^8$. 
1.2 Objectives of the Dissertation

The objectives of this dissertation are

a) Development of a synthetic strategy toward the novel small molecule anticancer drug candidates - iso-phidianidines, the analogs of recently discovered indole and unusual 1,2,4-oxadiazole containing marine natural product (Chapter 2)
This include the solution phase and the solid phase synthesis protocols development for the iso-phidianidines and their anticancer structure activity relationship study on cervical HeLa cells line

b) Preparation, characterization and the evaluation of “One-for-All” multiple theranostic modalities of polydopamine coated iron-cobalt magnetic nanocubes (Chapter 3) This include wet synthesis protocol development for highly magnetic Fe_{65}Co_{35}@Fe_{0.65}Co_{0.35}O_y nanocubes, coating of this nanoparticles with nature inspired biocompatible polymer polydopamine, their electronic microscopic, electron diffraction, thermogravimetric, spectroscopic, magnetic characterization and test on biocompatibility.

c) Ultra-high-field magnetic resonance, X-Ray CT contrasting properties and cellular imaging with FITC-attached nanocomposite as triple imaging diagnostic modalities study. Study on microwave induced chemotoxicity of this nanocomposite as low dose non-thermic microwave cancer treatment potential, as well as, study on near infrared wavelength laser induced hyperthermia,
alternating magnetic field hyperthermia, doxorubicin loading and intracellular delivery as quadruple therapeutic modalities.
1.3. Literature Background

1.3.1 Theranostic Nanoparticles

Since the visionary lecture, “There is plenty of room at the bottom” given by Richard Feynman on 26 December 1959, at the annual meeting of the American Physical Society (APS) at the California Institute of Technology, nanotechnology or more specifically nanomedicinal chemistry field developed dramatically allowing to manipulate nanosized objects with a high level of accuracy. Nanoparticles have been under close investigation in the biomedical research in recent years. The numerous and extensive studies of their application in medicine based on believe, that nanoparticles hold great potential to apply Paul Ehrlich’s more than 100 years old, but still unreached “magic bullet” concept9. These nanoengineered platforms provide many new opportunities for highly tunable and smart design of biomedical systems. The most characteristic feature and distinctive properties of nanoparticles are their small size, which allow them to penetrate to deep sites of biological tissues. Ability to control their size experimentally makes it possible to finely tune the biodistribution and clearance profile. Nowadays it is well known that diameter of nanoparticles should be superior to 7-8 nm to escape rapid and intensive filtration by the kidneys (renal filtration) and as consequence - urinary excretion. This fact was established by extensive studies done on example of quantum dots10. This comes from the properties of slit diaphragm at the level of podocyte foot of the kidney glomerulas, which
evolutionally designed by nature to prevent the filtration of the globular (having a spherical geometry) plasma proteins above 7nm size\textsuperscript{11}. Thus because of this size dependent renal filtration restriction, the nanoparticles differs dramatically from the small molecule drugs by their ability to escape the disadvantage of rapid clearance from the body and they are able to be retained for longer periods. From the diagnostic and therapeutic points of view, this modulation of pharmacokinetics allows more controlled dosage and administration of diagnostic and therapeutic agents. At the same time, it is also well established, that diameter of nanoparticles should not exceed 200-400 nm size, to avoid their intensive uptake and rapid clearance by the macrophages and monocytes\textsuperscript{12} - the guards of reticular endothelial system. Macrophages and monocytes are the types of immune blood cells, evolutionally designed to seek and destroy foreign invaders, such as bacteria. A third level restriction in size – the diameter of nanoparticles should be less than 50-80 nm to be able to extravasate (cross through) trough capillary walls\textsuperscript{13}.

Another distinctive feature of nanoparticles is their high surface-to-volume ratio, which allows the use of various surface modifications strategies, resulting in enhanced targeting, altered pharmacokinetic and toxicological profiles by changing the protein-nanoparticle and the cell-nanoparticle interactions\textsuperscript{14}. This information results in the development of one of the most well-known and studied surface modification strategy – PEGylation (surface covering by polyethylene glycol), which is known to delay the action of the reticular endothelial system clearance\textsuperscript{15}. The key mechanism of rapid
clearance by macrophages is the opsonization (tagging with signaling antibodies to attract macrophages) of uncoated nanoparticles by the circulating antibodies\textsuperscript{16}. Additionally, surface modification opens many possibilities for carrying and delivering of the therapeutic and diagnostic agents\textsuperscript{17}.

The variation of the core material of nanoparticles is another characteristic, which brings new diversity of applications, such as magnetic properties highlighted in the latest reviews and references therein\textsuperscript{18-23} and optical properties\textsuperscript{24-33}. Thus, so many advantageous properties of nanoparticle make them ideal for application in medicine, especially with the aim to use at the same time multiple combinations of their advantageous properties. The latest emerging and very promising field of research, which arose from the convergence of chemistry, biology, physics, nanotechnology and medicine is - Theranostic Nanomedicine.

1.3.2 “One-for All” nanoparticles

The Theranostic Nanomedicine (TNM) is a part of Theranostics, which is simply defined in \textit{The Scientist} journal (experts opinion from Roche, Wyeth, Astra Zeneca) as diagnostic plus therapy\textsuperscript{34}. It is a highly integrated system also known as nanotheranostic system, which is capable of dosage, targeted therapy and monitoring the response to therapy, a so called \textit{All-in-One System}\textsuperscript{35}.
So far, until very recently “All-in-One” approach for nanoparticle design was the dominating concept in theranostic nanomedicine. To design the theranostic nanoparticles utilizing this approach, chemists should unite within a single system many types of nanoparticles, each bearing unique modalities. Thus, quadripeutics technology\textsuperscript{36} combines four clinically validated therapeutic components: encapsulated drugs, colloidal gold nanoparticles and gold nanoshells (plasmonic nanobubbles) for

![Figure 1.3.1. All-in-One and One-for-All approaches in nanomedicine. Adapted from ref [37]](image)

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NIR laser hyperthermia and X-Ray radiosensitization. These nanoparticles with four modalities demonstrated outstanding *in vitro* and *in vivo* performance, and constitutes good examples of “All-in-One” systems. For this approach combination of various imaging units provides enhanced diagnostic information and the combination of many therapeutic agents increases the treatment efficiency and leading to synergic effect of multiplied activity. But the complexity of system also increases parallel with increase in number of components. Thus, to receive gain in number of modalities, keeping the complexity of system low, design of nanoparticle for theranostic nanomedicine should be based on easily synthesizable core unit, capable to serve for many applications – both, multiple imaging and multiple therapeutic modalities together. This approach
called by authors “One-for-All”, may overcome the complexity barrier of All-in-One systems by intrinsically designed multimodality of core. Thus specifically, magnetic core (either iron oxide or metallic iron, iron-cobalt) is intrinsically predisposed for MRI contrasting\textsuperscript{38} and PET/SPECT tracers bearing potential, because of relative easiness of surface functionalization. Moreover, they have alternating magnetic field hyperthermia mediator properties and may be coated with organic or inorganic polymers, capable of bearing other imaging agents and drugs, as well, which will characterize their multimodal therapeutic properties. Various coating, such as polyethylene glycol, dextran, albumin, peptides, antibodies, small molecules\textsuperscript{39} and very recently polydopamine\textsuperscript{40-45} is possible because of easiness of surface functionalization of iron oxide.

The main object of study of TNM is design and application of the smart, targeted and controllable multifunctional theranostic nanoparticles. This field requires wide range of knowledge of detection and therapy molecular mechanisms, diagnostic strategies and toxicity prediction\textsuperscript{46-48}. As for example in cancer medicine multifunctional nanoparticles have been highlighted in the following review\textsuperscript{49} by their unique properties, which distinguish them from other cancer treatment options:

TNMs can themselves have therapeutic - drug delivery, gene delivery, drug targeting photo- and hyperthermal properties, which further achieves synergistic effect by blocking of various receptors. They can be attached to multivalent different targeting ligand, which results in high affinity and specificity for different markers. They can be
made to carry multiple drug molecules that simultaneously enable combinatorial therapy. They can bypass traditional drug resistance, molecular heterogeneity and adoptive resistance mechanisms. They can achieve increased intracellular concentrations by using both passive and active targeting strategies. They can carry diagnostic molecules to provide imaging, prior, and after therapy.

Various established multifunctional nanoparticle platforms have been highlighted in literature recently: liposomes\textsuperscript{50-57}, polymer micelles\textsuperscript{58-61}, dendrimers\textsuperscript{62-68}, quantum dots\textsuperscript{69-72}, carbon nanotubes\textsuperscript{73-80}, silica nanoparticles\textsuperscript{81-88}, gold nanoparticles\textsuperscript{89-96}, graphene\textsuperscript{97-112} and many other systems reviewed recently\textsuperscript{37, 49, 113-126}.

Discovery of medicinal application of magnetic nanoparticles, as intracellular hyperthermia agents by Gordon et al\textsuperscript{127} and MRI contrasting agents by Kellar et al\textsuperscript{128} and the pioneering works of Widder at al\textsuperscript{129-131} on magnetically targeted drug delivery has affected medicinal chemistry dramatically. MNPs, oldest and still most actively studied in the application of both: as therapeutics and diagnostics. Huge amount of research are related to superparamagnetic iron oxide nanoparticles - SPIONs. Some of SPIONs are under the late stage clinical investigations\textsuperscript{132}, such as Feridex\textsuperscript{®} IV (ferumoxides; Advanced Magnetic Pharmaceuticals, MA, USA) for MRI of the liver and spleen), Endorem\textsuperscript{®} (ferumoxides; Advanced Magnetic Pharmaceuticals for MRI of liver lesions, cardiac infarcts and brain lesions), Resovist\textsuperscript{®} (ferucarbotran; Bayer Schering Pharma AG, Leverkusen, Germany for MRI of liver lesions, cardiac infarcts and brain lesions) Lumiren\textsuperscript{®} (SPIONs; Advanced Magnetic Pharmaceuticals for MRI
of the bowel) Combidex® (SPIONs; Advanced Magnetic Pharmaceuticals for MRI of lymph node metastases, Magnetofluid MFL 082AS (SPIONs; Magforce AG, Berlin, Germany for Hyperthermia treatment of prostate tumors), NanoTherm® AS1 (magnetic fluid MFL AS1; SPIONs; Magforce AG, for hyperthermia treatment of glioblastoma multiforme), Ferrofluid epirubicin (Nano-Technologies GBR, Berlin, Germany for Magnetic targeted delivery of 4’-epidoxorubicin into solid tumors) Meanwhile, considering the main reason that SPIONs didn’t demonstrate good clinical profile for magnetic drug targeting, because of rapid diminishing of magnetic field generated by permanent or electromagnets as function of distance from the target tissue, pure iron core based superparamagnetic particles are more promising. The magnetic susceptibility $\chi$ of metallic iron and especially iron-cobalt alloy is more than 10 times higher, than that of oxides (magnetite, maghemite, ferrite) and iron particles can be retained magnetically at tissue depths of 10cm with fields of 15kOe or less (1500 Oe/cm)$^{133}$.

Main problem associated with iron nanoparticles is their very strong instability, which can be easily overcome by formation of protective oxide shell, which prevents them from further oxidation. Thus, just simply iron core iron oxide shell nanosystem will hold the following three modalities: magnetically separable, mediators for vibrating magnetic field hyperthermia, as well, as contrast for magnetic resonance and X-Ray computed tomography.
1.4 Magnetic resonance imaging (MRI)

It is difficult to underestimate the role of MRI in modern medicine. MRI is probably the least invasive diagnostic method of high resolution imaging of soft tissues with deep penetration. Along with positron-emission tomography (PET), single-photon-emission tomography (SPECT) and ultrasound, magnetic resonance imaging (MRI) is one of the

![Diagram of MRI principles](image)

Figure 1.4.1: The theory of MRI. a) Spins of electrons align under the external large magnetic field $B$ in a parallel and antiparallel manner and take precession with Larmor frequency $\omega$, $\gamma$ is gyromagnetic constant. b) The second coil of MRI machine provides radiofrequency pulses with resonant frequency, which decrease net magnetization and induces appearance of magnetization in perpendicular plane $xy$. c) Once the RF pulse is discontinued, the net magnetization return to the initial value, d) induced magnetization in $xy$ plane disappear with time.
«Molecular Imaging» techniques\textsuperscript{134} In contrast to X-Ray CT, PET and SPECT imaging techniques MRI uses magnetic field instead of radiation, which is less obviously harmful for the human body and environment. The magnetic resonance is physical phenomena of resonant absorption of external magnetic energy by hydrogen nuclei, protons magnetic moments – spins of abundant in organism mostly water, lipid and other biomolecules. Although other nuclei besides hydrogen are also used in MRI, the hydrogen based imaging method is more widespread because of near hundred percent of its abundance in the human body. Additionally, the protons from the different tissues will behave differently, even if tissue is the same pathologically transformed cells and their components will differ from the healthy counterparts. The spins of protons align with the strong external magnetic field $B_0$ in parallel and antiparallel manner and take precession around it with Larmor frequency $\omega_0$ (Figure 1.4.1, redrawn from the references\textsuperscript{135, 136}). This process takes place under the strong static magnetic field produced by the one of the coils of MRI machine. Another electromagnetic coil is for generation of oscillating radiofrequency magnetic pulses with the resonant frequency $\omega$, which changes the net magnetization moments of spins. The net magnetization moment is vector and has $M_z$ and $M_{xy}$ components, the values of which are changing in response to the radiofrequency pulses. Excited spins are recovering to their values after some time passed in the absence of radiofrequency pulses and this process is called relaxation.
There are two types of relaxation, which is measured by the third coil of MRI machine: Longitudinal (spin-lattice) or T₁ relaxation, which is net magnetization, Mz is coming back to the initial state after the excitement by the radiofrequency pulse and the transverse (spin-spin) or T₂ relaxation, which is decrease to zero. In the case of longitudinal T₁ relaxation, it is driven by the process of thermal loss, from the spins to their surrounding lattice through dipolar coupling. The induced change and recovery (spin-lattice) in net magnetization M₂ is explained by the energy transfer In the case of that in M_xy is because of the spin dephasing process, that is, the “de-alignment of the magnetization of excited spins in the plane xy which are in the same phase coherence. The strong interaction between the spins of protons within their close vicinity, are the cause of the rapid loss of coherence and the characteristic feature of tissues transverse relaxation T₂. Any inhomogeneity of surroundings will influence T₂ value by the following formula:

\[
\frac{1}{T_2^*} = \frac{1}{T_2} + \gamma \frac{\Delta B_0}{2}
\]

Thus, to enhance the resolution and sensitivity of this imaging method different contrast agents have been developed. The vast majority of nanoparticle containing MRI contrast agents are based on superparamagnetic iron oxide nanoparticles – SPIONs and metallic iron, cobalt or ally nanoparticles. The presence of nanoparticles in close proximity to excited protons shorten the relaxation time, by increasing inhomogeneity surroundings of the protons magnetic spins. This helps to break coherence faster.
because of the strong magnetization of these nanoparticles. Therefore, in the tissues with more concentration of nanoparticles transverse relaxation will be shortened compared to the tissues with less nanoparticles and much more compared to the tissues lacking nanoparticles. All this provided the base for use of nanoparticles as contrast agents in MRI. Vast majority of research on nanoparticle contrast agents are focused on SPION, but recently it have been shown, that metallic nanoparticles, such as iron nanoparticles, can be more advantageous MRI contrasts providing strong evidence that iron core brings great advance in MRI contrast agents development. The authors of this article have demonstrated that 16nm iron/iron oxide core shell nanoparticles are 5 times better contrast agents for nodal micromethastasis visualization in murine breast cancer model than SPIONS – accuracy was 1mm compared to 5mm with SPIONs. And the explanation of this enhancement is simply the larger magnetic moment of metallic nanoparticles compared to their oxide counterparts.
1.5 X-Ray computed tomography (X-Ray CT)

Conventional X-Ray (projection radiography) imaging and X-Ray CT imaging (3D projection radiography slices) is two of the most convenient non-invasive methods for in vivo imaging. It is routinely used in hospitals due to its lower cost compared to MRI. However, X-Ray CT and especially X-Ray, along with the ultrasound imaging are inferior to magnetic resonance imaging\textsuperscript{139} and especially positron emission tomography in tandem with MRI (PET/MRI) by preciseness of imaging\textsuperscript{140}. It is well known that by using of X-Ray imaging it is quite difficult to distinguish small size tumors even if advanced computed tomography with the beam anti-scattering filters used to increase the accuracy\textsuperscript{141}.

The principle of X-Ray imaging is simply based on difference in X-Rays attenuation coefficients, $\mu$, of tissues, because of the difference in concentration and composition of atom nuclei, comprising the tissues. Heavier the atomic mass, $Z$, of nucleus the more attenuated will be $\gamma$-photons passing through it. The negligible difference in $\mu$ values between different types of soft tissues doesn’t allow efficiently differentiate between them\textsuperscript{142}. The formula describes

![First ever X-Ray image. Wilhelm Röntgen’s wife, Anna Bertha Ludwig’s hand](image)
how mass attenuation coefficient $\mu$ used for differentiation of heterogeneous tissue types, where $\omega_i$ is the fraction of each atom with number $Z$, and the $\rho$ is the density. The units are cm$^2$/g. Thus, two key parameters are important for contrast the $Z$ mass and the density.

$$\frac{\mu}{\rho} = \sum_{i}^{n} \omega_i \frac{\mu_i}{\rho_i}$$

Because of the difference in attenuation the incident intensity $I_0$ of X-Rays are decreasing to $I$ exponentially depending from the mass attenuation coefficient: where $x$ is the thickness of the layer.

$$I = I_0 e^{-\mu x}$$

The attenuation is a cumulative result of three events which happen when $\gamma$-photons pass through material: coherent scattering ($\omega$), the photoelectron effect ($\tau$), and Compton scattering ($\delta$).

$$\mu = \omega + \tau + \delta$$

Compton scattering prevails when high energy X-rays interact with tissues mainly comprising from low atomic weight elements. In the case of heavier elemental composition photoelectron effect becomes predominating scattering mechanism.
Because organs and tissues normally contain predominantly light elements, Compton scattering is the main cause of bad contrasting and phantom noise appearance in radiograms. The quantity of Compton scattering gradually diminishes as the X-ray photon energy increases, so that high energy photons are more likely to pass through the body than low-energy photons. Because of this, the overall radiation exposure of human body with huge energy X-Rays are lower than that with low energy. But there are major issue which doesn’t allow to increase the energy. When energy of X-Rays are

![Chemical Structures]

**Ultravist® (iopromide)    Hypaque® (diatrizoate)**

![Chemical Structures]

**Omnipaque® Iohexol    Iopydol**

**Figure 1.5.2. Some clinically used iodinated X-Ray CT contrasts**

high the difference between tissues decreases. Therefore to keep the radiation levels in acceptable range and in the same time to improve detection limit of this imaging
technique new paradigm recently appeared in medicine – the use of heavy atomic number (Z) contrast agents and the K-edge contrasting agents\textsuperscript{143}. Z – Contrast agents are mainly iodine-, gold-, thorium-, bismuth- and tungsten-based. Among them most widely used are barium (as sulfate suspensions, for gastrointestinal (GI) tract imaging) and iodinated organic contrast agents. Less common contrast agents are cesium-, tin-, silver-, tantalum- and rare earth element-based\textsuperscript{144}.

