Experimental platform for characterization and high-throughput screening of acetylcholinesterase inhibitors.

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Acetylcholinesterase (AChE) enzyme as a part of the cholinergic neuronal pathways is involved in learning and memory. As such, AChE is utilized as a drug target in treatment of neurodegenerative diseases such as Alzheimer's disease (AD). The disease is officially listed as the sixth-leading cause of death. Due to lack of effective therapy and demographic trends it is expected that the number of patients with AD will continue to increase dramatically. Since the AChE inhibitors currently used in treatment of AD have limited effectiveness there is a great need for new therapeutics. This, however, creates a demand for techniques that will allow rapid and effective selection of new drug candidates and facilitate the development of new drugs. Here we present novel experimental platform which combines affinity chromatography (AC) and isothermal titration calorimetry (ITC) for comprehensive characterization of new AChE inhibitors.

We propose the AC as a tool for rapid selection of drug candidates acting on AChE from large chemical libraries. For this, human recombinant AChE was immobilized on a chromatographic support, i.e. ethylenediamine monolithic convective interaction media (CIM) disk. The obtained micro-immobilized-enzyme reactor (IMER) was connected to a standard HPLC system. The system was validated and obtained results showed very good correlation with the data from conventional method. The optimized analysis protocols allowed for rapid on-line screening of AChE inhibitors, determination of their activity with simultaneous evaluation of their binding kinetics. Moreover, immobilized enzyme showed increased stability compared to the in-solution form. Consequently, assay costs were reduced and data reproducibility was increased. We manage also to reduce influence of any nonspecific interactions between the screened compounds and the chromatographic support. The described instrumental set-up can be easily automated making the method suitable for high-throughput screening.

The ITC technique constitutes the second component of the platform, which facilitates further development of the selected drug candidates. The ITC experimental protocols were tuned up for comprehensive and tailored characterization of AChE-inhibitor interaction, including determination of AChE kinetics, potency and affinity evaluation of AChE inhibitors, determination of their mechanism of action and stoichiometry of the interaction. For the first time, we also reported thermodynamic parameters of AChE-inhibitor

interaction, which are crucial for rational drug design and can be used as a predictor of the drug selectivity. The method allowed to study interactions in conditions resembling physiological ones with macromolecules in their native form. Since the method excluded the need for secondary reactions and protein chemical modifications (i.e. labelling) we manage to increase the reproducibility of the inhibitor potency assay and eliminate usual false positive results.

The developed experimental protocols can be easily modified to study other inhibitor-enzyme systems and considered as a universal methodology. Enzymes are targets of 47% marketed small-molecule drugs. They also represent the second largest group of novel target structures. As such, enzymes appear to be one of the most pharmacologically important class of biomolecules. Therefore, obtained results besides application in AChE-inhibitor interaction studies can have a broader impact in the field of drug development.

Structural and biological studies of *trans*-platinum(II) complex with 3-aminoflavone

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For many years it was believed that the platinum complexes of *trans* geometry were non-active as antitumor agents because of inactivity *in vivo* and less cytotoxicity *in vitro* than cisplatin. However, since the 1990s scientists discovered numerous biologically active platinum complexes with *trans* geometry and different substitutes instead of ammine groups, e.g. planar amines, iminoethers, aliphatic amines or non-planar heterocyclic ligands.

The aim of our studies is to synthesis a new *trans* platinum(II) complex of 3-aminoflavone (TCAP, *trans*-$Pt(af)_2Cl_2$) and estimate its cytotoxic and proapoptotic properties. Cisplatin was used as a reference compound. The *in vitro* anticancer activity of the compounds was evaluated against nine cancer cell lines and human lymphocytes.

The obtained results indicated that the novel *trans*-platinum(II) complex effectively inhibited cancer cells growth with IC_{50} values in the 4.6–16.3 μM range. TCAP was found to be slightly less cytotoxic to the tested cancer cell line than cisplatin. Furthermore, TCAP was also less toxic for normal lymphocytes in comparison with cisplatin, which is especially desirable for the prevention of potential drug side-effects. TCAP has the ability to retain cytotoxic activity against cisplatin-resistant cell lines, which could be explained as alternative mechanisms of action.

