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Synthesis, spectral characterization, cytotoxicity and enzyme-inhibiting activity of new ferrocene–indole hybrids

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Dedicated to Professor Vukadin Leovac on the occasion of his 70th birthday.

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ABSTRACT

Incorporation of a ferrocene moiety into the 2-phenylindole scaffold has been recently reported to drastically improve the cytotoxic activity of the parent compounds. In our search for new promising cytotoxic agents we designed and prepared two new ferrocene–indole hybrids, 2-(3-ferrocenylphenyl)-1*H*-indole and 2-(4-ferrocenylphenyl)-1*H*-indole, utilizing the Fischer indole synthesis as the key step. Detailed spectral analyses, including 1D and 2D NMR in various solvents, have been carried out to corroborate the structures of the synthesized compounds. In this work, we also present the first results of biological studies of these two compounds. Both compounds showed weak anticholinesterase activity but high cytotoxicity against rat peritoneal macrophages and the crustacean *Artemia salina*. Also, both compounds showed significant myeloperoxidase inhibiting activity, thus suggesting a potential use in inflammatory disorders. The results of these tests are very encouraging as they also suggest possible cytotoxic activities of these compounds against human cancer cell lines.

1. Introduction

Today a growing number of medicinal and synthetic chemists devote their time and effort in the search for new and/or more potent pharmacologically active compounds that might eventually represent therapeutic agents in the combat against human illnesses of the modern world. It is not enough to arrive at a compound possessing the desired activity but we need a compound that exerts this beneficial effect without being toxic in the applied dose [1]. Hence, it is paramount to know the pharmacological window of the potential drug and this starts by determining its toxicity versus targeted activity. Among a vast number of possible biological assays, one can argue that a "screening" that includes cytotoxicity tests against (*in vivo*) an animal species and (*in vitro*) specialized cell culture of a higher organism (murine), as well as an *in vitro* enzyme inhibiting assays should convey a wide spectrum of relevant data. For example, the brine shrimp cytotoxicity assay, cytotoxicity against rat peritoneal macrophages and acetylcholinesterase inhibition test would nicely suit this screening purpose.

Artemia salina is one of the standard organisms for testing the cytotoxicity of chemicals [2,3]. The *Artemia* bioassay is attractive to researchers due to the commercial availability and possible long storage of the cysts, quickness of the assay and since it complies with animal ethics guidelines in many countries [3].

Macrophages are a heterogeneous population of mononuclear phagocytes present in all tissues of the body. They play a crucial role in regulating and executing most homeostatic, immunological and inflammatory processes [4–6]. Tissue macrophages are the first line of defense against infection by pathogens prior to the migration of polymorphonuclear neutrophils and monocytes. Generally, macrophages' major functions include engulfing, degradation of self or foreign materials and the regulated production of inflammatory mediators such as pro-inflammatory cytokines, prostaglandins and reactive oxygen and nitrogen species. The production of oxygen species, often referred to as respiratory burst, is a tightly regulated mechanism, involving NADPH-oxidase and myeloperoxidase (MPO) as key enzymes. Previous studies have shown that MPO, like enzyme found in all phagocytes (neutrophils and macrophages) [7], exerts both antimicrobial and cytotoxic properties [8,9]. MPO reacts with hydrogen peroxide (H_2O_2) converted from the extra oxygen consumed in the respiratory burst to form a complex that can oxidize a large variety of substances. Among the latter are the chloride ions, which are oxidized to hypochlorous acid, with the subsequent formation of chlorine and chloramines. These products are powerful oxidants that have important roles in host defense by destroying a variety of targets

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including bacteria, fungi and viruses. However, the oxidant activity of MPO might contribute to tumor initiation as it is known to cause DNA damage [10]. Namely, MPO is released by neutrophils and macrophages, which are known to invade and infiltrate tumors, and this enzyme can be a useful specific marker of phagocytes infiltration in premalignant and neoplastic lesions [11]. On the other hand, macrophages are, also, professional antigen-presenting cells and play an important role in inducing of T cell activation and regulating the adaptive immune response [12].

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes acetylcholine at neuromuscular junctions and cholinergic synapses in the brain [13]. AChE activity controls the transmission of nerve impulses and prevents continuous activation of postsynaptic cholinergic receptors. It is vital in maintaining normal function of the entire nervous system [14]. Interestingly, the expression of AChE is not restricted to cholinergic nervous tissues and can even be found in different types of tumors [15]. Moreover, AChE may have some basic functions such as cell differentiation and cell adhesion [16].

Modern organometallic chemistry started with the recognition of the structure of ferrocene [17,18]. Ferrocene or its derivatives are used, among other things, as non-toxic anti-knocking fuel additives [19], as ligand scaffolds [20] and in the synthesis of carbon nanotubes [21]. It has gained a lot of interest in medicinal chemistry and is frequently used as a substituent for the phenyl or alkyl groups [22]. There are several traits that make ferrocene an attractive building block: lipophilicity (it is more lipophilic than benzene, thus it is expected that ferrocene derivatives have greater bioavailability than the parent phenyl compounds), stability (due to an 18 π electron configuration of the iron(II) center) and the reputation of being a "safe" metallocene (unsubstituted ferrocene is a particularly non-toxic compound–abnormally high doses of ferrocene were tested on dogs for 3 months in the study by Yeary [23]. There was no observed acute toxicity even at the doses of 1 g kg^{-1}).