Despite the wide use in clinical medicine of iodinated organic and inorganic compounds, because of high X-ray absorption coefficient of iodine atom, the lack of specificity and acute toxicity for kidneys and liver and short imaging time for operator, because of rapid clearance from the body through kidneys\textsuperscript{145, 146} other safer contrast media needed.

The one of the first attempts to find stronger contrast agents than iodine was the bismuth sulfide nanoparticles\textsuperscript{147}. The mentioned particles gave five-fold increase in contrasting compared to iodinated contrasts, but the inherent toxicity of bismuth ions (in the case of release) may lead to safety issues for their further successful clinical development. Later, Kim et al. reported that 30nm PEG coated gold nanoparticles could be perfect contrast agents, due to 5.7 times higher X-Ray attenuation of gold atom compared to widely used Ultravist\textsuperscript{®}\textsuperscript{148}. They have demonstrated that PEG-gold nanoparticles also have 24 times longer blood circulation time. Authors also demonstrated 2 fold contrast enhancement of rat hepatoma and normal liver tissue. One year later another group advanced gold nanoparticle based X-Ray contrast concept by
making them targeted.\textsuperscript{149,150} They attached UM-A9 antibody (anti α6-β4 integrin mAb) on gold surface through polyacrylic acid linker. They presented an \textit{in vitro} and \textit{in vivo} proof of 5 times higher contrasting of head and neck cancer cells compared to the healthy ones.
1.6 Magnetic Hyperthermia

“I would cure all diseases if I only could produce fever”

Parmenides, 515-460 BC

The therapeutic effect of the body temperature above physiological levels - hyperthermia, is well known since ancient times. More than half-a-century ago it had been demonstrated that alternating magnetic field could induce hyperthermia when applied to magnetic nanoparticles. This therapeutic technique is based on local and selective heating of tumor leading to death of its composing cancer cells. The success of this technique depends on relative transparency of human body tissues for the wide range of alternating frequency magnetic fields and also on higher vulnerability of tumor and its comprising cancer cells toward high temperatures and oxidative stress compared to the healthy tissues and cells. This phenomena was reported back in 1977 and

![Electromagnetic spectrum](image)

**Figure 1.6.1. Electromagnetic spectrum**
continue to be essential for modern magnetic and other hyperthermia cancer treatment regimens. In generally two main regimens are known for the delivery of heat to diseased sites. One in a range from 41 to 46 °C which separated and called itself “hyperthermia” and another – with aggressive heating above 46 up to 56 °C, named as “thermoablation”. Thermoablation leads to necrosis, carbonization and ultimately destruction of tumor. Meanwhile hyperthermia, a milder heating method, induces somehow reversible changes in cellular level, and sometimes used as adjuvant to radiation therapy or chemotherapy, potentiating the response of cancer cells to the mentioned treatment regimens.\[153\].

There are many ways of high temperature administration to diseased sites and they are divided by the electromagnetic energy spectrum used – microwave hyperthermia (gigahertz range, typically 2.47 GHz) and thermal ablation, radiofrequency capacitive

![Figure 1.6.2: Electric and magnetic components of electromagnetic waves](image)

- 46 -
hyperthermia (2 to 30 megahertz range, typically 13 MHz) and the alternating magnetic field hyperthermia (100 -1000 kilohertz range, typically 300-500 KHz). The ways how materials interact with varying wavelengths are quite different. While for the microwave and radiofrequency (RF) wavelengths electric component $E$ of electromagnetic wave are dominating factor, which heat high conductivity components and ions, in the case of kilohertz frequencies magnetic component $H$ (or $B$) of electromagnetic wave become dominating factor. In contrast to MW and RF frequency, at this specific range magnetic properties of materials starts to play the role in conversion of EM energy to heat. Whereas human body contains many ions which can generate heat while exposed to MW and RF field, they are lacking detectible magnetic components and it makes tissues relatively transparent in this range of frequencies. This mean, that cellular components will not heat significantly in the absence of magnetic material and will heat when the last will be specially introduced. More dense will be represented magnetic components in tumor volume, higher will be heat generated. But because there are a limit of how much magnetic material could be

**Figure 1.6.3. Heat dissipation mechanisms under applied AC by magnetic nanoparticle**
accumulated in tumor research are focused in development of efficient magnetic materials as heat mediators.

As the hyperthermia is more controllable regimen compared to thermoablation it is more preferred in modern medicine and therefore most of the research are focused on development of efficient heat transducers for localized treatment. This transducers act as mediators of magnetic energy dissipation as heat. Since the discovery of this method mediators mainly were magnetic nanoparticles, which are because of their size and surface chemistry tunability allows selective targeting and accumulation into the tumor site, thus providing the necessary difference for localized heating. Moreover they could be introduced by intra-tumoral or intravenous injection, which makes easier procedure of heat delivery compared to other routes, such as optical probes, microwave antennas, radiofrequency generating electrodes, infrared devices, intraperitoneal heated fluid circulation or laparoscopic delivery\textsuperscript{155}.

There are four mechanism of dissipation of electromagnetic energy as heat in case of single magnetic domain superparamagnetic nanoparticles. When external magnetic fields $H$ is applied antiparallel to the $M$ magnetic moment of nanoparticle, magnetic domain of it tends to rotate in parallel direction toward external field $H$ to lower energy (form metastable state). This mechanism is called Neel relaxation. Alternatively, when whole nanoparticle rotates in parallel direction of $H$ it called Brownian relaxation. Both processes lead to heating. The third mechanism is hysteresis loss, which is contributing when multidomain nanoparticles are used. Forth mechanism is eddy currents loss, are
applicable only for naked conductive metallic particles and related to heating through
perpendicular to $H$ circular currents $E_{ec}$ generation on surface of particles. Any
insulator coating on surface suppress this process and leave only Neel an Brownian
losses. Both mechanisms generate power loss $P$ (hysteresis losses) in anisotropy field
$H_K$. In the following formula $f$ is the frequency of alternating current and $\omega$ is weight of
magnetic particles. From this formula follows that higher will be frequency of cycles
and bigger will be $H_K$ (ideally equal to $H$) higher will be power losses

$$P_H = 4H_Kf \frac{\mu_0}{\omega}$$

Each relaxation process is characterized by its relaxation time. For the nanoparticles
with size above 10 nm Neel and Brownian relaxations are equally contributing to the
power loss.

$$\frac{1}{\tau} = \frac{1}{\tau_N} + \frac{1}{\tau_B} = f_0 e^{-\frac{KV}{Tk_B}} + 3\eta \frac{V_H}{Tk_B}$$

Where $\tau_N$ is Neel relaxation time and $\tau_B$ is Brownian relaxation time, $f_0$ is the attempt
frequency of $10^9$ s$^{-1}$, $k_B$ is the Boltzmann constant, $T$ is the temperature, $\eta$ is viscosity
of media and the $V$ is hydrodynamic volume of nanoparticle$^{154}$. As it was noted before
for small superparamagnetic nanoparticles, $\tau_N$ component dominates and power loss
will be expressed as:
\[ P_H = \pi \mu_0 \chi'' f \frac{H_{AC}^2}{\omega} \]

From this equation follows that to maximize the power loss the frequency \( f \) should be adjusted to \( \tau^{-1} \) and the amplitude of alternating current magnetic field \( H_{AC} \) maximized.

In practice to measure heat generation efficiency for various magnetic nanoparticles equivalent to specific loss power concept (SLP, which has theoretical value) specific absorption rate, SAR concept is used. SAR could be measured directly by measuring of rise of temperature \( T \) over the time \( t \) when known amount of nanoparticles \( m_{NP} \) are dispersed in the \( M_i \) total mass of media (water or organic solvent) with specific heat capacity \( C_{Pi} \) and the density \( \rho_i \) for each component. Simply SAR calculation formula is the following \(^{156} \).

\[ SAR = \sum_{i=1}^{n} \frac{C_{Pi} \rho_i M_i}{m_{NP}} \frac{dT}{dt} \]

Thus specific absorption rate will be higher in the case if small amount of nanoparticles will give higher temperature rise. This is crucial for biological application, because of naturally high rate of temperature dissipation inside of body and also difficulties of high loading of nanoparticles in specific area.
Despite the vast majority of magnetic particles studied for magnetic hyperthermia are metal oxide based, currently, some attention shifted on iron and combined iron/iron oxide based nanosystems. Because of the fact that saturation magnetization of iron more than twice that of any iron oxide, iron nanoparticles have twice higher specific absorption rates\textsuperscript{157}. Higher specific absorption rate allows minimization the overall amounts of nanoparticles necessary to undergo hyperthermia along with minimization of damage of the surrounding untargeted tissue\textsuperscript{158}.
1.7 Near-Infrared Photothermal therapy

“Where drugs do not cure, iron does; where iron does not cure, heat does; where real heat does not cure, cure is impossible”,

Hippocrates, 470-377 BC

Another thermal therapy method which gained significant attention in cancer treatment is based on use of near infrared (NIR) region of electromagnetic wavelengths. Because the most efficient way of delivery high energy light in a highly spatially controlled way is laser technology, various NIR-lasers were utilized for photodynamic (PDT, conversion of light energy to singlet oxygen) and photothermal treatment and ablation (PTA) of cancer. While photothermal ablation is extreme rise of temperature, much far from its physiological ranges (> 48 °C), which causes irreversible injury to the cells, the photothermal therapy (PTT) is a moderate hyperthermia method (41-48 °C) (PTT)\textsuperscript{159}. PTT method is based on phenomenon of near infrared energy absorption and conversion to thermal energy by materials, having specific absorption at this region. The first material used successfully for this property was gold nanorods\textsuperscript{160}, since there, the success of combination of NIR-laser and NIR-absorbing materials in cancer treatment catalyzed very intensive research in this field\textsuperscript{161-168}. Before the pioneering work with gold nanorods, laser-light induced photothermal therapy, has been traditionally considered as a non-reliable technique because of the fact that human tissues has large absorption coefficients in the visible range of the electromagnetic spectrum. It were limiting factor for the photothermal treatments to superficial tumors.
Moreover the visible spectrum laser energy was measured to be absorbed by all tissues, healthy and cancerous, limiting the differentiation until the concept of “optical windows” for biological tissues have been discovered\textsuperscript{169}. Within this range of wavelengths not only thermal effect but also imaging became possible\textsuperscript{170}. Simply, there are three regions at which laser light penetrate at maximum depth. First “optical window” is from 650 to 950 nm wavelength (some authors estimated as from 700 to 980 nm\textsuperscript{159}). It is the most widely used region and correspond to the minimum absorptions range of Hemoglobin and water. The second biological “optical window” is from 1000 to 1400 nm and limited from below and above by the water characteristic absorption. In generally, in tissue, the major absorber is hemoglobin, accounting for 39–64% of total absorbance at NIR laser wavelengths\textsuperscript{171}. Both regions have advantages
and disadvantages. For the first “window” the advantage is the absence of significant background absorption by hemoglobin or water, but the disadvantage is high level of scattering. For the second “window” scattering is low which makes it good for imaging, but the background absorption of water is more compared to the first “window”. There are also third “optical window”, which ranges from 1500 to 1800 nm and mainly used for NIR-imaging purposes. Minimization of background absorption and scattering is prerequisite for successful application of laser light in biological systems. The monochromatic and highly focused nature of lasers allows application strictly narrow range of wavelength of choice to minimize undesired interactions with surrounding tumor healthy tissue. Additionally, it also allows deeper penetration of the laser energy inside the tissue. Moreover, if specific light-to-heat converting mediator materials used, the selectivity may be increased dramatically. There are clear advantages of use of nanoparticles as mediators for this purpose. The absorption cross section of nanoparticles is the orders of magnitude larger than that of organic dye molecules\textsuperscript{172}. 
1.8 Microwave ablation and microwave hyperthermia

Microwave cancer ablation and microwave hyperthermia technology is relatively new compared to other thermal treatment regimens, such as radiofrequency, alternating magnetic hyperthermia and NIR-laser photothermal therapy methods\(^\text{173}\). It was tested for use in clinics as a non-invasive ablation technique for advanced stage non-operable cancers, such as melanoma,\(^\text{174, 175}\) hepatic tumors,\(^\text{176-181}\) advanced invasive and early stage breast cancers,\(^\text{182-185}\) osteosarcoma,\(^\text{186}\) lung cancer,\(^\text{187-194}\) renal carcinomas,\(^\text{195-197}\) model of pediatric brain cancer,\(^\text{198}\) adrenal carcinomas,\(^\text{199}\) pancreatic cancer,\(^\text{200, 201}\) bladder cancer,\(^\text{202}\) colorectal cancer,\(^\text{203}\) head and neck cancer\(^\text{204}\).

<table>
<thead>
<tr>
<th>Company/Product</th>
<th>Certification</th>
<th>Frequency/Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covidien (USA) Evident</td>
<td>FDA/CE</td>
<td>915 MHz / 45 W</td>
</tr>
<tr>
<td>MedWaves (USA) – MedWaves</td>
<td>FDA/CE</td>
<td>915 MHz / 45 W</td>
</tr>
<tr>
<td>BSD Medical (USA) Micro Therm</td>
<td>In process</td>
<td>915 MHz / 60 W 3 Antennas</td>
</tr>
<tr>
<td>NeuWave (USA) Certus 140</td>
<td>In process</td>
<td>2450 MHz / 140 W3 antennas</td>
</tr>
<tr>
<td>Hospital Service (Italy) Amica</td>
<td>FDA/CE</td>
<td>2450 MHz / 100</td>
</tr>
<tr>
<td>Acculis (UK) – Acculis</td>
<td>CE</td>
<td>2450 MHz / 100</td>
</tr>
</tbody>
</table>

Table 1.8. Microwave systems on market. Data from ref [205]
Systems for microwave ablation and hyperthermia comprise a generator, power distribution system, and an interstitial or focused applicator. In the case of interstitial applicator it has a single or multiple antennas. The most widespread are focused microwave systems and the systems with coaxial multiple antennas.

The mechanism of microwave interaction with tissue is based on cause of very fast rotational energy dissipation as heat (due to friction) of molecules having large dipole moments in response to high frequency oscillation of electric $E$ component of electromagnetic field$^{173}$. This process is called dielectric hysteresis. The specific absorption rate or heat generation rate $Q_h$ depends on second order power of amplitude of $E$, effective conductivity $\sigma$ and the mass density $\rho$ of tissue$^{175}$:

$$SAR = \frac{1}{2\rho} Q_h = \sigma |E|^2$$
The typical frequencies used in cancer treatment are 915 MHz and 2.45 GHz\textsuperscript{205}, although lower power and more cancer selective minimally invasive higher frequencies (2 − 18 GHz) are also under experimental testing\textsuperscript{206}. Thus more molecules with high dipole moments will present in media, more will be heating effect. It is known that in this case the dielectric permittivity $\varepsilon$ of media will be high. Moreover tumor is more dense, acidic and contains more ions in cellular media, increasing conductivity.

$$\varepsilon = \varepsilon_r\varepsilon_0 = (1 + \chi)$$

Thus, one of the reasons of successful use of microwave irradiation in cancer treatment is some degree of differential interaction of tumor tissue with microwave compared to the healthy tissues.

It has been shown that dielectric permittivity of tumor is higher compared to the healthy tissue\textsuperscript{207}. Differential interaction is a key factor, therefore tissue that respond best to microwave hyperthermia and ablation are those with significant differences in permittivity values between tumors and the surrounding healthy environment. The relatively frequent use of microwave ablation in treatment of breast and lung cancers is because in these particular cases, breast tumor are surrounded with fat, which has much lower dielectric permittivity and the lung tumors are surrounded by air\textsuperscript{205}.

As can be seen from the above literature review unlike magnetic hyperthermia technique, microwave hyperthermia and ablation technique are solely based just on
differential interaction of microwaves with tissues and there are no mediator nanoparticles studied to increase the specificity and effectivity of this process. Moreover, only the microwave radical ablation was considered for the cancer treatment, because the microwave moderate hyperthermia had no advantages over other techniques, especially over the alternating magnetic field hyperthermia in terms of controllability. Developing new nanomaterials that can be triggered by external stimuli without extreme temperature elevation can provide a safer route to utilize radiation techniques for cancer therapy.
Chapter 2

Synthetic Medicinal Chemistry of *iso*-Phidianidines

2.1 Background

Oxadiazoles are five membered heterocyclic compounds which can exist in three regioisomeric forms: 1,2,4-oxadiazoles are the most common forms of oxadiazoles (50%), 1,3,4 oxadiazoles (42%). Less common forms are 1,2,5-oxadiazoles (7%) and 1,2,3-oxadiazoles (1%) \(^{208,209}\).

![Regioisomeric forms of oxadiazoles](image)

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**Figure 2.1.1: Regioisomeric forms of oxadiazoles**

The compounds bearing 1,2,4-oxadiazole heterocyclic units are very attractive for the medicinal chemistry. Particularly, many reports published about the synthesis of 1,2,4-oxadiazoles with various modes of anticancer activity \(^{210-236}\). Despite such a generous
variety of studies on synthetic medicinal chemistry of 1,2,4-oxadiazoles, only two natural products, bearing this unit are known. Specifically, the Phidianidines as a versatile target for synthetic chemistry attracted our attention.

Phidianidines are a new class of marine indole alkaloids recently isolated from the aeolid opisthobranches shell-less mollusk Phidiana militaris by Gavagnin M. et al. Their structure is composed 1,2,4-oxadiazole heterocyclic ring connected to indole heterocyclic ring through methylene bridge. The authors note that the only known natural product possessing a ring related to the 1,2,4-oxadiazole core before their discovery of phidianidines was the quisqualic acid (Figure 2.1.4)- strong agonist for AMPA (R-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and group I metabotropic glutamate receptors. It was isolated from the seeds of Quisqualis indica and Q. fructus.