Topoisomerases have become popular targets for cancer chemotherapy treatments. As the cytotoxicity may be a result of topoisomerase-1 inhibition, enzyme-compound interaction was tested.

Several different methods were used to compare the activities of *trans*-$Pt(3\text{-}af)_2Cl_2$ and cisplatin in inducing apoptosis in model cancer cell lines. A new *trans* platinum(II) complex caused apoptosis. Apoptotic cell death involves a series of morphological and biochemical changes, which include DNA degradation, phosphatidylserine externalization, collapse of mitochondrial transmembrane potential (Ψm) and activation of caspase-3. Interestingly, *trans*-$Pt(af)_2Cl_2$ was a stronger inducer of apoptosis in contrast to *cis*platin despite its lower cytotoxicity.

These results are very promising, as they indicate that TCAP has beneficial features for potential anticancer agent.

Synthesis and applications of new fluorophores based on pyridine or quinolone core

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Fluorescence techniques are commonly used for sensing chemical species in biology, medicine, pharmaceutics, environment and biotechnology. Analytical methods based on fluorescence are quick, specific, highly sensitive and convenient to use. Furthermore in a particular experiment can be determined many of the analytes using different probes with exclusive emission spectra. Development of optical probe technology requires the design of new fluorophores with advantageous physicochemical and optical properties, such as good water solubility when applied in medical research, high photostability, large Stokes shifts, high quantum yields as well as narrow emission spectra.

The aim of this work was to synthesize new fluorescent dyes based on quinoline core with different optical properties. Next step was to label amino acids with obtained dyes and use fluorophores to improve the sensitivity of detection mass spectrometry analysis of proteins with stable, quaternary ammonium ionized tags.

New fluorescent dyes were synthesized based on tandem Mannich – electrophilic amination reaction. Profluorophores reacted with formaldehyde and secondary amines to form fluorescent betaines. 1,2,4-triazolo[4,3-a]pyridin-2-ium-8-carboxylates were further converted into the amine-reactive N-hydroxysuccinimide esters which, in turn, served for labeling free amino groups of amino acids and lysine-containing proteins.

Excitation/emission spectra of the dyes can be tuned in the ranges 350-400 nm and 465-515 nm (blue-green), respectively. The obtained dyes exhibit high quantum yields, large Stokes shift, remarkable water solubility and adjustable polarity (achieved by introducing alkyl chain of various size). The dyes showed no solvatochromism as the optical properties in polar and nonpolar solvents were very similar.

Synthesis of novel 4-chloropicolinonitrile derivatives with tuberculostaticactivitiy

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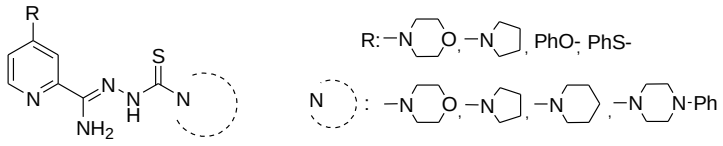
Despite the dramatic growth of bacterial resistance to used antibiotics, the number of new approved antibacterial drugs declined. Also in the case of tuberculosis we observed an increase in the prevalence of MDR-TB and XDR-TB. Merely 50% patients with MDR-TB and 26% patients with XDR-TB completed treatment successfully[1]. Drug development for TB was stagnated for decades, until 2012 when FDA approved bedaquiline as a new antitubercular drug[2]. The crisis of microbial resistance is serious issue of public health in the world. It is necessary to obtain the new drugs with new mechanism of action.