It is impossible to predict the effect of incorporation of ferrocene in molecules that possess biological activity. For example, Loev and Flores [24] reported the first synthesis of ferrocenylated pharmacologically active compounds – yet the prepared ferrocene analogues of amphetamine and phenytoin showed no activity at all. Fortunately, a number of studies exist where ferrocene introduction improved the activity of the parent compound, or changed the activity profile [22]. Ferroquine is 35 times more active than chloroquine against drug-resistant strains of *Plasmodium falciparium* [25]. In addition to the primary mechanism of quinoline action, fluorescent probe studies in infected red blood cells showed another mechanism based on the production of HO· (by the metallocenic moiety) at work [26,27]. Inclusion of ferrocene in the selective estrogen receptor modulator tamoxifen (anti-tumor agent) is reported to have fixed some of the shortcomings of the parent compound. For other examples of successful ferrocene-aryl substitutions, the reader is referred to a review by Fouda et al. [28].

The indole nucleus is present in an array of compounds possessing interesting biological activities [29]. There are a very limited number of reported compounds that contain both the indole and ferrocene moieties [30,31], which is surprising due to an expected contribution of both structural fragments to the overall possible activity of such hybrids. Prompted by the lack of such chemical/biological investigations and motivated by the work of Quirante et al. [31], we recently engaged in a study of the synthesis and biological/toxicological evaluation of ferrocene–indole hybrids. The authors of the aforementioned paper noted that the 2-phenylindole scaffold represented a potent antimitotic agent and prepared a series of 3-ferrocenylmethyl derivatives – two of the synthesized compounds showed 25-fold increase in the cytotoxic activity compared to 2-phenylindole. While Quirante et al. [31] introduced a ferrocene moiety in the position 3 of the indole ring, we decided to prepare a ferrocene–indole hybrid tethered by an aromatic ring. We arrived at the first amounts of these new compounds using the traditional Fischer indole synthesis. Detailed spectral analyses, including 1D and 2D NMR in various solvents, have been carried out to corroborate the structures of the synthesized compounds.

Hence, in this work we report the first results on the biological/pharmacological activity of two new ferrocene–indole hybrids, 2-(3-ferrocenylphenyl)-1*H*-indole and 2-(4-ferrocenylphenyl)-1*H*-indole. We evaluated the biological activity of the two compounds through a cytotoxicity assay using *A. salina* and AChE inhibitory activity. Also, the direct influences of ferrocene–indole hybrids on cells involved in the inflammatory reactions were studied by means of viability and MPO enzymatic activity in rat peritoneal macrophages.

2. Results and discussion

2.1. Synthesis

Ferrocenylacetophenones **2a** and **2b** were prepared by coupling of ferrocene with appropriate diazonium salts (Scheme 1). A reaction of these ketones with phenylhydrazine yielded hydrazones which were directly subjected to a Fischer indolization to give compounds **3a** and **3b**. Fisher indolization has not been performed on a ferrocene-containing hydrazone thus far. Hence, this is the first report of a *de novo* synthesis of indoles in the presence of a ferrocene moiety. While Fischer indole synthesis is known to be catalyzed be various acids [29], only polyphosphoric acid gave reasonable yields. The use of conc. H_2SO_4, *p*-toluenesulfonic acid, glacial acetic acid and $BF_3\cdot Et_2O$ gave no desired product at all. Perhaps this is the reason why previous reports are lacking. Also, please note that we tried to synthesize the third regioisomer (2-(2-ferrocenylphenyl)-1*H*-indole) but failed – only traces of the product were detected. In spite of the general notion that the ferrocene moiety is stable under acidic conditions (an array of ferrocene derivatives are obtained in acidic media), the combination of high temperature and high acidity needed to drive the Fischer indole synthesis turned out to be detrimental to the metallocene (we observed a high degree of decomposition to inorganic iron compounds during these reactions). Although the reaction yields were poor (ca. 20%), this synthetic methodology provides a means of acquiring such new structures of ferrocene–indole hybrids-the ferrocene containing substituent is bonded through carbon-2 of the indole core and tethered by an aromatic ring, whereas only 3-ferrocenyl substituted indoles and non-tethered derivatives have been synthesized so far [30,31]. Currently, new higher yielding synthetic approaches are being tried out in our laboratory.

2.2. Spectral characterization

Mass spectrometry of the two synthesized compounds confirmed their molecular formula ($C_{24}H_{19}FeN$). The mass spectra (fully shown in Supplemental material) were dominated by the molecular ion and its isotopic ions (expected for compounds with high degree of aromaticity). The monoisotopic mass of the molecular ion (377) corresponded to the masses of ions containing only the most abundant isotopes (^{12}C, ^{1}H, ^{56}Fe and ^{14}N). The spectra were also characterized by high intensities of [M−2] ions, a result of the presence of ^{54}Fe atoms. Experimentally determined intensities of ions (from [M+3] to [M−2]) correlated well with the theoretically calculated values for a compound of the molecular formula $C_{24}H_{19}FeN$. Fragment ions were of very low intensities, but the ion at *m*/*z* 56 (Fe^+) found in almost all ferrocene-containing compounds was present in both of the compounds.