Figure 2.1.2: Phidianidines A and B structures and the photos of Phidiana militaris mollusks from where they have been isolated.
The interesting properties of phidianidines are their cell type-selective cytotoxicity profile. The tested cell lines were C6 rat glioma cells, HeLa human epithelial cervical cancer cells, CaCo-2 human epithelial colorectal adenocarcinoma cells and non-cancerous H9c2 rat embryonic cardiac myoblasts and 3T3-L1 murine embryonic fibroblasts. As could be seen from the activity profile phidianidines were much more potent towards highly proliferating glioma and epithelial cervical cancer cells and embryonic fibroblasts that on slowly proliferating colorectal adenocarcinoma cells.

<table>
<thead>
<tr>
<th>cell line</th>
<th>phidianidine A (1)</th>
<th>phidianidine B (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6</td>
<td>0.642 ± 0.2</td>
<td>0.98 ± 0.3</td>
</tr>
<tr>
<td>HeLa</td>
<td>1.52 ± 0.3</td>
<td>0.417 ± 0.4</td>
</tr>
<tr>
<td>CaCo-2</td>
<td>35.5 ± 4</td>
<td>100.2 ± 8.5</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>0.14 ± 0.2</td>
<td>0.786 ± 0.3</td>
</tr>
<tr>
<td>H9c2</td>
<td>2.26 ± 0.6</td>
<td>5.42 ± 0.8</td>
</tr>
</tbody>
</table>

*IC₅₀ values are expressed as mean ± SEM (n = 24) of three independent experiments. Bold values show IC₅₀ of less than 1 μM.

**Figure 2.1.3. Cytotoxicity profile of phidianidine A and phidianidine B on cancer and healthy cell lines, IC₅₀ (μM). Reprinted from ref [237]**

![Quisqualic acid](image)
cells and cardiac myoblasts.

Although the outstanding property of phidianidines is known for 4 years, until now there are no many reports done on understanding the mechanism of action underlying the cell selective profile. The only study, undertaken on evaluation of pharmacological target for phidianidines were done by Amodeo P. et al\textsuperscript{241}. The authors have developed new computational docking model based on minimal hybrid ligand/ receptor-based pharmacophore for CXCR4 receptor and screened for the virtual library of natural products. Screening resulted in prediction of good docking for phidianidine A which they have proven experimentally. The CXCR4 is CXCL12 (SDF-1) chemokine receptor involved in cancer survival, progression, proliferation, gene transcription, metastasis, angiogenesis (formation of tumor feeding blood vessels network)\textsuperscript{242} Thus CXCR4 is excellent target for anticancer medicinal chemistry.

Besides the anticancer action, central nervous system (CNS) activity for phidianidines and their derivatives were reported. Thus, Guo, Y. W et al\textsuperscript{243} reported neuroprotective
properties of some phidianidines analogs (Figure 2.1.3). The authors suggest for their potential candidacy for the treatment of Alzheimer’s disease.

Another mode of biological activity of phidianidine analogs were reported by Lindsley, C. W. et al\textsuperscript{244}. The authors evaluated the selective inhibition profile of phidianidines A and B of dopamine transporter (DAT), without affecting the serotonin (SERT) and norepinephrine (NET) transporters function (Figure 2.1.6.)

\[
\begin{array}{c|c|c|c|c}
\text{Compd} & \text{DAT} & \text{Opioid-\(\mu\)} \\
 & K_i (nM) & IC_{50} (nM) & K_i (nM) & EC_{50}^a \\
\hline
\text{A} & 310 & 390 & 230 & 17\% \\
\text{B} & 680 & 860 & 340 & 12\% \\
\end{array}
\]

*\(\%\) activation at 10 \(\mu\)M reported in a GTP\(\gamma\)S assay relative to 10 \(\mu\)M DAMGO control, suggest potential weak, partial agonism of \(\mu\)OR.

Figure 2.1.6\textsuperscript{45}: DAT and Opioid-\(\mu\) inhibition profile of Phidianidines A and B

Moreover, the natural compounds had also selective and potent ligand properties and agonistic activity for \(\mu\)-Opioid receptor, without any affinity toward \(\delta\)- and \(\kappa\)-Opioid

Figure 2.1.7 Snider’s scheme: hydroxyguanidine precursor
receptors.

Recently, Guo et al.\textsuperscript{245} reported neuroprotective effect of phidianidine-analogs against amyloid-\(\beta\)25–35 (A\(\beta\)25–35)-, hydrogen peroxide (H\(_2\)O\(_2\))- and oxygen–glucose deprivation (OGD)-induced neurotoxicity in SH-SY5Y. This property may serve as

![Figure 2.1.8. Snider’s scheme: 1,2,4-oxadiazole through acid chloride route](image)

**Figure 2.1.8. Snider’s scheme: 1,2,4-oxadiazole through acid chloride route**

amyloid-\(\beta\)25–35 (A\(\beta\)25–35)-, hydrogen peroxide (H\(_2\)O\(_2\))- and oxygen–glucose deprivation (OGD)-induced neurotoxicity in SH-SY5Y. This property may serve as

![Figure 2.1.9 Snider’s scheme: azide reduction and guanidylation](image)

**Figure 2.1.9 Snider’s scheme: azide reduction and guanidylation**
alternative strategy toward management of Alzheimer disease.

The synthetic studies on phidianidines and their analogs are also few. First total synthesis in eight steps and 19% overall yield were reported by Snider, B. B. et al\textsuperscript{246}. On a Snider’s synthesis scheme (Figure 0.8) starting material is pentamethylene diazide which is converted to 5-azido-1-pentanamine in 85% yield by selective reduction with triphenylphosphine in Et\textsubscript{2}O/EtOAc, Selectively reduced amine reacted further with cyanogen bromide in dichloromethane and aqueous NaHCO\textsubscript{3} solution leading to formation of unstable cyanamide. Freshly formed cyanamide was immediately converted to hydroxyguanidine, which was also by reacting with hydroxylamine hydrochloride in ethanol using potassium carbonate as basic catalysis. Another precursor toward phidianidine was prepared though conversion of indole-3-acetic acid to chloroanhydride by reacting with oxalyl chloride in dichloromethane containing catalytic amounts of dimethylformamid.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.1.10.png}
\caption{Lindsley’s scheme: Boc protected hydroxyguanidine precursor}
\end{figure}
Freshly formed acid chloride and hydroxyguanidine precursors were coupled in dichloromethane at room temperature leading to the linear product, which were further cyclized to 1,2,4-oxadiazole by heating in dichloroethane (Figure 2.1.8). Then authors reduced azide with activated zinc and ammonium formate in methanol at room temperature. The obtained amine was reacted with Boc-protected S-methylisothiourea without further purification using silver nitrate in dimethylformamide with the trimethylamine catalyst leading to the formation of protected guanidine, deprotection of

![Chemical reaction diagram]

**Figure 2.1.11 Lindsley’s route toward phidianidines**
which by trifluoroacetetic acid in dichloromethane afforded phidianidine B trifluoroacetic salt (Figure 2.1.9).

The interesting findings reported by the authors: amino-terminated phidianidines were averagely 2.45 times more potent on National Cancer Institute (NCI) 60 cell line growth inhibition, compared to guanidine terminated analogs. And the presence of bromine in Phidianidine A were making it and its amine precursor more potent compared with non-brominated Phidianinde B. The strongest activity at single dose 10 µM were reported for amino-terminated Phidianidine A for the following cell lines (in a percent’s of cell death) OVCAR-3 (ovarian cancer) – 50.18%, HL-60TB (leukemia) – 48.83%, NCI-H522 (non-small cell lung cancer) – 38.26%, MDA-MB-435 (melanoma) – 36.97%, M14 (melanoma) – 26.33% and SF-539 (CNS cancer) – 23%. For the same dose Phidianidine B demonstrated the following activity (in a percent’s of growth inhibition) – HL 60TB (leukemia) – 39.54%, MDA-MB345 (melanoma) 33.08%, UO-31 (renal cancer) – 31.1%, HOP-92 (non-small cell lung cancer) – 28.86% and MALME-3M (melanoma) 26.62%. Later Lindsley, C. W. et al.244 reported improved total synthesis scheme for phidianidines A and B - in eight steps (the authors claimed 6 steps considering the availability of mono Boc protected 1,5-pentanediocidmine and one pot coupling and condensation of the partner precursors) starting from indole acetic acids in overall yields 39.9% for Phidianidine A and 21% for Phidianidine. Lindsley group’s synthesis scheme starts from the 1,5-diaminopentane starting material (Figure 2.1.11) instead of the pentamethylene diazide as in the Snider’s scheme. First authors
are selectively protecting one amino group of commercially available 1,5-diaminopentane with butoxycarbonyl (Boc) then reacted with cyanogen bromide to form unstable intermediate which were converted to hydroxyguanidine by reacting with hydroxylamine in ethanol.

The hydroxyguanidine precursor were coupled with indole acetic acids using HATU-mediated coupling affording linear coupling product, which were later cyclized by heating in dichloroethane affording the desired 1,2,4-oxadizoles. Subsequently Boc deprotection, guanidination and another deprotection leaded to formation of phidianidines. (Figure 2.1.12)

Figure 2.1.12 Chemberland’s concise synthesis of phidianidines
Authors reported the absence of cytotoxicity of Phidianidines A and B on HEK293 cells (human epithelial kidney healthy cell line) after 24 and 48 h incubation measured
by WST-1 cell proliferation assay at concentrations of 10 μM, relative to the DMSO control, the same concentration used by Snider et al. Shortly after Snider’s and Lindley’s groups publications another synthetic route toward Phidianidines were reported by Gavagnin, M. et al. The difference is in the absence of use of toxic cyanogen bromide and manipulation with highly unstable intermediates (Figure 0.13). The most concise synthesis was developed by Chemberland, S. et al. with five steps through 1,5-pentanediamine and yield for Phidianidine A and B 5.4% and 5.7%, respectively. On their synthesis scheme key reaction is coupling of indole-3-acteic acids chlorides with Boc protected hydroxyguanidine precursor, which have been prepared in 88% yield from 1,5-pentanediamine by the route developed by Botta, M et al. The authors also mentioned the absence of necessity to use small molecule azide.
intermediates compared to the previous schemes, as another benefit of their synthetic strategy from the safety concerns.

Shortly after these works on the total synthesis of phidianidines and some analogs, D. Kumar et al\textsuperscript{250} reported the synthesis and biological evaluation of 2-arylamino-5-(3-Indolyl)-1,3,4-oxadiazoles, which are regioisomeric analogs of phidianidines with directly attached 1,3,4-oxadiazole to indole, as potent cytotoxic agents. The authors discusses the interesting structural similarity of phidianidines and their analogs with Labradorins (Figure 2.1.15), which are tested to be an inhibitors of non-small-cell lung carcinoma with GI value 40.8 µm and pancreatic adenocarcinoma with 50% growth inhibition value 40.8 µM and pancreatic adenocarcinoma with 50% growth inhibition value 25.µM\textsuperscript{251} and Topsentsins (cytotoxic on multidrug resistant cancer cell lines) and Nortopsentins, which are potent inhibitors of human and murine leukemia cells\textsuperscript{252-255}. They also note that replacement of other functional groups on indole pharmacophore unit may yield strong cytotoxic agents because of unique physicochemical and

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Exchange of pyridyl, aryl pharmacophores on indole increases potency}
\end{figure}
pharmacological properties of indole directly attached to oxadiazoles. Such in the case of 1,3,4-oxadiazoles and 1,3-oxazoles exchange of aryl unit on indoles dramatically increases cytotoxicity (on compound IMC-094332, Figure 2.10.15)\textsuperscript{256}. Biological activities of these new compounds, phidianidines regioisomers lacking methylene bridge between two heterocyclic systems, were very high. Thus for some compounds inhibitory activity selectively against the HeLa (human cervical cancer cell line) and the MCF-7 (human breast cancer cell line), (IC\textsubscript{50}) less than 1 nm reported\textsuperscript{250} (Figure 0.17). Remarkable cytotoxicity was detected also against PC-3, LNCaP (human prostate cancer cell lines) and MDA-MB-231 (human breast cancer cell line). Study on

![Figure 2.1.16: In vitro cytotoxicity data for 2-arylamo-5-(3'-indolyl)-1,3,4-oxadiazoles](image)

<table>
<thead>
<tr>
<th>Compd</th>
<th>R\textsuperscript{1}</th>
<th>R\textsuperscript{2}</th>
<th>HEK293</th>
<th>HeLa</th>
<th>LNCaP</th>
<th>MCF-7</th>
<th>PC3</th>
<th>MDA-MB-231</th>
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</thead>
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<td>11a</td>
<td>H</td>
<td>C\textsubscript{4}H\textsubscript{6}</td>
<td>1.5±0.01</td>
<td>2.2±0.04</td>
<td>3.0±0.01</td>
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<td>4.7±0.04</td>
<td>9.8±0.01</td>
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<tr>
<td>11b</td>
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<td>4-CH\textsubscript{2}CH\textsubscript{2}</td>
<td>2.4±0.04</td>
<td>0.0001</td>
<td>2.8±0.01</td>
<td>0.2±0.01</td>
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<td>9.8±0.01</td>
</tr>
<tr>
<td>11c</td>
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<td>4-OCH\textsubscript{2}CH\textsubscript{2}</td>
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<td>3.1±0.01</td>
<td>6.5±0.01</td>
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<td>0.9±0.01</td>
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<td>Dextromethorphan</td>
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<td>-</td>
<td>0.4±0.02</td>
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<td>1.4±0.06</td>
<td>7.0±0.02</td>
<td>1.3±0.01</td>
</tr>
</tbody>
</table>

Figure 2.1.16: In vitro cytotoxicity data for 2-arylamo-5-(3'-indolyl)-1,3,4-oxadiazoles

mechanism of cell death on MDA-MB-231 by propidium iodide assay revealed apoptotic nuclei after the 48 h of incubation, which were consistent to the previous study\textsuperscript{222} relating the 1,3,5-oxadiazoles.
2.2 Experimental section

2.2.1 Design of iso-Phidianidines

Combining the information from literature one can easily come to the conclusion, that bi-heterocyclic systems comprising of indoles and oxadiazoles linked together are well proven models for synthetic medicinal chemistry study as anticancer and antiproliferative drug candidates. Many research works reviewed in the section 2.2 were focused on synthesis and medicinal chemistry of phidianidines and some of their direct analogs, as well, as 1,3,4-oxadiazoles synthetic medicinal chemistry as one of

![Diagram of Phidianidine and iso-Phidianidine](image-url)

Figure 2.2.1. Design of synthetically easy accessible novel compounds with anticancer activity – iso-Phidianidines
regioisomeric analogs of phidianidines. But, to the best of our knowledge, up to day, there were no studies on 1,2,4-regioisomers of phidianidines.

With the aim to develop short and high yielding synthetic route toward regioisomeric phidianidines, first we have designed the structure of \textit{iso}-phidianidines. Taking into account that molecules are aromatic, so they are flat, sterically the change of N and O should not affect significantly the overall geometry of molecule. Moreover, oxadiazole moiety of the biologically active compounds have been widely considered as metabolically stable isosteric groups of esters and amides, as if there is no any specific “key-lock” type specific interaction with biological substrates. Computer aided energy minimization (MM2 method in ChemBio3D Ultra software) for both molecules demonstrated exactly the same geometry, thus providing rationale for further synthetic studies.

![Figure 2.2.2 Snapshot after the energy minimizations for both phidianidines and iso-phidianidines in ChemiBio3D Ultra demonstrating identical geometry](image)
2.2 Experimental section

2.2.2 Synthetic chemistry

Reagents obtained from the commercial suppliers (Sigma Aldrich, Alfa Aesar, TCI) and were used without further purification. Dry solvents with deep degree of drying was achieved by incubating of organic solvents over molecular sieves for 72 hours. Molecular sieves (3Å) were used to dry polar solvents such as methanol, ethanol, dimethylformamide, dimethylsulfoxide. Molecular sieves (4 Å) were used for drying of apolar organic solvents such as dichloromethane, chloroform, dichloroethane, hexane, ethyl acetate. All glassware was dried by heat gun heating under vacuum. All reactions were performed in heat gun-dried flasks under nitrogen and monitored by thin layer chromatography (TLC). TLC was performed on silica coated aluminum sheets (60 F254) supplied by Merck. Compounds were visualized with UV light and para-anise aldehyde stain followed by gentle heating with heat gun. Flash column chromatography was performed using Flash Silica 60 Å (230–400 mesh) 9385 supplied by Merck. \(^1\)H and \(^13\)C NMR spectra were recorded using Bruker 400, 500, 600 and 700 MHz instruments, as indicated. Deuterochloroform, d6-dimethyl sulfoxide, d4-methanol and D\(_2\)O were used as the solvents, and chemical shifts (δ) are given in parts per million (ppm) relative to the standard reference TMS or residual peaks of protonated solvent. Peak assignment was achieved using two-dimensional, \(^1\)H–\(^1\)H and \(^1\)H–\(^13\)C NMR experiments. Infrared spectra were recorded on a Thermo Scientific Nicolet iS10
spectrometer. Samples were prepared by mixing of neat compound with KBr in mortar. High resolution mass spectra were carried out on a Thermo Sci.