We used phenotypic screening which is one of methods of searching for new biologically active substances. This method is mainly used to search for drugs first-in-class with a new mechanism of action. The aim of our study was design, synthesis and evaluation of biological activity of novel derivatives of 4-chloropicolinonitrile.Pyridine ring frequently occurs in the structure of various therapeutic agents. It is also present in the isoniazid one of the most potent antitubercular drug. Polyfunctional pyridines are characterized by high activity and provide a lot of synthetic opportunities.

Previously we obtained derivatives of 4-methylipicolinonitrile and 4-phenylpicolinonitrile. Obtained compounds showed variety tuberculostatic activity. The most potent were compounds with the thiosemicarbazide structure[3].Promising results of biological activity prompted us to the synthesis of 4-chloropicolinonitriles derivatives with thiosemicarbazide structure.The first step of the synthesis was the nucleophilic substitution of chlorine atom of 4-chloropicolinonitrile by morpholine, pyrrolidine, phenol or thiophenol. Subsequently 4-substituted picolinonitriles were conducted into 4-substituted picolinimidates in the presence of methanol and catalytic amounts of DBU. In the last step picolinonitriles reacted with cycloalkylamino-1-carbothiohydrazides. As a result of the synthesis 16 novel derivatives of 4-chloropicolinonitrile have been obtained. All compounds were characterized by IR, ^{1}H NMR spectra and elemental analysis.



They have been tested for tuberculostatic activity in vitro against M. tuberculosis strains: H_{37}Rv, Spec. 210, Spec. 192. The highest activity exhibited compounds that contained a morpholine ring in the thiosemicarbaizide moiety (<3,1 μg/mL). Activity at the same level also showed compound which contained phenoxy moiety at C-4 position of pyridine ring and pyrrolidine ring in the thiosemicarbazide moiety. For the most active compounds cytotoxic activity was also evaluated. The cytotoxic activity was determined by MTT method on mouse melanoma cell line B16-F10 and human dermal fibroblasts HDF. The findings indicate that the supplied derivatives are good leading structure for discovery of new tuberculostatic agents. A further modification of these structures will allow to determine the structure-activity relationship and the structural elements essential for activity against M. tuberculosis.

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Sulfanyl tribenzoporphyrazines with dendrimeric periphery and their promising photocytotoxicity *in vitro*

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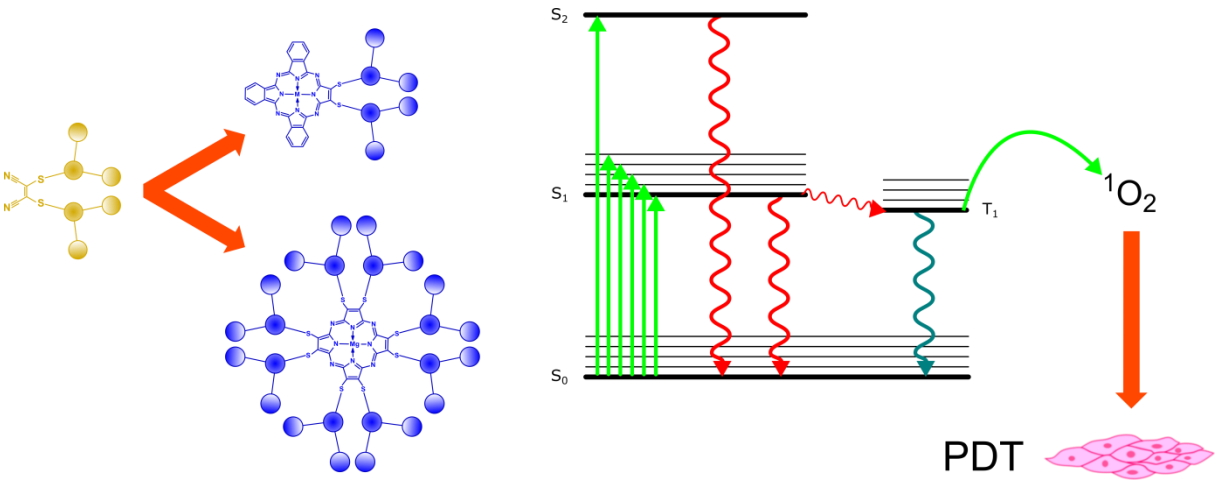
Cancer has become the main cause of death in developed countries. Therefore, there is a constant need to develop new methods for treatment of this disease. One of the modern approaches constitutes photodynamic therapy (PDT), which involves activation of photosensitizer with light of an appropriate wavelength within photodynamic reactions (Figure). PDT is based on *in situ* generation of reactive oxygen species, especially singlet oxygen ($^1O_2$), which induce either necrotic or apoptotic tumor cells death. The main advantage of PDT is the inability of cancer cells to develop specific resistance against this treatment method.