Chemistry: General scheme of synthesis of the 3-indolylmethyl substituted 1,2,4-oxadiazoles at the position of 3' of the oxadiazole ring is described here. In generally, first reaction is formation of indolyl-3-methylamidoxime by refluxing the indolyl-3-acetonitrile with hydroxylamine hydrochloride and sodium bicarbonate in methanol which after 8 hour leded to the product with 90 % yield after recrystallization from methanol/DCM. Though indolyl-3-acetonitrile is commercially available, we prepared it quantitative yield in two steps from indole. Briefly, A 50-mL round flask was charged with secondary amine (3, 2 mol), EtOH (3 mL), zinc chloride (3mmol), formaldehyde (2, 2 mmol, 36% aq.), and indole (1, 2mmol). The mixture was stirred for a period of 1.5 h. After dilution with H₂O (7 mL), the mixture was made basic with NaOH (6 mmol, 20% aq.) and extracted three times with ethyl acetate (3x10 mL). Ethyl acetate was evaporated under reduced pressure. The residue was diluted with H₂O (8 mL) and acidified with HCl (4 mmol, 20% aq.). After filtration, the mother liquor was

![Figure 2.2.1. Amidoxime formation through commercially available 3-indolyl acetonitrile](Image)
basified with NaOH (7 mmol, 20% aq.), the resulting suspension was cooled in an ice bath. The product was collected by filtration and washed with water. If the product was oil, the product was extracted three times with diethyl ether (3x10 mL), the combined organic layers were dried over anhydrous MgSO₄, and the solvent was evaporated under reduced pressure. All the products were confirmed by comparing their melting points (165 - 167 °C), ¹H and ¹³C-NMR data with data reported in literature²⁵⁷. The second reaction may be done without deep chromatographic purification of obtained

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**Figure 2.2.2. First step toward indole-3-acetonitrile. Formation of Mannich’s base with zinc chloride catalyst**

**Figure 2.2.3. Formation of indole-3-acetonitrile**
Mannich’s base in quantitative yield\textsuperscript{258}. Thus, obtained product were dissolved in methanol, diluted with water and cooled to -10\(^\circ\)C. Methyl iodide was then gently syringed using syringe pump in a period of one hour, maintaining temperature of solution low. After the completion of addition ice bath was removed and the reaction was allowed to stir overnight at room temperature. Then mixture was diluted with triple amount of ethyl acetate and washed with water three times, then with brine. Ethyl acetate was removed by rotovap and residue was chromatographed in silica column using hexane-ethyl acetate solvent system. Next reaction is the novel variation of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Figure 2.2.4. Formation of oxadiazole from cyclocondensation of amidoxime with trichloracetic acid}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Figure 2.2.5. Nucleoplyic aromatic substitution with various diamines. Trichloromethyl as excellent leaving group}
\end{figure}

\textsuperscript{258}Reference number.
known formation of oxadiazole through condensation of amidoxime with trichloroacetic acid anhydride. The typical reaction procedure is following: trichloroacetic acid anhydride have been dissolved in dry ethyl acetate and di-isopropyl ethyl amine have been added. The reaction mass have been cooled to ice-temperature and amidoxime added dropwise previously dissolved in ethyl acetate. Then temperature allowed to rise until ambient and after stirring additional 2 hours reaction have been carefully quenched by addition of 10% solution of sodium bicarbonate and worked-up with sat. NaHCO₃ and brine solutions, dried over anhydrous magnesium sulfate evaporated and chromatographed on silica using ethyl acetate hexane system to lead pure compound with 91% yield. This is the best yield for trichloro-substituted compound. Previous attempts to get it through coupling with HATU with trichloroacetic acid have been unsuccessful in the terms of yields. HATU coupling gave only 36% yield. Amidoxime have demonstrated its incompatibility with acids. Reaction of aromatic nucleophilic substitution of indolyl-trichloromethyl oxadiazole with various diamines have been tried in methanol and in ethyl acetate. Methanol as solvent leaded to decomposition of starting material. Ethyl acetate gave 80-91% yields of pure

Figure 2.2.6. Guadinylation with trifly activated Boc protected guanidine

```plaintext
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```plaintext
and amidoxime added dropwise previously dissolved in ethyl acetate. Then temperature allowed to rise until ambient and after stirring additional 2 hours reaction have been carefully quenched by addition of 10% solution of sodium bicarbonate and worked-up with sat. NaHCO₃ and brine solutions, dried over anhydrous magnesium sulfate evaporated and chromatographed on silica using ethyl acetate hexane system to lead pure compound with 91% yield. This is the best yield for trichloro-substituted compound. Previous attempts to get it through coupling with HATU with trichloroacetic acid have been unsuccessful in the terms of yields. HATU coupling gave only 36% yield. Amidoxime have demonstrated its incompatibility with acids. Reaction of aromatic nucleophilic substitution of indolyl-trichloromethyl oxadiazole with various diamines have been tried in methanol and in ethyl acetate. Methanol as solvent leaded to decomposition of starting material. Ethyl acetate gave 80-91% yields of pure

```plaintext
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products after flash chromatography on silica. Various amino-terminated iso phidianidine analogs have been synthesized.

The next reaction is near-quantitative conversion of amine to di-Boc-protected alkyl guanidine by N-tryflyl-diBoc-guanidine which have been used for the next reaction without further purification. And finally, deprotection were done using trifluoroacetic acid dichloromethane 1 to 10 mixture leading to the target compounds after 2 hours with 86-92% yield over two steps. The compounds have been purified using flash column chromatography with using DCM/MeOH/NH₄OH system since all of them have demonstrated high polarity. Protected guadinylo-terminated analogs of phidianidine was afforded in good yields according to the procedure described above using a series of amino-terminated analogs as starting materials. Deprotection of these analogs leaded to the guanidine-terminated compounds with moderate yields. The comparative structure - activity relationship profile is described in results and discussion section.

![Figure 2.2.7. Deprotection with of Boc protective groups with trifluoroacetic acid in dichloromethane](image)
2.2.3 Optimization of synthesis of *iso*-Phidianidines

All the optimization procedure were studied on a pentamethylenediamine. For the amidoxime formation, it was found that longer time of reflux has leaded to higher yields. Moreover, if ethanol were used instead of methanol, as in original procedure, higher yields was obtained. This may be due to elevated temperature of boiling of ethanol compared to the methanol.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>4h</td>
<td>86%</td>
</tr>
<tr>
<td>MeOH</td>
<td>8h</td>
<td>90%</td>
</tr>
<tr>
<td>EtOH</td>
<td>24h</td>
<td>96%</td>
</tr>
<tr>
<td>EtOH</td>
<td>6h</td>
<td>76%</td>
</tr>
<tr>
<td>EtOH</td>
<td>24h</td>
<td>82%</td>
</tr>
<tr>
<td>MeOH/EtOH</td>
<td>24h</td>
<td>85%</td>
</tr>
</tbody>
</table>

Table 2.2.1: Amidoxime formation conditions and yields
Figure 2.2.9. Formation of oxadiazole from cyclocondensation of amidoxime with trichloracetic acid

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>2h</td>
<td>75%</td>
</tr>
<tr>
<td>DCM 3A MS</td>
<td>2h</td>
<td>79%</td>
</tr>
<tr>
<td>EtOAc</td>
<td>2h</td>
<td>83%</td>
</tr>
<tr>
<td>EtOAc 3A MS</td>
<td>2h</td>
<td>90%</td>
</tr>
<tr>
<td>EtOAc 3A MS</td>
<td>4h</td>
<td>96%</td>
</tr>
<tr>
<td>EtOAc 3A MS DIPEA</td>
<td>4h</td>
<td>64%</td>
</tr>
</tbody>
</table>

Table 2.2.2 Oxadiazole formation conditions and yields
For the oxadiazole formation best solvent found to be the ethylacetate. The highest yields are obtained when ethylacetate was extremely dried over freshly activated 3A molecular sieves after more than 72h of staying. The addition of Hunig’s base, di-isopropyl ethylamine has not increased the yield, opposite, it leaded to formation of many byproducts.

The best yield for nucleophilic aromatic substitution of trichloromethyl group with cadaverine (pentamethylene diamine) was achieved in ethylacetate dried in the same way as described for cyclization reaction. The amine was used in large excess. Though it complicated further purification of product, but it leaded to near-quantitative conversion of trichlorooxadiazole to amine substituted product. The product was polar but can be purified by flash chromatography or preparative TLC plates using.

Figure 2.2.10. **Nucleopylic aromatic substitution with various diamines. Trichloromethyl as excellent leaving group**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temp</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc</td>
<td>50</td>
<td>91%</td>
</tr>
<tr>
<td>THF</td>
<td>50</td>
<td>90%</td>
</tr>
</tbody>
</table>

Table 2.2.3 Nucleophilic aromatic substitution best conditions and yields
Small library of amino-terminated iso-Phidianidines (Figure 2.3.7) was obtained for structure – activity relationship study. It was previously found that aminoterminated compounds have higher overall anticancer activities compared to guanidine-terminated compounds. Also libraries of Boc-protected guanidine terminated compounds and the guanidine-terminated compounds were prepared, by the developed methodology by using amino-terminated compounds as starting materials.
Figure 2.2.12. Library of Boc-protected guanidino-terminated iso-Phidianidines via guadinylation of amino-terminated compounds

Figure 2.2.13. Library of guanidino-terminated iso-Phidianidines obtained using developed synthetic route
2.3 Other synthetic studies

2.3.1 Solid phase synthesis protocol toward iso-Phidianidines

Experiencing significant difficulties with purification of highly polar amino-compounds solid phase on resin synthesis protocol were developed using 2-chlorotryptil Merrifield resin.

![Diagram of solid phase synthesis protocol](image)

**Figure 2.3.1. Solid phase synthesis protocol developed for iso-Phidianidines**

For this purpose first reaction is set to be attachment of diamine to resin. 10 equivalents of cheap and commercially available diamines were shaken in a plastic solid phase synthesis tubes with 1 eq. of resin for overnight in dry THF. Prior to this, resin was swelled in dry THF for 2 hours. After the completion of reaction solvent and excess of reagent were filtered out through a fritted bottom of a plastic synthesized and washes sequentially 3 times with THF, 3 times with DMF and 3 times with DCM. Then to block unreacted groups on a resin, if any, dry methanol were added and shaken for
another 8h. Upon completion resin were washed with DCM three times and dried under vacuum. Then, 5-(3-indolylmethyl-3’-trichloro-1,2,4-oxadiazol were added to the resin previously dissolved in THF and shaken for two hours in a presence of DIPEA. Because in this case formation of bi-substituted byproduct is sterically impossible (each molecule is restricted in his free movement on resin) base could be used, in contrast to the solution phase synthesis, to catalyze faster formation of product. Thus, in 2h reaction was finished and the resin was washed with THF three times, three times with DMF and three times with DCM. Then, previously prepared mixture of one part acetic acid to ten parts of trifluoroethanol in dichloromethane part was passed through resin each 10 min (three times overall) and the product was obtained by removing solvents in vacuum evaporator as acetates. The products were compared with the same from solution phase synthesis protocol and were found to be identical to them.

In conclusion, short and efficient synthetic protocol toward novel heterocyclic compounds were successfully developed using both, solution, and solid phase strategy.
2.3.2 Total synthesis of natural Phidianidines

Prior to the Gavagnin’s, Lindsley’s and Snider’s reports on total synthesis of natural phidianidines we were working on synthetic route based on 1,3-dipolar addition of dirbomoamidoxime to 3-indolylmethyl acetonitrile, with proposed further substitution of bromine with diamines. But unfortunately many conditions (Figure 2.3.10) tried didn’t lead to oxadiazole formation. 3-indolylmethyl acetonitrile demonstrated unusually bad dipolarophytic properties. On a synthetic scheme (Figure 2.3.2.) unaccomplished reactions highlighted with green color.

![Total Synthesis of Phidianidine](image)

Figure 2.3.2. Synthetic route toward natural Phidianidines based on 1,3-dipolar cycloaddition of dibromoformaldoxime to very bad dipolarophile 3-indolylmethyl acetonitrile
dirbomoamidoxime to 3-indolylmethyl acetonitrile, with proposed further substitution of bromine with diamines. But unfortunately many conditions (Figure 2.3.10) tried didn’t lead to oxadiazole formation. 3-indolylmethyl acetonitrile demonstrated unusually bad dipolarophytic properties. On a synthetic scheme (Figure 2.3.2.) unaccomplished reactions highlighted with green color.
Figure 2.3.3. Conditions tried for 1,3-dipolar cycloaddition reaction.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Catalyst/Additive</th>
<th>Solvent 1ml</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>no</td>
<td>Benzene</td>
<td>80 °C</td>
</tr>
<tr>
<td>B</td>
<td>MS 3A</td>
<td>Toluene</td>
<td>80 °C</td>
</tr>
<tr>
<td>C</td>
<td>MS 4A</td>
<td>Toluene</td>
<td>80 °C</td>
</tr>
<tr>
<td>D</td>
<td>BF$_3$-Et$_2$O 0.02 ml</td>
<td>Et$_2$O</td>
<td>-5°C</td>
</tr>
<tr>
<td>E</td>
<td>BF$_3$-Et$_2$O 0.02 ml</td>
<td>Toluene</td>
<td>-5°C</td>
</tr>
<tr>
<td>F</td>
<td>MS 4A vacuum</td>
<td>no</td>
<td>55 °C</td>
</tr>
<tr>
<td>G</td>
<td>MS 3A vacuum</td>
<td>no</td>
<td>55 °C</td>
</tr>
<tr>
<td>H</td>
<td>vacuum</td>
<td>no</td>
<td>55 °C</td>
</tr>
<tr>
<td>I</td>
<td>KHCO$_3$</td>
<td>no</td>
<td>55 °C</td>
</tr>
</tbody>
</table>
2.4. *In vitro* Evaluation of Biological Activities

Cell Culture: Human cervical adenocarcinoma, HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Human mammary carcinoma MCF-7 cells were cultured in DMEM supplemented with 10% FBS. Cytotoxicity evaluation: Antiproliferative Properties. To evaluate antiproliferative properties of the phidianidine analogues, the MTT assay was used. The HeLa cell lines were assessed by trypsinizing cell culture and seeding $5 \times 10^3$ cells per well into 96 well microtiter plates. All compounds were dissolved in DMSO at a concentration of either 100 mM prior to cell treatment. The cells were grown for 24 h before treatment at the concentrations ranging from 0.001 to 100 μM and incubated for 24 h in 200 μL of media. Twenty microliters of MTT reagent in serum free medium (5 mg/mL) was added to each well and incubated further for 8 h. Media was removed, and the resulting formazan crystals were re-solubilized in 100 μL of DMSO. Absorption values at 490 nm were measured using a Thermomax MolecularDevice™ plate reader. The experiments were performed in hexaplicate. Four values out of six were selected. Cells was treated with 0.1% DMSO was used as a negative control, and doxorubicin hydrochloride (DOX) was used as a positive killing control. The IC$_{50}$ values were calculated and the range of activities was from the most potent 11 μM (spermidine derivative) and 21 μM (phenylene diamine substituted) to 350 μM less potent (piperazine derivaive).
Figure 2.4.1. In vitro structure activity comparison chart for amino-terminated iso-Phidianidines
2.5 Conclusions

A small library of iso-Phidiane analogs the following structure-activity relationship was synthesized and characterized. In generally all the diamino-substituted compounds has good anticancer activities, they was synthesized in a short route with high yields and good purity.

![Structure-activity relationship results](image)

1) the strict importance of presence of proton near nitrogen at 5’-position of 1,2,4-oxadiazole ring
2) Amino-terminated compounds were more potent
3) longer hydrocarbon chain in diamine subunit
4) phenylene versus hydrocarbon chain
5) polyamines versus hydrocarbon chain.
6) disubstituted nitrogen at 5 – posit. of oxadiazole makes it less cytotoxic and makes compounds interesting for CNS activity tests

Figure 2.5.1 Structure-activity relationship results
Chapter 3

Synthesis and Evaluation of Multiple Theranostic Modalities of Polydopamine Coated Magnetic Iron-Cobalt Nanocubes

3.1 Background

3.1.1 Iron-cobalt nanoparticles synthesis and application for cancer diagnosis and treatment

Metallic iron and relatively recently iron-cobalt nanoparticles have been studied as therapeutic and diagnostic agents with application in cancer nanomedicine for magnetic hyperthermia, magnetic separation, magnetic resonance imaging (MRI) contrast enhancement, and ultrahigh-density data storage [Nanoscale Research Letters 2013, 8:540] separately, to the best of our knowledge there are no real theranostic agents developed based on this nanocomposite system.

Thus the Afghahi et al.\textsuperscript{262} Evaluated FeCo alloy about 7 nm (which is below the good limit for biological application) spherical magnetic nanoparticle fluids, prepared by reducing FeCl$_3$$\times$7H$_2$O and FeSO$_4$$\times$7H$_2$O with sodium borohydride in a water/CTAB/hexanol reverse micelle system for application in magnetic hyperthermia treatment. Using Stoner-Wohlfarth (SW)-based models, which neglect thermal activation and assume a square hysteresis area, the authors defined Specific Absorption
Rate (SAR) as a function from strength of applied field $H_c$, frequency $f$, magnetic saturation $M_s$:

$$SAR = 2f\mu H_c M_s$$

The key factor to obtain the maximum SAR in conventional clinical hyperthermia treatments ($f = 120$-$500$ kHz, $\mu H_{\text{max}} = 20$ mT) is the anisotropy of synthesized nanoparticles. Calculations of SAR as a function of anisotropy in several size regimes reveal that the maximal SAR would be obtained at the single-domain ferromagnetic size regime. Thus synthesized nanoparticles demonstrated 165 W/g loss power, which meets experimental model calculations. The authors explain this high value by high intrinsic magnetic saturation of this type of nanoparticles. Among the iron compounds, FeCo alloys are known to exhibit the highest magnetic properties. Iron and cobalt are both near the peak of the Slater-Pauling curve and have maximum saturation magnetization when combined together. $Fe_{0.7}Co_{0.3}$ and $Fe_{0.65}Co_{0.35}$ has the highest saturation magnetization among all magnetic alloys$^{263}$. Later McHenry et al$^{264}$. Prepared iron-cobalt 18 nm nanoparticles plated carbon nanotubes via one-step palladium catalyzed electroless deposition (CNT@FeCo) for hyperthermia purposes. The ratio of iron to cobalt was 1:1.05. Thus prepared nanocomposite demonstrated very low magnetic saturation SAR values, only in concentrations 33-100 mg/ml they have clinically relevant rise of temperature. Separate for this experimental work on the magnetic hyperthermia application of iron-cobalt nanoparticles and composites on their basis, some theoretic sturdies recently published. McHenry et al reported that for oxide
stabilized (for better biocompatibility, stability) 35 nm iron-cobalt nanoparticles theoretical prediction of 480 W/g SAR power may be achieved, which is larger than for the same composition oxide based nanoparticles\textsuperscript{265}. Another computational approach for comparative evaluation different magnetic nanoparticles on their applicability for magnetic hyperthermia was published by Puri et al.\textsuperscript{266} They compared six different types of ferrofluids, namely those containing magnetite (FeO-Fe\textsubscript{2}O\textsubscript{3}), maghemite (γ-Fe\textsubscript{2}O\textsubscript{3}), iron-platinum (FePt), iron-cobalt (FeCo, size 17 nm), barium-ferrite (BaFe\textsubscript{2}O\textsubscript{4}), and cobalt-ferrite (CoFe\textsubscript{2}O\textsubscript{4}). Among this materials FeCo nanoparticles demonstrated highest heating rate, though based on this authors concluded that with larger field strength heating may be too rapid and because of this not safe in clinical applications. But the other works report that higher heating rates guarantees rapid tumor destruction without induction of resistance to hyperthermia. Summarizing, the number of reports on use of FeCo in hyperthermia are scarce, but the conclusion is that FeCo nanoparticles because of their high magnetic saturation compared to metal oxide nanoparticles has greater promise in application of magnetic hyperthermia.

There are also scarce about the synthesis of iron cobalt nanoparticles with the aim to use them as MRI contrast agents. Thus McConnel et al. synthesized biocompatible graphite carbon coated iron cobalt nanocrystals. 7nm size nanocomposite was prepared by thermal decomposition of metal salts in with organic solution. Then carbon shell was functionalized with PEG-NH\textsubscript{2} and fluorescent dye Cy5.5 was attached to terminal amino group via N-hydroxysuccinimide activation. This material was tested for
vascular macrophage uptake and imaging in vivo using fluorescence and MRI. Better contrasting was shown after 72h of injection (for comparison in our work it just 2h). Martel et al.\textsuperscript{267} prepared big 60 µm poly (D,L-lactic-co-glycolic acid) (PLGA) coated iron cobalt nanoparticles with 73 emu/g saturation magnetization value by the procedure developed by Dai et al.\textsuperscript{268} The core was prepared by refluxing of iron and cobalt pentacarbonyls in solution of dichlorobenzene, tri-\textit{n}-octylphosphine oxide and oleic acid at 285 °C. During high temperature annealing thin layer of graphite was formed on surface of nanoparticles. Then FeCo nanoparticles were sonicated in the presence of PLGA obtaining biocompatible polymer coated FeCo@PLGA nanocomposite, which they have tested on MRI contrasting property. They have compared this nanocomposite with Fe@PLGA and Co@PLGA – FeCo@PLGA outperforms it counterparts by magnetic saturation value. Moreover they have demonstrated excellent MRI contrasting properties. Interestingly, Fe and Co non-coated nanoparticles released more ions in physiological solution compared to FeCo nanoparticles. Magnetic saturation values was 140, 180 and 220 \text{emu/g} respectively (the highest value for any nanoparticles previously used in biomedical studies, but still 6 \text{emu/g} less than our PDFC nanoparticles). Dai et al.\textsuperscript{268} demonstrated even higher MRI angiocontrasting ability of 7 nm graphitic carbon coated iron-cobalt nanoparticles. The authors also report excellent biocompatibility of this material both, in vivo and in vitro on rabbits. By the similar synthesis procedure, but with 182 nm of diameter PLGA coated graphitic coated iron cobalt nanoparticles were synthesized and evaluated for
Magneto-resonance steering and contrasting by Martel et al.\textsuperscript{269} All this reports suggests excellent biocompatibility, magnetic targeting feasibility, good mediator property for magnetic hyperthermia and excellent MRI contrasting ability.

Other synthesis procedures without biological studies also used in preparation of FeCo nanoparticles. Thus, sodium borohydride reduction of iron and cobalt chlorides emulsion in ethanol reported by van Lierop et al.\textsuperscript{270} Johnson et al\textsuperscript{271}. Developed procedure for high temperature reduction of iron and cobalt bromides by lithium triethylborohydride, obtaining 5-10 nm nanoparticles with 210 emu/g Ms value. Sol-gel method for FeCo nanoparticles with the size range 20-30 nm and 890 Oe large coercivity was obtained by citric acid reduction of iron and cobalt nitrates with further heating of formed gel up to 400 °C.\textsuperscript{272} Iron-cobalt nanowires with varying ratio of elements was obtained by electrodeposition on gold coated aluminum film from the cobalt and iron sulfates aqueous solution under slightly acidic conditions.\textsuperscript{273} bcc-FeCo shell AuCu core spherical nanoparticles with Ms 211 emu/g and coercitivity 846 Oe was obtained by thermal decomposition of metals pentacarbonyls on AuCu seeds.\textsuperscript{274} Inert gas evaporation of bulk FeCo alloy and condensation with pulse laser deposition technique also used to produce iron-cobalt nanoparticles.\textsuperscript{275} But the most earliest and well-studied method for their preparation is called polyol method – using of polyethyleneglycol as a solvent and reducing agent of iron chloride and cobalt acetate under the basi conditions and 300 °C.\textsuperscript{276} Also sodium borohydride reduction of metal salts in N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB)/water/hexanol system
as a soft template has routinely used to prepare spherical nanoparticles with 68/32 elements ratio and 235 emu/g Ms.\textsuperscript{277} Organometallic approach, decomposition of organic precursors Fe and Co(N(SiMe\textsubscript{3})\textsubscript{2})\textsubscript{2} is an alternative technique recently developed for synthesis of millimeter sized super-crystals of organized 20 nm nanoparticles.\textsuperscript{278}

### 3.1.2 Polydopamine coating

![Figure 3.1.1.](image)

Figure 3.1.1. (A) Photograph of a mussel attached to commercial PTFE. (B and C) Schematic illustrations of the interfacial location of Mefp-5 and a simplified molecular representation of characteristic amine and catechol groups. (D) The amino acid sequence of Mefp-5 (13, 34). (E) Dopamine contains both amine and catechol functional groups found in Mefp-5 and were used as a molecular building block for polymer coatings. (F) A schematic illustration of thin film deposition of polydopamine by dip-coating an object in an alkaline dopamine solution. (G) Thickness evolution of polydopamine coating on Si as measured by AFM of patterned surfaces. Reprinted from Ref [279]
Formation of the polydopamine coating on various surfaces using of procedure of in situ oxidative polymerization of dopamine under the slightly basic aqueous buffer was reported by Messersmith et al.\textsuperscript{279} The inspiration for the discovery of this biocompatible polymer coating was the adhesive proteins expressed by the mussel for the attachment on wet surfaces. The mussels are able to attach on any type of organic and inorganic surfaces, even on polyfluorinated polymers, which are the known least adhesive prone surface coating materials. The authors noticed that this high adhesivity property is because of the unique chemical composition of protein – richness in 4-dihydroxy-L-phenylalanine (DOPA) and lysine amino acids. Authors based their design of the new polymer coating on Hypothesis, that the co-existence of catechol (DOPA) and amine (lysine) groups plays crucial role in achieving adhesion to a broad spectrum of materials. They identified dopamine as a small-molecule compound that contains both functionalities -catechols and amino groups, thus mimicking natural *Mytilus edulis* mussel’s foot protein (Mefp-5) (Figure 3.1.1).

![Figure 3.1.2 Aqueous chemical equilibrium between catechol (left) and quinone (middle). The equilibrium shifts toward quinone under alkaline conditions, conferring latent reactivity toward amine groups of proteins (right) Ref [280]](image)

- 99 -
The mechanism of oxidative polymerization was simply described by the Messersmith et al. as the reactivity of chinoid form of dopamine toward its own amino groups or amino groups from proteins, which dominate over the cathecholic form in basic aqueous medium (Figure 3.1.2). Similarly, polydopamine is reactive toward thiol terminated compounds. Thus, the authors successfully attached PEG-NH$_2$ and PEG-SH to polydopamine activated surfaces. Tests on M07e and mouse fibroblasts cell lines demonstrated complete biocompatibility of polydopamine. Moreover, cells were...
increasingly adhesive toward polydopamine coated surfaces. But the structure of polydopamine is not completely deciphered. There are many works focused on revealing of elementary units of this new nature inspired polymer. Computational studies of Buehler et al.\textsuperscript{281} describes the mechanism of polymerization as a formation of oxidized reactive intermediate compound – dehydroxyindole (DHI) and the further oxidative polymerization of it (Figure 3.1.3).

The authors validate their results by synthesizing of polydopamine thin films and perform experimental nanoindentations on the film. Observations of linear behavior for Young's modulus as a function of the degree of cross-linking demonstrated the enhancing the mechanical robustness of polydopamine materials by increasing the

\textbf{Figure 3.1.4} Natureal pigment eumelanin tetrameric forms as a result of copolymerization of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carbonxylic acid
extent of polymerization. At the highest degree of polymerization considered (70%), the model mimics the linear tetrameric model for polydopamine and melanin. At this degree of polymerization, they found, that a Young's modulus of 4.1–4.4 GPa, in agreement with our nanoindentation results of 4.3–10.5 GPa, previous experiments for natural melanin, as well as simulation results for the cyclic tetrameric melanin model. This model was suggested as a proposed structure for the polydopamine earlier by the Park et al.\textsuperscript{282} (Figure 3.1.4).

Figure 3.1.5 Intra- and interchain noncovalent interactions, including hydrogen bonding, \(\pi\)-stacking, and charge transfer as a proposed structure for polydopamine formed through reaction of dopamine in Tris buffer under the aerobic conditions. Ref [283]
Alternative mechanism was proposed by the Bielawski et al.\textsuperscript{283} [Langmuir 2012, 28, 6428 – 6435] (Figure 3.1.5). The authors suggested hydrogen bonding and π-stacking as a main driving force of polymeric chain organization of dihydroxyindole.

Despite the disputes on polydopamine structure between Messersmith and Beliawski groups, both experimental data, such as 3.8 Å interspace between the polymer chains organized one over the another and the participation of hydrogen bonding explains well the loading of aromatic ring containing doxorubicin anticancer drug and its pH responsive release as a some results of this dissertation. Thus, doxorubicin may intercalate between the chains by the same π-stacking mechanism and the acidification of media may disrupt the interchain hydrogen bonding.
3.2 Experimental Section: Design of the System

With the aim to design the “One-for-All” system, which will have high magnetic saturation value of magnetic core, iron-cobalt alloy system was chosen, because for bulk alloy it is well known that at ration of iron to cobalt 65 to 35 material has highest saturation magnetization. So, the same ratio of elements we proposed for the nanoparticles. Moreover it was demonstrated, that cubic shape has big influence on magnetic properties, because at this crystal structure – body centered cubic, for iron, cobalt and their alloys they have closest packing. Close packing lead to higher density of core, which is 8.3g/cm³ this plays crucial role in X-Ray CT good contrasting. Also, magnetic shape anisotropy increases MRI contrasting and hyperthermia specific loss power. Moreover, both iron and cobalt are trace elements in human body and compared to other metals (such as barium or lantanides) have good biocompatibility range at relatively high concentrations. Thus, highly magnetic cubic iron-cobalt nanoparticles designed to be excellent material for the core of nanoparticles. For the shell design, the most important is the high biocompatibility and the ability to carry drug and dye molecules. For this purpose polydopamine were chosen as excellent candidate. It is nature inspired, biocompatible polymer, which is able to carry different loads. As a imaging dye fluorescein isocyanate were chosen and as drug model doxorubicin was selected. Thus, on a Figure 3.2.1 major rationale of a design is summarized for the polydopamine coated iron-cobalt nanocubes.
Iron-Cobalt (high Ms) core is for

- MRI contrasting
- X-Ray CT contrasting
- Magnetic targeting

Polydopamine coating is for

- Biocompatibility
- Better dispersabilty
- NIR-photothermal therapy
- Drug loading
- Dye attachment

**Figure 3.2.1 Rationale in design of polydopamine coated iron cobalt nanocubes**
3.3 Synthesis of polydopamine coated iron cobalt nanocubes

3.3.1 Wet synthesis of iron-cobalt nanocubes

![Scheme 3.3.1 The typical scheme of synthesis of polydopamine coated iron cobalt iron cobalt nanocubes in oil/water emulsion using PEG as surfactant and further oxidation and polymer coating.](image)
The typical wet chemistry procedure of synthesis single crystalline iron-cobalt metallic nanocubes, developed by Wei X.-W. et al\textsuperscript{284} was used with minor modifications as described for synthesis of the FeCo nanocubes with atomic ratio of Fe/Co to 65/35, which is well known ratio at which alloy has the highest saturation magnetization. First solution A was prepared: 6.88 g of PEG-200 (34.4 mmol) were dissolved in 50 ml of deionized and degassed water (DDW) (3 cycles of freezing under N\textsubscript{2} atmosphere / warming up under vacuum) following the addition of 720 mg of FeSO\textsubscript{4}\cdot7H\textsubscript{2}O (2.589 mmol) and 392 mg of CoSO\textsubscript{4}\cdot7H\textsubscript{2}O (1.394 mmol). The solution A was stirred under N\textsubscript{2} atmosphere until complete dissolution of salts. Then 0.8 ml of heptane added via syringe and emulsion was placed in bath sonicator for 80 min. Solution B was prepared in another flask: 2.45 g of sodium hydroxide (61.2 mmol) was dissolved in 20 ml of

Figure 3.3.1. TEM images of as synthesized nanocubes demonstrating strictly cubic shape of nanoparticles with good polydispersity.
hydrazine hydrate 85% water solution (0.34 mol) and purged with N₂ for 15 min followed by the addition of 0.1 ml of heptane and sonication for 1h. Solution A was heated with stirring to 78 ⁰C and Solution B was added within 1 min under N₂ atmosphere. The color of solution changed from light pink to green first and then within seconds darkened to brown. After 1.5 h of stirring, the obtained nanoparticles were separated by rare earth magnet and washed 5 times with degassed and deionized water (DDW) until the pH of the washings became neutral and finally washed twice with absolute ethanol and dried under vacuum for 4 h at 40 ⁰C.

TEM images show the alignment of nanocubes in long chains and bigger agglomerates because of the remaining magnetization after separation with magnet. For all next steps including controlled oxidation, polymer coating, and characterization experiments, mechanical stirring and separation on centrifuge at 4000 rpm for 5 min were used to

Figure 3.3.2. HR-TEM images of iron-cobalt nanocubes after the controlled oxidation with trimethylamine N-oxide in toluene.
obtain non-magnetic material for better dispersion. It is crucial to keep this material far from magnetic fields to prevent their rapid agglomeration prior to use in biological experiments. For the formation of the protective oxide layer the nanoparticles were dispersed with sonication in the solution of 30 mg of trimethylamine N-oxide in toluene (0.4 mmol), purged previously with N₂ for 2h, and stirred with mechanical stirrer for 2 h at 70 °C. Nanocubes were then isolated by centrifugation at 4000 rpm for 5 min, washed with absolute ethanol for 5 times and dried for 4 h under vacuum.

3.3.2 Polydopamine coating of FeCo nanocubes – PDFC

The procedure of coating was done through polymerization of dopamine where the following: 60 mg of FeCo nanoparticles were dispersed in 120 ml of deionized water containing 120 mg of dopamine hydrochloride with intensive sonication. After 30 min of sonication with shaking, dispersion showed no visible aggregates (dark grey but transparent under the light). pH of the solution was adjusted with potassium hydroxide 0.01 M solution to 8.5 and mechanically stirred under ambient conditions. After 12 h of stirring pH dropped to 6.4, which was again adjusted to 8.5 and continued stirring for another 12 h. Obtained nanoparticles were separated by 10 min centrifugation at 4000 rpm and washed 5 times with DDW, two times with ethanol and dried in freeze drier for 12 h prior to characterization. Remaining solution containing polydopamine without FeCo nanoparticles was transferred in dialysis membrane with MW cutoff 1000 and dialyzed against DI water for 2 days to remove low molecular weight by-products and dopamine. After purification water was removed on freeze drier and dark brown-to-
black sticky polymer was used to compare with PDFC nanoparticles. According to the thermogravimetric analysis 26% w/w PDA containing composite was obtained. Varying the amount of polydopamine versus FeCo nanocubes different degrees of coating may be achieved (Figure 3.3.1).

The optimal ratio of polydopamine coating for biomedical application 26% was chosen. It is based on the competition of two factors. First, more polymer will decrease the overall magnetic property of nanocomposite, but the least amount will not allow carrying of necessary amount of dye or drug loads. Thus, the middle ration composition has enough material for FITC fluorescent dye loading for imaging and doxorubicin loading for drug delivery, but at the same time it retain strong magnetic properties for other applications, such as MRI contrasting, microwave and alternating current hyperthermia.

Figure 3.3.3. Various degrees of polydopamine coating over the FeCo nanocubes. The central image represents the optimum ratio of polymer over the nanocubes chosen for all experiments.
3.3.3 FITC Dye Attachment - PDFC-FITC

Scheme 3.3.4. Attachment of FITC-isocyanate to PDFC with formation of fluorescently trackable nanoparticles

Figure 3.3.5. CLSM images of PDFC-FITC nanoparticles after their incubation with HeLa cells for 12 h
1 mg of PDFC were dissolved in degassed and deionized water containing 1 mg of FITC isocyanate by intense sonication for 15 min in ultrasonic bath, then one drop of

Figure 3.3.6. CLSM images obtained for PDFC-FITC nanoaprticles incubated with HeMembrane stained with CellMask™ Deep Red Plasma stain
10 % sodium bicarbonate solution was added and left to shake intensively overnight. The nanoparticles were separated by centrifugation for (4000 rpm) 3 min and washed with water 6 times to remove excess of FITC. PDFC-FITC nanoparticles were dried under vacuum for 2 days.

Figure 3.3.7 CLSM Z-positioning images demonstrating that the PDFC-FITC is localized in the internal compartment of the cells in close proximity to cell nucleus
Then confocal laser scanning microscopy (CLSM) images (Figure 3.4.1.) was obtained for PDFC-FITC nanoparticles having two aim: first to visualize internalization of nanoparticles and the second, to get proof of concept of their use as cell tracking by fluorescent imaging. Additionally, membrane staining with Z-positioning was done in another experiment to proof internalization (Figure 3.4.2).

Briefly, HeLa cells were seeded on glass cover slides, and cultured in EMEM medium containing 10% FBS and 0.1% penicillin-streptomycin at 37 °C in a humidified 5% CO2 atmosphere. After cells attachment, the medium was replaced by fresh medium containing 10 µg/ml of PDFC-FITC, followed by incubation for 12 h. Cells on cover slides were washed twice with DPBS, then fixed with 4% paraformaldehyde for 1 h and washed 3 times with DPBS. Then, nuclei were stained with DAPI for 3 min and then washed for 3 times with DPBS. Finally, cells were observed with confocal laser scanning microscopy (CLSM, Zeiss LSM 710 upright confocal microscope, with excitation and emission wavelengths at 488 and 500-600 nm respectively).
3.3.4 Doxorubicin attachment and release rates - PDFC-DOX

Scheme 3.3.4. Doxorubicin attachment on PDFC nanoparticles through formation of pH responsible Schiff base.