Our study was focused on the synthesis and characterization of novel photosensitizers suitable for PDT. Symmetrical and unsymmetrical porphyrazines were synthesized and characterized using spectral techniques with special emphasis on their photophysical properties. Effectiveness of macrocycles was assessed in photodynamic activity assays against human oral squamous carcinoma cell lines. Among the sulfanyl tribenzoporphyrazines screened, the highest photocytotoxicity with nanomolar IC_{50} values was found for an analogue with 4-[3,5-di(hydroxymethyl)phenoxy]butyl substituents.

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Serendipity discoveries in the development of 5-HT_6 receptor ligands – bioisosterism, organic synthesis, crystal structure and molecular modeling

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Background

One of the most recently identified serotonin receptor subtypes – the 5-HT_6 receptor,[1,2] localized practically only in the brain,[3] is a very promising target for different new psychotropic drugs. These receptors are supposed to be responsible mainly for motor control, memory and learning and it is suggested that ligands of the 5-HT_6R can be used to treat cognitive impairments.[4,5,6] So far, several thousand of ligands have been synthesized and their structural diversity makes consensus binding mode very difficult to be defined.

Aim of the research

The main objective was to investigate ligand-receptor interactions through the use of bioisosteric replacement, crystal structures and molecular modeling. In the course of research some additional findings were further explored.

Results

A number of bioisosteres of the known 5-HT_6R ligands were designed, synthesized, assayed in vitro, crystallized, and docked to homology models of the 5-HT_6R. It was found that aromatic/hydrophobic interactions between ligand and the receptor are of utmost importance while simultaneously the role of a basic nitrogen atom is probably less important.

Within one series of derivatives an interesting relationship between introduction of a halogen atom (chlorine, bromine, iodine) and affinity to receptors led to the discovery of putative halogen binding pockets in 5-HT_6, 5-HT_7 and D_2 receptors.

Crystal structures of bioisosteric pairs of compounds containing arylsulfonyl, benzoyl and benzyl group were used to analyze the role of sulfonyl group in the ligand-receptor interaction. An intramolecular hydrogen bond between oxygen of a sulfonyl group and a hydrogen in the ortho position of an aromatic ring was found to strongly stabilize bioactive conformation of a ligand.

Conclusions

As a result, besides finding new 5-HT_6R ligands, several additional discoveries were made: (i) a new pharmacophore model for the 5-HT_6 receptor ligands, (ii) a new, potential anchoring point for a protonated nitrogen atom, (iii) putative halogen binding pockets and (iv) the intramolecular hydrogen bond stabilizing ligand conformation. None of the above was assumed in the initial aim of the research. These additional findings may provide valuable support in designing new better therapeutics and to improve our understanding of ligand-receptor interactions.

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SYNTHESIS OF NOVEL 4-ARYL-PYRIDO[1,2-C]PYRIMIDINE DERIVATIVES WITH DUAL SERT AND 5-HT_{1A} ACTIVITY

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Our earlier research resulted in the synthesis of a series of pyrido[1,2-c]pyrimidine derivatives, characterized by a double mechanism of action: the inhibition of 5-HT reuptake and agonistic activity towards both pre- and postsynaptic 5-HT_{1A} receptors[1–5]. These compounds can be considered a good entry point for novel drug candidates. The aim of our present study was to synthesize and assess the biological properties of new tetrahydro-pyrido[1,2-c]pyrimidine derivatives with dual activity. New ligands were obtained after the modification of previously synthesized pyrido[1,2-c]pyrimidine derivatives with 3-(piperidin-3-yl)-1H-indole residue in the pharmacophore element. Modifications were performed in the terminal portion, where the pirydo-part of the pyrido[1,2c]pyrimidine ring system was saturated.