0.5 mg of PDFC were dissolved in 10 ml of degassed and deionized water containing 0.5 mg of doxorubicin hydrochloride (DOX) by intense sonication for 15 min in ultrasonic bath, then of 10 % sodium bicarbonate solution was added dropwise until pH increased to 8.5 - 9 and left to shake intensively overnight. The nanoparticles were separated by centrifugation for (4000 rpm) 3 min and washed with water 10 times to remove excess of doxorubicin. Then all the washing were combined and the amount of doxorubicin loaded on nanoparticles were calculated from the difference of doxorubicin in washings and initial. For this purpose the calibration curve (Figure 3.5.1) for doxorubicin solutions in water have been created form serial dilutions and the absorbance at 497 nm on UV-VIS spectroscopy were measured. Later the value of doxorubicin in washings was calculated from the formula obtained from the calibration experiment.
For the nanoparticles with as low as 16% PDA loading amount were 5% by weight, for the 26% polydopamine content the loading was calculated to be 10%. The release of doxorubicin from nanocomposite was in pH-responsive manner. At pH 7.4, which corresponds to the physiological pH of blood, release was much lower than for pH 4.5, which corresponds to acidic compartments of cancer cells. Moreover, release was highly attenuated in time. Within 10 days only 65% of loaded doxorubicin was released. This is advantageous property, because in classic chemotherapy strategy high systemic toxicity of drug is associated with burst release in organism after the administration. In case of attenuated release doxorubicin supplied metronomically.

![Doxorubicin calibration curve](image)

**Figure 3.3.8. Calibration curve and the formula for measurement of amount of doxorubicin in aqueous solutions.**
Figure 3.3.9. Doxorubicin release rates at pH 7.4 and 4.5 calculated by measurement of absorption value at 497 nm of aliquots of buffers in time intervals.
3.4 Characterization

Figure 3.4.1 FTIR spectra for PDFC (coated) and uncoated iron-cobalt nanoparticles

Figure 3.4.2 TGA curve for the sample prepared by using 1:1 initial ratio of nanocubes to dopamine. With 16% organic content
To monitor the polydopamine coating FTIR spectroscopy was used. The uncoated and polydopamine coated nanoparticles was mixed with dry KBr (FTIR grade), stirred in mortar and thin film tablet was obtained after pressure. The empty KBr was used as background spectra and the samples spectra have been taken sequentially. On spectra (Figure 3.4.1) broad band at 3140 and aromatic stretching at 1043 and 974 are all characteristic for polydopamine. The thermogravimetric analysis was done to measure the ratio of polydopamine.

![Figure 3.4.3 TGA curves for the sample prepared by using 1:2 initial ratio of nanocubes to dopamine. With 26% organic content](image)

**Figure 3.4.3** TGA curves for the sample prepared by using 1:2 initial ratio of nanocubes to dopamine. With 26% organic content
For the main sample used in biomedical applications, thermogravimetric curves and differential scanning calorimetry analysis was done on a sample and for polydopamine

Table 3.4.5 HR-TEM EDAX analysis demonstrated the designed ratio of iron to cobalt elements in the core of nanoparticle 65:35
alone to see the difference if any in behavior. Interestingly, thermal stability of polydopamine increased dramatically, over the 270 °C, which could be explained with strong complexation of catecholic hydroxyl groups of polymer chains with the iron and cobalt oxides on surface. Strong chemical bonding prevents its low thermal degradation, as in the case of pure polydopamine.

The distribution of elements was measured by HR-TEM EDAX and SEM EDAX methods. Both demonstrated that initially designed ratio 65 to 35 of iron to cobalt element was achieved experimentally and was consistent for both and shell, confirmed by HR-TEM EDAX and oxide layer, confirmed by SEM - EDAX
Powder X-Ray diffraction (XRD) was done and three peaks were registered from the lattices 110, 200 and 211 corresponding to the body centered cubic crystal lattice structure of iron-cobalt nanocubes.

![XRD pattern of Fe<sub>65</sub>Co<sub>35</sub>@(Fe<sub>0.65</sub>Co<sub>0.35</sub>)<sub>3</sub>O<sub>4</sub>](image)

Figure 3.4.7 Powder XRD for iron-cobalt nanocubes
3.5. Microwave induced chemotoxicity

3.5.1 Methods

Transition electron microscopy (TEM) images were recorded on Tecnai Bio Tween T12 TEM microscope (FEI Co.), high resolution (HR) TEM and HR-TEM- energy dispersive X-ray spectra (EDX) analysis were carried out on Titan CT microscope (FEI Co.). The Scanning electron microscopy (SEM)-EDX analysis was carried out on Quanta 200 FEG microscope (FEI Co.). X-ray powder diffraction (XRD) was carried out on Bruker D8 Advance X-Ray diffractometer with Cu Kα (λ=1.5406 Å) in the 2θ ranges. Magnetization was measured on Magnetic Property Measurement System (MPMS® SQUID VSM), UV-VIS-NIR spectra was recorded on Varian 5000 spectrophotometer. FT-IR spectra was recorded on Thermo Nicolet iS10 spectrophotometer. Thermal gravimetric analyses (TGA) were done on TG 209 F1 machine. Confocal microscopy images were recorded on Zeiss 7MP multiphoton microscope.
3.5.2. Heating rates measurement

Figure 3.6.1 Parallel measurement of SAR for electrolite, FeCo nanoparticles in electrolyte, PDFC nanoparticles in electrolyte, polydopamine in electrolyte

Figure 3.6.2: Heating rates in °C/sec for two identical vials containing 0.1 M KCl in each, calculated from Figure 3.6.3
As it was already mentioned in Chapter 1, microwave ablation has been clinically evaluated for use in the treatment of many types of cancer. Focused microwave irradiation over a desired area leads to local application of heating to induce irreversible cell injury. However, indirect heating and killing of healthy tissues remains one of the major drawbacks of microwave therapy, as well as magnetic hyperthermia and near-infrared photothermal therapy.

Developing new nanomaterials that can be triggered by external stimuli without extreme temperature elevation can provide a safer route to utilize radiation techniques for cancer therapy. Herein, we report the fabrication of polydopamine coated FeCo

**Figure 3.6.3:** Typical heating rates curve for two vials shows identical behavior, with $R^2 = 0.99765$
nanocubes with low inherent toxicity. Microwave irradiation of 2.45 GHz frequencies (similar to Bluetooth and microwave ovens) provided significantly enhanced toxicity. To the best of my knowledge, this represents the first study of low energy microwave radiation on such systems.

To understand the influence of the material in the heating rates of model electrolyte solution, first, in were studied the influence of the nanoparticles presence in electrolyte in change of heating rate, if any. In this case electrolyte solution represent a model of intra- and extracellular fluids, which comprises of many electrolytes, which are along

Figure 3.6.4 Absence of difference in the heating rates in °C/sec for 1 mg/ml polydopamine in 0.1 KCl electrolyte and empty 0.1 M KCl
with polar water molecules considered as main absorbers of electromagnetic energy of microwave range.

Heating rates were measured via parallel registration of temperature rises by two or four optical fiber temperature probes placed deep in vials containing electrolyte solution with dispersions of polydopamine, as-synthesized FeCo nanocubes and PDFC nanocomposite. The plastic test tubes or glass vials were weighted and the closest mass vials (with the accuracy 0.1 mg) were chosen. If the mass of vials will be different it will affect the accuracy of SAR measurement. Despite in many microwave ovens

![Figure 3.6.5 Absence of difference in the heating rates in °C/sec for two identical vials containing 0.1 M KCl in each, calculated from](image)

distribution of power is inhomogenous inside the cavity laboratory microwave tissue processor BS-111-RS has advanced power distribution profile in inner cavity. And the positioning of the tubes was done after test thus, during measurement power distribution was homogeneous while two or four (the highest accuracy registered for two) identical electrolyte samples with the same masses in identical vials were tested.
Although, within one experiment power of microwave was distributed uniformly, after switching on and off power was slightly different. This happen because the magnetron generates the microwaves of slightly different power when it is on first run, compared with the continuous run.

Thus, for accurate control within every measurement, control sample of 0.1 M KCl electrolyte was used as standard and results of all heating rates were normalized according to this standard. The heating rate result from one experiment was considered as one and the rest of other repetition experiments were divided on a value of the difference of standards.

Figure 3.6.6 Absence of difference in the heating rates between empty 0.1 KCl electrolyte and the same electrolyte containing non-coated FeCo nanocubes
Figure 3.6.6 Absolute heating rates comparison between empty 0.1 KCl electrolyte and the same electrolyte containing polydopamine coated FeCo nanocubes.

Figure 3.6.7 Comparative normalized heating rates calculated at 0.8 W/cm$^2$ of microwave power for 0.1 M KCl electrolyte, which is Control, polydopamine (PDA), 10 and 100 µg/ml of polydopamine coated FeCo nanocubes (PDFC).
3.5.3. Cyclic voltammetry

Given the lack of differential heating of the nanocubes solution, an alternative mechanism to simple hyperthermia is required. We hypothesized that microwave treatment could induce redox processes that would release toxic ions. Evidence for this release is provided by cyclic voltammetry, using a well-known electroactive compound, namely methyl viologen. The experiment have been done with minor modifications as described in reported procedure. 128.575 mg of methylviologen hydrochloride...
hydrate (MV) were dissolved in deionized water containing 3.72755 g of KCl leading to formation of 0.1 mM solution of MV in 0.1 M KCl. Then solution was degassed by purging nitrogen for 2 hours. Reference electrode was Ag/AgCl and the working electrode was a graphite carbon. Reading electrode was a platinum wire. Scans were done in the range from 0V to -1.4 and then back to 0V in reverse current. Scan step was 0.05 V/s⁻¹. 14 ml of this solution were used in each experiment for cyclic voltammogram (10 experiments total) and all experiments was done in triplicate. The MV solution showed no change in voltammetric behavior during microwave exposure.

Figure 3.6.9 Control cyclic voltammetry experiments with 1 mM methyl viologen in 0.1 M KCl with polydopamine only exposed to the microwaves for 6 min, uncoated FeCo nanocubes exposed to the microwaves for 6 min and FeCo@PDA nanocomposite without microwave exposure demonstrated no changes in oxidation states.
Similarly, no changes were shown in solution containing dispersed polydopamine coated iron-cobalt nanocubes. But the gradual and time-of-exposure dependent changes in peaks behavior was registered when solution containing the dispersed nanocomposite was exposed to microwave irradiation. Additional set of control experiments was done to make sure wither the presence or the absence of polydopamine influence that change. For this purpose uncoated FeCo nanoparticles, polydopamine, and empty methyl viologen electrolyte was exposed to the 6 min of microwaves (corresponds to the maximum duration of experiment on Figure 3.5.9). It was found, that naked nanoparticles, and polydopamine itself are not responsible for this effect.

Thus, no changes registered when just electrolyte solution was exposed to microwaves. Similar results were observed when PDFCs were dispersed in MV solution without microwave radiation. In contrast, obvious changes were detected in the set of experiments with suspensions of nanocubes exposed to microwave (Figure 3.5.8). Moreover, these changes were dependent on irradiation exposure, indicating that the nanocubes either generated interfering species or served directly as redox units for the MV. It is clear that organic species are involved in this toxicity, as only trace amounts of metal was released to the media after as long, as ten 10 min of exposure to the microwaves (0.01 ppm for Co and 0.1 ppm for Fe, respectively), around four orders of magnitude less than the established toxic concentrations of these ions\textsuperscript{286, 287}. The release of ions was measured by the following procedure. Nanocomposite was dispersed in the cell culture media (MEM Alfa) in a same concentration used for cytotoxicity
measurement (10 µg/ml) - NPM. Following the explosion to microwaves nanocomposite was separated from media and the concentration of iron and cobalt ions in the remaining media were measured by inductively coupled plasma optical emission spectroscopy technique along with the reference media used as control and compared with it. The same was done also with the dispersion of nanoparticles in water (NPW).

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Table 3.6. ICP-OES measurement of changes in concentration of iron and cobalt ions in water and cell culture media after 10 min of exposure to the microwave irradiation. Values in parts per million
3.5.4. Cell cytotoxicity study with microwave

HeLa cells were grown in 96 well plates at a density 5000 cells per well in EMEM cell culture media containing 10% of FBS and 0.1% of penicillin–streptomycin at 37°C in a humidified 5% CO2 atmosphere. For the cell viability test using microwave irradiation, HeLa cells were incubated with PDFCs at the concentrations - 10, 1, 0.1, 0.01 and 0.001 µg/ml and incubated for 12h prior to addition of WST-8 dye - \(2-(2\text{-methoxy-4-nitrophenyl})-3-(4\text{-nitrophenyl})-5-(2,4\text{-disulfophenyl})-2\text{H-tetrazolium \cdot} \). Then the non-internalized cells were removed along with media and fresh media was added, thus the remaining viable cells (non-viable cells are unable to attach to the bottom of the plates and were removed along non-internalized nanoparticles) in plates are with internalized...
nanoparticles monosodium salt) Then the cells was exposed to the 2.45 GHz microwaves for 60 second at 0.86 W/cm², the power found to the addition of WST-8 dye incubated for additional 3h according to the CCK-8 standard protocol for colorimetric measurement of cell viability. In the case of the cell internalization study of PDFCs by ICP-OES, cells were incubated with 10, 1, 0.1 and 0.01 µg/ml of nanoparticles for 6h. Consequently media containing non-internalized PDFCs were removed and cells were washed with fresh media, which were combined with the first portion. Then the cells, containing the internalized PDFCs were harvested by trypsination and the amount of Co and Fe were analyzed by ICP-OES along with the supernatant, containing non-internalized PDFCs.

Figure 3.6.11. Linear dose response of cytotoxicity on HeLa cells for 60 seconds irradiation

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As could be seen form the graph at the density of power 0.86 W/cm² microwaves of 2.45 GHz frequency itself didn’t affect the viability of cells. But starting from the concentrations, as low as 0.01 µg/ml the cell viability decreased dramatically, when exposed to the microwaves. The concentration versus viability dose response curve shows good linear dose response behavior (Figure 3.6.11). This mean that with increase of concentration of polydopamine coated magnetic iron-cobalt nanoparticles to 10 µg/ml and exposure to very low dose of microwaves, which are themselves didn’t lead to heating or decreased viability of cells very strong toxic effect (more than 73 % of HeLa cells died at this concentration) on cancer cells may be achieved.

**3.6. Conclusion**

Microwave cancer hyperthermia and ablation plays very important role in a modern cancer management strategy. But the absence of mediator nanoparticles limits selectivity of microwave effect on cancer. It requires extremely elevated temperature to ablate diseased sites, which lead to destruction of surrounding tissue. Thus novel approach in microwave use for cancer management, escaping thermal effect may lead to more controlled influence.

In summary, we have demonstrated that polydopamine coated iron-cobalt single crystalline nanoparticles at very low concentration led to nonthermal cancer cell death under 2.45 GHz microwave irradiation at low power. Specific absorption rate
measurements demonstrated that the mechanism of death was not related to the rise of temperature (hyperthermia). We hypothesized that microwave irradiation is inducing a degradation of the polydopamine monolayer, inducing toxicity. We are further exploring the mechanism and application of this novel process.
3.7. Therapeutic Evaluation of Doxorubicin loaded PDFC-DOX

3.7.1 Cell cytotoxicity study with PDFC-DOX

Doxorubicin is one of the most widely studied hydrophobic drug model in drug delivery research area. Since 1950’s it was used in clinic to treat many forms of cancer, such as acute leukemia, lymphomas, breast cancer, lung cancer, hepatocellular cancers, testicular and bladder cancers.\(^\text{288}\) It is produced by the genetically modified *Streptomices spp.* and was comparatively less cardiotoxic compared to its parent natural compound, daunorubicin\(^\text{289}\). The mechanism of action of doxorubicin is the inhibition of macromolecular biosynthesis through intercalation of DNA and RNA intercalation.\(^\text{290, 291}\) The major systemic toxicity related to the use of doxorubicin is the acute and chronic cardiotoxicity, which acts as dose limiting factor.\(^\text{292}\) To overcome the concerns of cardiotoxicity many formulations of doxorubicin was successfully developed for selective drug delivery. The earliest and until now most widespread formulations are lyposomal.\(^\text{288, 293-295}\) Recently, polymeric delivery vectors gain high attention\(^\text{296}\) especially polydopamine found to be good material for doxorubicin loading, selective delivery and pH responsive release in intracellular environment\(^\text{296-305}\).

Thus, we have evaluated our nanocomposite on its ability to carry and delivery doxorubicin to cancer cells. With this aim HeLa cells were grown in 96 well plates at a density 5000 cells per well in EMEM cell culture media containing 10% of FBS and 0.1% of penicillin–streptomycin at 37°C in a humidified 5% CO2 atmosphere. For the
cell viability test using microwave irradiation, HeLa cells were incubated with PDFC-DOX at the concentrations - 30, 10, 1, 0.1 and 0.01 µg/ml and incubated for 8h prior to addition of WST-8 dye - (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium. Then the non-internalized cells were washed out fresh and media added. After the addition of WST-8 dye cells were incubated for 3h according to the protocol prior to colorimetric measurement. At the concentration 10 µg/ml nearly 45% of cancer cells died reaching to the 60% cell death at the concentration 30 µg/ml.

Figure 3.7.1. The concentration dependent cytotoxic effect of PDFC-DOX on HeLa cells after the 8h of incubation
3.7.2. Cell internalization study with PDFC-DOX

Figure 3.7.2. CLSM images of HeLa cells after 12h incubation with PDFC-DOX. Blue is DAPI channel (nuclei), red is DOX channel.
HeLa cells were grown in 96 well plates at a density 5000 cells per well in EMEM cell culture media containing 10% of FBS and 0.1% of penicillin–streptomycin at 37°C in a humidified 5% CO₂ atmosphere. For the cell internalization and imaging studies after HeLa cells attachment to the bottom of 96 well plates, the medium was replaced by

Figure 3.7.3. CSM imaging with Z-stack positioning for proof of internalization of PDFC-DOX in HeLa cells after 12h of incubation
fresh medium containing 10 µg/ml of PDFC-DOX, followed by incubation for 12 h. Cells on cover slides were washed twice with DPBS, then fixed with 4% paraformaldehyde for 1 h and washed 3 times with DPBS. Then, nuclei were stained with DAPI for 3 min and then washed for 3 times with DPBS. Finally, cells were observed with confocal laser scanning microscopy (CLSM, Zeiss LSM 710 upright confocal microscope, with excitation and emission wavelengths at 480 and 560-590 nm respectively). Thus, confocal imaging demonstrated good internalization and delivery of doxorubicin into HeLa cells.
3.8. Magnetic Characterizations

Figure 3.8.1. SQUID magnetization versus applied field (MH) curve for PDFC at 300K

Figure 3.8.2. SQUID magnetization versus applied field (MH) curve for PDFC at 2K
Characterization of PDFC were done in superconducting quantum interference device and the results was computed using MPMS 3 MultiView software. For this purpose 14 mg of material were weighted in a magneto-compatible weighting paper and wrapped tightly with magneto-compatible self-adhesive tape. Dimensions of final sample were

Figure 3.8.4. Zero-field-cooling and field-cooling magnetization versus temperature curves

mg of material were weighted in a magneto-compatible weighting paper and wrapped tightly with magneto-compatible self-adhesive tape. Dimensions of final sample were
not exceeded 3X4 mm. The sample were placed in special sample holder and again fixed very tightly with self-adhesive tape. Different conditions were chosen – two temperatures at 2K and 300 K for the response of magnetization change at Field Cooling (FC) and Zero Field Cooling (ZFC). Saturation magnetization gave 163.2 emu/g at 300 K. Considering the fact that magnetic core of nanocomposite is 74% of total weight (TGA for this sample showed 26% of polydopamine content PDA) recalculation of magnetization of core gives 226.6 emu/g. This value meets the value of vibrating sample magnetometer measurement (VSM) measurement done on uncoated nanoparticles. Low coercivity value could be explained as good dispersibility of sample – in case of more interaction between two nanocubes (contact) higher coercivity will be. This experiment showed that magnetic cores are separated from each other by non-magnetic material, polydopamine. For the zero-field-cooled and field-cooled MH curves centering by VSM method was done and the heating rate 2K/min from 2 to 350 K. According to the curves PDFC has a typical superparamagnetic behavior.
3.9. Magnetic resonance imaging with PDFCs

3.9.1 Molar relaxivities on agar phantoms

To study the contrasting properties of PDFCs nanoparticles was dispersed at different concentrations in agar gel (agar gel is mimicking animal tissue compositions). For this purpose 2% agar gel was prepared by dissolving agar in deionized and degassed water under the intensive microwave irradiation. Then equal aliquots of PDFCs dispersions were added along with the equal amount of warm gel in a small Eppendorf plastic tubes fixed in a big diameter plastic tube with dimensions 3x3.5 cm as a carrier. All the empty space and one tube in every sample was filled with empty agar gel, which was the reference background. The results from different tubes were normalized to the reference values. The measurements were carried out on 500 MHz SWB Bruker Avance III imaging system. Transverse ($T_2$) and longitudinal ($T_1$) relaxivities was measured using machine software protocol. The relaxation shortening times were

Figure 3.9.1 Typical agar phantom used to measure relaxivities of PDFCs
plotted versus total metal concentrations (concentrations were measured by ICP-OES and the Co molar concentration was added to the Fe molar concentration providing the total Me molar values). Echo time curves demonstrated excellent fitting profile for both, transverse and longitudinal relaxation experiments (Figures 3.11.2 and 3.11.3).

**Figure 3.9.2.** T2 relaxation curves for PDFC agar phantoms.

**Figure 3.9.3** T1 saturation recovery curves for PDFC agar phantoms.
Thus to calculate molar transverse relaxivity value for PDFCs, $r_2$ reverse of $T_2$

**Figure 3.9.4.** Molar transverse relaxivity for PDFC’s

**Figure 3.9.5.** Molar longitudinal relaxivity for PDFC’s
shortening time were plotted against total metal concentration and the high value 186.44 mM$^{-1}$s$^{-1}$ was obtained. The same measurement was done on molar longitudinal relaxivity measurement and the value 4.55 mM$^{-1}$s$^{-1}$ was obtained. Molar transverse relaxivity are exceeding the values of commercially used agents such as, Ferridex$^\text{TM}$ and CLIQ$^\text{TM}$ as T$_2$ contrast examples (the values are 110 mM$^{-1}$s$^{-1}$ and 62mM$^{-1}$s$^{-1}$ respectively$^{306}$). Although the r$_1$ value is compatible with commercially used agents, but the ratio of r$_2$/r$_1$ are over 41, which mean that PDFC’s could be considered as excellent T$_2$ contrast agent.

3.9.2 Mice in vivo MRI imaging with PDFCs

To test the feasibility of MRI contrasting in animals we have chosen mice as a model with the following procedure: female Balb/c mice (20-25g) were obtained from King Saud University’s main animal care center. All animal procedures were performed in accordance with the National guidelines for the care of laboratory animals and the study was approved by the Ethical Committee of the College of Applied Medical Sciences (agreement number: CAMS07/3334). During the different imaging protocols, each animal was anesthetized by intramuscular administration of a mixture of 0.1mL of 4mL of ketamine (50mg/ml), 1mL of xylazine (2%) and 5mL of physiological serum.

Magnetic Resonance Imaging (MRI) was performed on a 4.7T Pharmascan 47/16 Bruker magnet interfaced to ParaVision 5.1 software (Bruker Biospin GmbH,
Rheinstetten, Germany). Mice were imaged using a noninvasive free-breathing protocol before and at 2h, 24h, 68h, 1-week, 2-weeks and 1-month following intravenous injection of nanoparticles at concentration of 100 µg/mice in 100 µL water suspension.

To assess the biodistribution of nanoparticles in systemic abdominal organs, axial slices with 1mm thickness (n=20) were positioned over organs of interest such as liver, spleen and kidneys. MR imaging was performed using a susceptibility-weighted gradient echo sequence with the following parameters: TR/TE = 300/3ms, in plane pixel resolution=100x100µm, flip angle=30°, 4 averages.

An ultrapure water tube was positioned over the body of the mice as external reference to normalize the MR signal and allow measurement of contrast-to-noise ratio (CNR) variation in the different regions of interests.

CNR was defined as:

\[
CNR = \frac{SNR_{ROI} - SNR_{ref}}{SNR_{ref}}
\]

with

\[
SNR = \frac{Mean_{Signal}}{SD_{Noise}}
\]
where $\text{ref}$ denotes the water tube reference and $\text{SD}$ is the standard deviation of the noise in the image. A water tube, used as a reference, was positioned near the mice to enable

![Figure 3.9.6. MRI images with timing intervals on female Balb/C mice after 100 \(\mu\text{g/mice}\) PDFC injection](image)

CNR measurement and proton NMR signal normalization. Within the liver, regions of interest (ROIs) were drawn around apparent vascular structures and these regions were
subtracted from the map to retain liver parenchyma only. ROIs encompassing the whole spleen or the two kidneys were manually selected for signal measurement.

To allow tracking of nanoparticles biodistribution in the lung, noninvasive free-breathing pulmonary MR imaging was performed using a radial ultra-short time of echo (UTE) sequence with the following parameters: TR/TE=100/0.4ms, in plane resolution=100x100µm, flip angle=15°, 4 averages. Axial slices with 1mm thickness (n=10) were positioned to cover the entire lung volume. CNR was measured in ROI drawn around apparent vascular structures in the lung and these regions were subtracted from the map to retain lung parenchyma only.

As an outcome it could be mentioned, that first all mice well tolerated the injection of PDFC’s at the concentration 100 µg/mice. Second, one month post-injection follow-up

- Female Balb/C mice

- Concentration 100 µg/mice

- Good contrasting at 100 µg/mice

- Complete clearance
showed complete clearance of PDFC’s from mice organisms (monitored by MRI). Third, excellent $T_2$ contrasting was achieved, especially after 2 hours of post-injection. Therefore, based on these preliminary observations we can note, that PDFC’s need to be studied *in vivo* further as highly contrasting agent for MRI.
3.10. X-Ray CT imaging with PDPCs

Similarly to MRI agar phantoms X-Ray CT phantoms were prepared, but with higher concentrations. Images on a GE machine from the KMC clinic were recorded. At 90 keV contrasting was better compared to the 100 and 120 keV, but considering the further safety reasons imaging for 100 keV was chosen.

Figure 3.10.1. X-Ray CT images 100 keV for PDFC’s agar phantoms at the concentrations in mg/ml

keV contrasting was better compared to the 100 and 120 keV, but considering the further safety reasons imaging for 100 keV was chosen.
Different imaging windows, such as brain sinus, extremity, cervical spine, bones, liver (X-Ray CT machine installed image processing software protocols) was tested. Concentration depending contrasting was detected by using PDFC’s starting from the 30 µg/ml which is very good value for barium-, iodine-, and gold-free containing X-Ray contrasts. This due to high density of PDFC’s comprising metallic core, which is 8.7 g/cm² for the ratio Fe₆₅Co₃₅ of alloy. Another factor is higher concentration of relatively heavier iron and cobalt atoms, compared to their natural body concentrations. Thus, PDFC’s may serve as additional, X-Ray diagnostic information provider to MRI imaging in combined X-Ray CT/MRI dual mode imaging machines and the

![Figure 3.10.2. X-Ray CT images with different image processing windows at 90 keV for PDFC’s agar phantoms at the concentrations in mg/ml](image)

- 155 -
combination of both imaging modalities may provide more detailed information for the operator.
3.11. NIR laser phothermal therapy *in vitro* effect on cancer cells

Among the nanoparticles used in cancer photothermal therapy with NIR laser the most widely studied systems are mainly based on using of gold, graphene, fullerene, and carbon nanotubes. But relatively very recently, in this year, polydopamine, as much more biocompatible material has gained significant attention as a photothermal mediator, which efficiently converts NIR laser light energy to the thermal.

Despite tens of papers have been published using polydopamine-coated or polydopamine itself nanoparticles as NIR photothermal
mediators, all this systems are lacking multimodality which has our PDFC nanoparticles. To evaluate the properties of PDFC’s to induce cancer cell death upon NIR laser irradiation we have designed the following in vitro studies with HeLa cells and 808 nm Fiber-Coupled 5mW LED laser.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Death %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained Hela (Negative Control)</td>
<td>0.87</td>
</tr>
<tr>
<td>HeLa cells Stained with PI (Positive Control)</td>
<td>25.35</td>
</tr>
<tr>
<td>HeLa cells + NIR (Control)</td>
<td>22.78</td>
</tr>
<tr>
<td>HeLa cells + PDFC + NIR (Main Experiment)</td>
<td>60.44</td>
</tr>
</tbody>
</table>

Table 3.11. Quantitative results of death by the HeLa cells count on FACS with propidium iodide staining. 808 nm 0.5 W/cm² laser for 15 min kills 60.44% of HeLa cell after incubation with 30 µg/ml PDFC’s

With this aim HeLa cells were grown in 96 well plates at a density 5000 cells per well in EMEM cell culture media containing 10% of FBS and 0.1% of penicillin–streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Then HeLa cells were incubated with PDFC- at the concentrations – 30 µg/ml (the same concentration, which was efficiently used in DOX delivery and the microwave chemotoxicity experiments) for 8h. Then the non-internalized cells were washed out fresh and media added prior exposure to the NIR laser and harvesting for flow cytometric (FACS) cell viability assay with propidium iodide staining. After the addition of WST-8 dye cells were incubated for 3h according to the protocol prior to colorimetric measurement. The
results demonstrated very good photothermal effect – 60.4 % percent cell death (Figure 3.13.1). Other lines are representing the control experiments, which are HeLa cells unstained, HeLa cells stained, HeLa cells with the same 15 min of NIR laser exposure, but without nanoparticles (Table 3.13.1)
3.12. Alternating magnetic field hyperthermia with PDFC

Next we evaluated the properties of PDFC’s under alternating magnetic field. Specifically specific absorption rates (SAR) were measured to proof feasibility of their use as magnetic hyperthermia treatment mediators. With this aim two widely used frequencies of alternating magnetic field were tested – 370 and 470 KHz. Different frequencies was applied by different water-cooled copper coils and the 30% of machine power was used for 370 KHz coil and the 100% of machine power was used for 470 KHz coil. The temperature rise versus time was registered by optical probe connected to the reading device with computer interface.

First, 1 mg/ml dispersion of PDFC’s in degassed and deionized water was prepared in a

![Figure 3.12.1. Specific absorption rate of PDFC's at 1mg/ml concentration and 370 KHz of working frequency.](image)

\[
\text{SAR} = 65 \text{ W/g}
\]
1 glass vial with rubber septum. This glass vial mass was measured and it was placed inside of bigger diameter hard wall glass vial with rubber septum cup in such a way that it was not contacting the walls of vials and was hanging solely on 0.5 mm diameter fiber optical temperature probe. This procedure minimizes heat exchange between sample and environment for accurate SAR measurement. The sample was placed in a center of the copper coil during measurement.

The SAR was calculated by the common formula and it was found that at 470 KHz

![Figure 3.12.2. Specific absorption rates for PDFC's at different concentrations and 470 KHz of working frequency.](image)

PDFC's has 180 W/g heating power and only 65 W/g at 370 KHz. Moreover, at 470 KHz the response was more linear at concentrations before 2.5 mg/ml (at high
concentrations, such as 5 mg/ml it is non-linear anymore because of intense aggregation) depending from the time and different concentrations. Thus, the conclusion is that PDFC's may be served as good alternating magnetic field hyperthermia treatment mediators at 470 KHz of working frequency with the 180 W/g SAR, which is higher than for commercially used Ferridex™ (only 83 W/g even at 1000 KHz).
Chapter 4

Conclusion

In summary two strategies in medicinal chemistry were united in this dissertation. First, the new class of anticancer compounds, iso-phidianidines, was designed inspired by natural phidianidines structure. Efficient and short synthetic route toward these compounds was developed and small library of compounds was obtained. Solid phase route was also developed for easier purification of final products. Biological activities for iso-phidianidines were tested on human cancer cervical HeLa cell lines, and the cytotoxicity was within micromolar range. One compound was identified as lead with 11 mM activity. Structure activity relationship on iso-phidianidines revealed increase of activity with increase of diamine side chain length, the importance of presence of proton at nitrogen near 5’ position of oxadiazole ring of iso-phidianidines. Polyamine side chain, and aromatic diamine versus aliphatic diamine showed increased activity. The presence of terminal guanidine group was not necessary for anticancer activity, because amino-terminated compounds were very active.

Second, the modern strategy in medicinal chemistry, nanomedicine was used to design, synthesize and evaluate polydopamine coated iron-cobalt nanocubes for their unprecedented seven theranostic modalities. These novel nanomedicines significantly enlarged the concept of “One-for-All” theranostic nanoparticles for multiple molecular image guided therapy of cancer. Novel cancer treatment method was developed by
combination of microwave irradiation with internalized polydopamine coated nanoparticles leading to induction of chemotoxicity and intensive death of cancer cells with low concentration of nanocomposite and low, non-thermic power of microwave irradiation.

Moreover, extensive and deep literature treatment was done and state of the art of phidianidines chemistry and nanomaterials chemistry for multiple cancer theranostic application was reviewed in the following dissertation.
References


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APPENDICES

Spectral Data for iso-Phidianidines

1.  N-(Indol-3-yl-methyl)piperidine: $^1$H-NMR (500 MHz, CDCl$_3$): 8.5 s [1N-H], 7.83-7.85 d [1CHar.], 7.43-7.41 d [1CHar.], 7.22-7.28 dd [2CHar.], 7.19 s [1CHar.], 3.75 s [2CH$_2$], 2.25-3.26 d [4CHpip.], 1.62-1.81 m [4CH$_2$pip.], 1.48-1.37 m [2CH$_2$pip.]:

$^{13}$C-NMR (125 MHz, CDCl$_3$) 136.8, 128.3, 122.8, 120.9, 118.6, 118.1, 111.9, 110.5, 58.1, 55.9, 25.2, 24.5

2.  Indoly-3-acetonitrile: $^1$H-NMR (500 MHz, CDCl$_3$): 6.69-6.71 d [1CHar.], 6.51-6.53 d [1CHar.], 6.45-6.46 d [1CHar.], 6.23-6.27 dt [1CHar.], 6.15-6.19 dt [1CHar.], 3.1 s [2CH$_2$]:

$^{13}$C-NMR (125 MHz, CDCl$_3$): 136.2, 126.0, 123.9, 121.6, 119.4, 118.9, 118.0, 111.7, 103.6, 13.2

3.  Indoly-3-yl-methyl amidoxime: $^1$H-NMR (500 MHz, CDCl$_3$): 7.94-7.96 d [1CHar.], 7.58 s [1CHar.], 7.36-7.38 d [1CHar.], 7.12-7.15 dt [1CHar.], 7.06-7.09 dt [1CHar.], 4.9 bs [4NH, OH], 3.34 s [2CH$_2$]:

$^{13}$C-NMR (125 MHz, CDCl$_3$): 153.3, 138.1, 126.2, 125.6, 123.1, 121.9, 121.0, 112.4, 109.3
4. 3-(Indoly-3-yl-methyl)-5-trichloromethyl-1,2,4-oxadiazole: \[^1\text{H-NMR}\] (500 MHz, CDCl\(_3\)): 8.07 bs [1NH], 7.59-7.60 d [1Char.], 7.25-7.27 d [1Char.], 7.14-7.15 d [1Char.], 7.11-7.13 dt [1Char.], 7.08 s [1Char.], 7.05-7.07 d [1Char.], 4.2 s [2CH\(_2\)];

\[^{13}\text{C-NMR}\] (125 MHz, CDCl\(_3\)): 174.3, 170.7, 136.2, 126.9, 123.3, 122.5, 119.9, 118.8, 111.4, 108.6, 83.5, 22.8

5. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(4'-aminoethyl)-5-amino-oxadiazole: \[^1\text{H-NMR}\] (500 MHz, CDCl\(_3\)): 7.53-7.58 d [1Char.], 7.23-7.25 d [1Char.], 7.21 s [1Char.], 7.04-7.06 dt [1Char.], 6.95-6.98 dt [1Char.], 3.9 s [2CH\(_2\)], 3.29-3.3 m [2CH\(_2\)], 2.73-2.76 m [2CH\(_2\)]

\[^{13}\text{C-NMR}\] (125 MHz, CDCl\(_3\)): 173.0, 171.1, 138.0, 128.4, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 46.8, 41.7, 23.5

6. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(4'-aminopropyl)-5-amino-oxadiazole: \[^1\text{H-NMR}\] (500 MHz, CDCl\(_3\)): 8.8 [mNH], 7.51-7.50 d [1Char.], 7.31-7.32 d [1Char.], 7.14 s [1Char.], 7.05-7.08 dt [1Char.], 6.96-6.99 dt [1Char.], 3.9 s [2CH\(_2\)], 3.57-3.95 m [2CH\(_2\)], 3.29-3.33 m [2CH\(_2\)], 2.61-2.64, 1.67-1.7, 1.15-1.27

\[^{13}\text{C-NMR}\] (125 MHz, CDCl\(_3\)): 172.9, 171.1, 138.0, 128.4, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 41.8, 41.7, 33.3, 23.5, 18.3

7. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(4'-aminobutyl)-5-amino-oxadiazole: \[^1\text{H-NMR}\] (500 MHz, CDCl\(_3\)): 8.3 bs, [mNH], 7.54-7.52 d [1Char.], 7.32-7.34 d [1Char.],
7.15 s [1CHar.], 7.07-7.10 dt [1CHar.], 6.97-7.00 dt [1CHar.], 3.97 s [2CH₂], 3.31-3.34 m [2CH₂], 2.28-2.91 m [2CH₂], 1.64-1.66