Combining a selective serotonin reuptake inhibitor with the 5-HT_{1A} autoreceptors antagonist was proposed in the 1990s as a therapeutic strategy leading to the faster onset of antidepressant action and the greater efficacy of treatment[6]. Several clinical trials proved that the coadministration of pindolol (5-HT_{1A} autoreceptor antagonist) may shorten the latency period of paroxetine and fluoxetine (well-known SSRIs). Concerns about simultaneous, unfavourable in the view of antidepressant activity, non-selective blocking of the postsynaptic receptors[7] generated the concept of combining SSRI activity with the 5-HT_{1A} agonism as another promising strategy for potential new antidepressants development. The FDA registration of vilazodone (Viibryd) in 2011 and vortioxetine (Brintellix) in 2013, as dual-acting antidepressants confirmed the validity of this approach[8,9].

The new 4-aryl-pyrido[1,2-c]pyrimidine derivatives were obtained by way of a multi-step chemical synthesis and subjected to analytical studies, using the methods of ^{1}H NMR and ^{13}C NMR spectroscopy as well as HRMS. The pharmacological profile of the obtained compounds was assessed in radioligands binding assays (5-HT1A, SERT). In vivo functional studies will be conducted in the Institute of Pharmacology, the Polish Academy of Sciences, and the Department of Pharmacobiology of the Jagiellonian University Medical College in Kraków; metabolic stability evaluation will be performed in the Department of Pharmaceutical Chemistry, Medical University of Gdańsk. The results of in vitro and in vivo studies will allow us to draw conclusions regarding structure-activity relationship in the tested group of compounds and to select compounds for further pre-clinical evaluation.

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(*R*,*R′*)-4′-methoxy-1-naphthylfenoterol acts as bitopic, GPR55 + β2-adrenergic receptor, ligand in various tumor cell lines

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Background: (*R*,*R′*)-4′-methoxy-1-naphthylfenoterol (MNF) was designed as an agonists of β2-adrenergic receptor (β2AR), but it is also a potent competitive inhibitor of the oncogenic GPR55 receptor.
Materials and methods: The antitumorigenic effects of MNF effect were investigated using [^{3}H]-thymidine incorporation, cell motility assays, western blotting, and internalization of the fluorescent GPR55 ligand – T1117.
Results: MNF inhibited the proliferation of 1321N1 and U118 astrocytoma cell lines and UACC-647, M93-047 and UACC-903 melanoma cell lines in a β2AR-dependent manner. These effects of MNF were mediated by the activation of adenylyl cyclase, accumulation of cAMP and PKA phosphorylation. Furthermore, MNF elicited rapid drop in the ERK phosphorylation and inducted the activation of eEF2, thus blocking proproliferatory signaling and inhibiting the protein synthesis, respectively.
MNF inhibited protumorigenic signaling in GRP55-positive human HepG2 hepatocarcinoma and PANC-1 pancreatic carcinoma cells. MNF attenuated the internalization of fluorescent GPR55 agonists T1117 and inhibited cell motility in *in vitro* hound healing assay. In the mouse xenograft model, daily MNF treatment for 21 days led to a ∼2-fold reduction in the expression of EGFR, PKM2, β-catenin in PANC-1 tumor tissue relative to vehicle-treated controls. These data suggest that MNF exerts its antitumor effects by inhibiting GPR55-mediated activation of selected cancer biomarkers.
Conclusions: In conclusion, MNF is a bitopic ligand that acts as an agonist of the β2-adrenergic receptor and an antagonist of GPR55 and, therefore, may have therapeutic potential for the management of cancer.
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