\(^{13}\text{C-NMR}\) (125 MHz, CDCl₃): 172.8, 171.1, 138.0, 128.3, 124.4, 122.4, 119.7, 119.3, 112.2, 109.7, 43.5, 40.1, 27.3, 25.6, 23.5

8. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(4'–aminoheptyl)-5-amino-oxadiazole: \(^{1}\text{H-NMR}\) (500 MHz, CDCl₃): 8.3 bs [mNH] 7.54-7.53 d [1CHar.], 7.32-7.34 d [1CHar.], 7.15 s [1CHar.], 7.07-7.10 dt [1CHar.], 6.97-7.00 dt [1CHar.], 3.97 s [2CH₂], 3.28-3.3 m [2CH₂] 1.58-1.61 m [4CH₂], 1.38-1.40 [2CH₂]

\(^{13}\text{C-NMR}\) (125 MHz, CDCl₃): 172.8, 171.1, 138.1, 128.4, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 44.1, 40.5, 30.2, 28.4, 27.0, 26.9, 23.5

9. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(4'-aminoheptyl)-5-amino-oxadiazole: \(^{1}\text{H-NMR}\) (500 MHz, CDCl₃): 8.2 bs [mNH] 7.54-7.53 d [1CHar.], 7.32-7.34 d [1CHar.], 7.15 s [1CHar.], 7.07-7.10 dt [1CHar.], 6.97-7.00 dt [1CHar.], 3.97 s [2CH₂], 3.26-3.31 m [2CH₂] 2.85-2.88 m [2CH₂], 1.57-1.62 [5CH₂]

\(^{13}\text{C-NMR}\) (125 MHz, CDCl₃): 172.8, 171.1, 138.0, 128.4, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 44.2, 40.6, 30.3, 29.7, 28.4, 27.3, 26.9, 23.5

10. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(1’,2’-diaminocyclohexyl)-5-amino-oxadiazole: \(^{1}\text{H-NMR}\) (500 MHz, CDCl₃): 8.32 bs [mNH] 7.53-7.55 d [1CHar.], 7.32-
7.34 d [1CHar.], 7.17 s [1CHar.], 7.07-7.10 dt [1CHar.], 6.97-7.00 dt [1CHar.], 3.99 s [2CH₂], 3.35-3.58 m [2CH₂], 3.05 s [2CH₂] 3.02 -3.06 m [2CH₂]

\(^{13}\)C-NMR (125 MHz, CDCl₃): 172.6, 171.2, 138.1, 128.3, 124.4, 122.5, 119.8, 119.3, 112.2, 109.8, 57.1, 55.5, 32.8, 31.04, 25.4, 24.7, 23.5

11. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(piperazyl)-5-amino-oxadiazole: \(^1\)H-NMR (500 MHz, CDCl₃): 8.21 bs [mNH] 7.53-7.55 d [1CHar.], 7.32-7.34 d [1CHar.], 7.16 s [1CHar.], 7.06-7.09 dt [1CHar.], 6.96-6.99 dt [1CHar.], 4.00 s [2CH₂], 3.80-3.82 m [2CH₂], 3.61-3.62 m [2CH₂] 2.58 -2.62. m [2CH₂]

\(^{13}\)C-NMR (125 MHz, CDCl₃): 171.9, 171.8, 138.0, 128.3, 124.5, 122.5, 119.7, 119.3, 112.2, 109.6, 48.4, 45.4, 23.6

12. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(phenylene)-5-amino-oxadiazole: \(^1\)H-NMR (500 MHz, CDCl₃): 8.5 bs [mNH] 7.57-7.97 d [2CHar.], 7.34-7.35 d [2CHar.] 7.32-7.34 d [1CHar.], 7.22 s [1CHar.], 7.09-7.1 dt [1CHar.], 7.01-7.07 dt [1CHar.], 6.6 -6.9 d [2CHar.] 4.6 s [2CH₂],

\(^{13}\)C-NMR (125 MHz, CDCl₃): 171.8, 170.9, 139.7, 137.9, 136.1, 128.3, 124.5, 122.5, 119.7, 119.3, 111.4, 109.8, 23.5

13. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(piperazyl)-5-amino-oxadiazole: \(^1\)H-NMR (500 MHz, CDCl₃): 8.6 bs [mNH] 7.87-7.88 d [1CHar.], 7.52-7.54 d [1CHar.], 7.13 s [1CHar.], 7.05-7.08 dt [1CHar.], 6.96-6.99 dt [1CHar.], 4.9 s [2CH₂], 3.33-3.29 m
[2CH₂], 2.70-2.73 m [2CH₂] 2.48 -2.62. m [6CH₂], 2.42-2.46 m [2CH₂] 1.72-1.75 m [2CH₂], 1.63-1.67 m [4CH₂]1.43-1.46 m [2CH₂]

13C-NMR (125 MHz, CDCl₃): 176.3, 172.9, 138.0, 128.4, 124.5, 122.5, 119.8, 119.4, 112.3, 109.9, 49.8, 47.2, 47.0, 42.1, 40.0, 30.7, 29.8, 27.6

14. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(metapyrydine)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 7.9 bs [mNH] 7.41-7.45 d [1CHar.], 7.33-7.35 dt [1CHar.], 7.27 – 7.28 d [2CHar.], 7.23 s [1CHar.] 7.19 -7.20 d [1CHar.], 7.08 – 7.11 dt [1CHar.], 6.99 – 7.02 dt [1CHar.], 4.3 s [2CH₂], 3.80-3.82 m [2CH₂], 3.61-3.62 m [2CH₂] 2.58 -2.62. m [2CH₂]

13C-NMR (125 MHz, CDCl₃): 172.1, 170.7, 164.8, 160.2, 153.1, 150.6, 140.6, 138.0, 128.2, 124.6, 122.6, 119.9, 119.6, 112.3, 109, 39 2

15. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinoethylene)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 8.54 bs [mNH] 7.50-7.52 d [1CHar.], 7.21-7.23 d [1CHar.], 7.06-7.09 dt [1CHar.], 6.99-7.06 dt [1CHar.], 7.00 s [1CHar.], 3.92 s [2CH₂], 3.41 m [2CH₂] 1.33 -1.42 s [9CH₃], 1.18 s [2CH]

13C-NMR (125 MHz, CDCl₃): 170.9, 169.8, 162.8, 157.1, 153.0, 136.2, 127.0, 123.3, 122.2, 119.6, 119.3, 118.8, 111.4, 109.4, 83.8, 80.0, 40.1, 31.1, 27.8-28.2

16. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinopropylene)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 8.54 bs [mNH] 7.50-7.52 d [1CHar.], 7.27-
7.28 d [1CHar.], 7.10 s [1CHar.], 7.02-7.05 dt [1CHar.], 6.93-6.96 dt [1CHar.] 3.93 s [2CH₂], 3.34-3.37 t [2N-CH₂], 3.27-3.31 t [2N-CH₂], 3.31 s [2CH], 1.73-1.75 m [CH₂] -1.41-1.45 s [9CH₃]

1³C-NMR (125 MHz, CDCl₃): 171.3, 169.8, 163.4, 156.6, 153.3, 136.4, 127.1, 122.9, 121.9, 119.3, 119.0, 111.5, 109.6, 83.5, 79.8, 42.3, 40.3, 28.2, 26.29, 22.99

17. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinoheptylene)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 8.2bs [mNH] 7.27-7.29 d [1CHar.], 7.04-7.11 d [1CHar.], 7.02 s [1CHar.], 6.93-6.96dt [1CHar.], 6.94-6.96 dt [1CHar.] 3.93 s [2CH₂], 3.31 s [4CH₂], 3.26-3.27 dt [2N-CH₂], 3.23-3.25 t [2N-CH₂], 3.31 s [2CH], 1.53-1.56 m [4CH₂], 1.49 s [6CH2]-1.42-1.47 s [9CH₃]

1³C-NMR (125 MHz, CDCl₃): 172.8, 171.1, 164.5, 157.5, 154.2, 138.0, 128.4, 124.4, 122.4, 119.7, 119.4, 112.2, 109.8, 84.4, 48.4, 44.2, 41.1, 30.3, 29.9, 28.5, 23.4

18. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinopropylene)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 8.54 bs [mNH] 7.50-7.52 d [1CHar.], 7.27-7.28 d [1CHar.], 7.10 s [1CHar.], 7.02-7.05 dt [1CHar.], 6.93-6.96 dt [1CHar.] 3.93 s [2CH₂], 3.34-3.37 t [2N-CH₂], 3.27-3.31 t [2N-CH₂], 3.31 s [2CH], 1.73-1.75 m [CH₂] -1.41-1.45 s [9CH₃]

1³C-NMR (125 MHz, CDCl₃): 170.9, 169.8, 162.8, 157.1, 153.0, 136.2, 127.0, 123.3, 122.2, 119.6, 119.3, 118.8, 111.4, 109.4, 83.8, 80.0, 40.1, 31.1, 27.8-28.2, 23.5
19. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinobutylene)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.3 bs [mNH] 7.31-7.33 d [1Char.], 7.09-7.015 d [1Char.], 7.08 s [1Char.], 7.00-7.06 dt [1Char.], 6.97-6.98 dt [1Char.] 3.96 s [2CH$_2$], 3.29-3.34 ss [4N-CH$_2$], 3.26 - 3.29 m [2CH$_2$], 3.31 s [2CH], 1.55-1.58 m [3CH$_2$] -1.49-1.45 s [9CH$_3$], 1.50 s [6CH]

$^{13}$C-NMR (125 MHz, CDCl$_3$): 172.8, 171.1, 164.5, 157.5, 154.2, 138.0, 128.4, 124.4, 122.4, 119.7, 119.4, 112.2, 109.8, 84.4, 80.3, 44.2, 41.7, 30.3, 29.8, 29.9, 28.8, 27.6, 23.5.

20. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinohexylene)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.3 bs [mNH] 7.32-7.33 d [1Char.], 7.11-7.14 d [1Char.], 7.10 s [1Char.], 7.07-7.08 dt [1Char.], 6.97-7.00 dt [1Char.] 3.95 – 3.96 d [2N-CH$_2$], 3.34 s [2CH$_2$], 2.03-2.05 d [2CH$_2$], 1.72-1.74 d [2CH], 1.47 s [6CH$_2$] -1.41-1.45 s [9CH$_3$], 1.36-1.38 m [CH2]

$^{13}$C-NMR (125 MHz, CDCl$_3$): 172.6, 170.9, 164.4, 157.7, 153.9, 138.4, 128.4, 124.3, 122.4, 119.8, 119.3, 112.2, 109.7, 84.5, 80.4, 58.7, 55.5, 33.2, 28.8, 25.5

21. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-bocguanidinopiperazine)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.2 bs [mNH] 7.43-7.42 d [1Char.], 7.22-7.25 d [1Char.], 7.15 s [1Char.], 7.10-7.12 dt [1Char.], 6.98-7.00 dt [1Char.] 3.65 – 3.67d [2N-CH$_2$], 3.34 – 3.36 d [2CH$_2$], 1.47 s [6CH$_2$] -2.41-1.45 s [2CH], 1.46-1.48 s [9CH$_3$]
$^{13}$C-NMR (125 MHz, CDCl$_3$): 171.9, 170.1, 164.3, 157.7, 153.9, 138.4, 128.4, 124.3, 122.5, 119.7, 119.7, 112.5, 109.4, 84.5, 80.4, 58.7, 51.5, 46.2, 23.4

22. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-boc-guanidinophenylene)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.4 bs [mNH] 7.60-7.61 d [2Char.], 7.36-7.37 d [2Char.] 7.34-7.36d [1Char.], 7.19 s [1Char.], 7.09-7.11 dt [1Char.], 7.01-7.07 dt [1Char.], 6.6 -6.9 d [2Char.] 4.1 s [2CH$_2$]

$^{13}$C-NMR (125 MHz, CDCl$_3$): 171.8, 170.9, 139.7, 137.9, 136.1, 128.3, 124.5, 122.5, 119.7, 119.3, 111.4, 109.8, 84.5, 80.4, 53.5, 49.2, 23.5

23. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-boc-guanidinocadaverine)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.3 bs [mNH] 7.65-7.67 d [2Char.], 7.317.32 d [2Char.] 7.15-7.18 dt [1Char.], 7.09-7.11 dt [1Char.], 7.09-7.11 dt [1Char.], 7.1 s [1Char.], 4.0 s [2CH$_2$] -3.53-3.63 mm [4Char. and 6 N-CH$_2$], 1.53 s [2CH], 1.44-1.9 ss [9CH$_3$]

$^{13}$C-NMR (125 MHz, CDCl$_3$): 170.8, 170.4, 155.6, 136.3, 127.2, 123.0, 122.2, 119.6 119.1, 111.1, 110.1, 45.39, 28.1-28-4, 23.5

24. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(di-boc-guanidinometapyrdine)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.2 bs [mNH] 7.43-7.48 d [1Char.], 7.39-7.40 dt [1Char.], 7.29– 7.32 d [2Char.], 7.19 s [1Char.] 7.16 -7.18 d [1Char.], 7.10 – 7.13dt [1Char.], 6.99 – 7.02 dt [1Char.], 4.1 s [2CH$_2$], 3.81-3.83 m [2CH$_2$], 3.59-3.60 m [2CH$_2$ ], 1.53 s [2CH], 1.41-1.5 ss [9CH$_3$]
13C-NMR (125 MHz, CDCl₃): 171.0, 169.7, 164.5, 161.2, 155.3, 151.6, 141.4, 137.2, 129.2, 125.2, 121.1, 120.0, 111.4, 85.5, 80.4, 55.1, 50.1, 23.9

25. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(guanidinometapyrydine)-5-amino-oxadiazole: ¹H-NMR (500 MHz, CDCl₃): 8.4 bs [mNH] 7.52-7.53 d [1CHar.], 7.32-7.34 d [1CHar.], 7.16 s [1CHar.] 7.08– 7.10 dt [2CHar.], 7.16 -7.18 dt [1CHar.], 6.98 – 7.00 dt [1CHar.], 4.1 s [2CH₂], 3.9 s [2CH₂], 3.34-3.36 m [2N-CH₂], 3.16 – 3.19 m [2N-CH₂], 1.81 – 1.84 dt [2CH₂]

13C-NMR (125 MHz, CDCl₃): 172.9, 171.1, 158.1, 138.1, 128.3, 124.4, 122.5, 119.3, 112.2, 109.8, 41.4, 39.5, 29.7, 23.5

26. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(guanidinopropylene)-5-amino-oxadiazole:

¹H-NMR (500 MHz, CDCl₃): 8.3 bs [mNH] 7.52-7.54 d [1CHar.], 7.32-7.34 d [1CHar.], 7.15 s [1CHar.] 7.07– 7.10 dt [2CHar.], 7.16 -7.18 dt [1CHar.], 6.97 – 7.00 dt [1CHar.], 3.9 s [2CH₂], 3.31-3.34 m [2N-CH₂], 3.13– 3.15 m [2N-CH₂], 1.60 – 1.61 dt [2CH₂]

13C-NMR (125 MHz, CDCl₃): 172.8, 171.1, 158.5, 138.1, 128.3, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 43.7, 41.9, 27.6, 26.9, 23.5

27. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(guanidinopropylene)-5-amino-oxadiazole:

¹H-NMR (500 MHz, CDCl₃): 8.3 bs [mNH] 7.52-7.54 d [1CHar.], 7.32-7.34 d [1CHar.], 7.15 s [1CHar.] 7.07– 7.10 dt [2CHar.], 7.16 -7.18 dt [1CHar.], 6.97 – 7.00
dt [1CHar.], 3.9 s [2CH2], 3.31-3.34 m [2N-CH2], 3.13– 3.15 m [2N-CH2], 1.60 – 1.61 dt [2CH2]

$^{13}$C-NMR (125 MHz, CDCl3): 172.8, 171.1, 158.5, 138.1, 128.3, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 43.7, 41.9, 27.6, 26.9, 23.5

28. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(guanidinobutylene)-5-amino-oxadiazole:

$^1$H-NMR (500 MHz, CDCl3): 8.3 bs [mNH] 7.52-7.54 d [1CHar.], 7.32-7.34 d [1CHar.], 7.15 s [1CHar.] 7.07 – 7.08 dt [2CHar.], 7.16 -7.18 dt [1CHar.], 6.98 – 7.00 dt [1CHar.], 3.9 s [2CH2], 3.27-3.3 m [2N-CH2], 3.10– 3.13 m [2N-CH2], 1.53 – 1.60 dt [3CH2]

$^{13}$C-NMR (125 MHz, CDCl3): 172.8, 171.1, 158.5, 138.1, 128.3, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 43.7, 41.9, 27.6, 26.9, 23.5

29. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(guanidinoheXylene)-5-amino-oxadiazole:

$^1$H-NMR (500 MHz, CDCl3): 8.3 bs [mNH] 7.53-7.54 d [1CHar.], 7.32-7.33 d [1CHar.], 7.15 s [1CHar.] 7.08– 7.10 dt [2CHar.], 6.97 – 7.00 dt [1CHar.], 3.9 s [2CH2], 3.26-3.28 m [2N-CH2], 3.10– 3.13 m [2N-CH2], 1.53 – 1.59 mm [4CH2], 1.37 ss [CH2]

$^{13}$C-NMR (125 MHz, CDCl3): 172.8, 171.1, 158.5, 138.0, 128.3, 124.4, 122.4, 119.7, 119.3, 112.2, 109.8, 44.7, 41.2, 42.3, 30.3, 29.8, 27.5, 23.5
30. 3-(Indoly-3-yl-methyl)-N-(1,2,4-(1’,2’-guanidino-diaminocyclohexyl)-5-amino-oxadiazole: $^1$H-NMR (500 MHz, CDCl$_3$): 8.3 bs [mNH] 7.52-7.54 d [1CHar.], 7.31-7.33 d [1CHar.], 7.15 s [1CHar.], 7.07-7.10 dt [1CHar.], 6.98-7.01 dt [1CHar.], 3.99 s [CH], 3.05 s [CH] 2.35-2.58 m [2CH$_2$], 2.02 -2.06 m [2CH$_2$]

$^{13}$C-NMR (125 MHz, CDCl$_3$): 172.6, 171.2, 138.1, 128.3, 124.4, 122.5, 119.8, 119.3, 112.2, 109.8, 57.1, 55.5, 32.8, 31.3, 25.3, 20.5, 23.5