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Synthesis and characterization of the anticancer and metal binding properties of novel pyrimidinylhydrazone derivatives

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ABSTRACT

Three novel pyrimidinylhydrazones substituted at either the aromatic moiety or at the imine carbon atom were synthesized and characterized by standard analytical methods. All compounds were found to be toxic in the micro- to submicromolar range against a diverse panel of cancer cell lines including multidrug resistant (MDR) derivatives expressing P-glycoprotein (Pgp). UV–visible spectrophotometry experiments demonstrated that the most active compound (**3**) forms highly stable complexes with iron(III) and copper(II) in a wide pH range with a stronger preference towards iron(III). The redox activity of the iron and copper complexes of ligand **3** was investigated using cyclic voltammetry and was tested with cellular reductants. The impact of reactive oxygen species (ROS) on the mechanism of toxicity was assessed using the ROS-sensitive cell permeable dye 2′,7′-dichlorofluorescin diacetate (DCFDA). Our results demonstrate that the studied pyrimidinylhydrazones form redox-active iron and copper complexes that are capable of producing intracellular ROS, which might lead to cellular damage and cell death in cancer cells regardless of their resistance status.

1. Introduction

The cellular homeostasis of metals is tightly regulated. As a result, only a minor fraction of the intracellular pools is freely available, and the majority of metal ions are bound to storage proteins or enzymes that utilize them as cofactors or as structural support elements [1,2]. It is estimated that at least one third of the human proteome consists of metalloproteins, many of which have been associated with various pathological conditions including infectious, cardiovascular and neurodegenerative diseases [3,4]. As compounds that can directly target the metal ion cofactors of metalloproteins are expected to have a significant biological effect, the development of chelators is a promising strategy in medicinal chemistry. Chelators have been traditionally used for the treatment of metal overload and diseases related to imbalanced metal homeostasis including hemochromatosis, β-thalassemia, and Alzheimer's or Parkinson's diseases [5,6]. Several studies have described the deregulation of iron homeostasis in cancer, suggesting that the altered metal homeostasis of cancer cells represents a vulnerability that can be targeted by chelation strategies [7,8]. Cancer cells increasingly rely on iron due to the crucial role of metalloproteins in proliferation. Since metal homeostasis is tightly linked to the regulation of the intracellular redox balance, anticancer metal complexes can also target cancer cells by the formation of redox active complexes giving rise to reactive oxygen species (ROS) [5,8].

Examples of chelators with antitumor potential include aroyl- and arylhydrazones, and variously substituted thiosemicarbazones [9–15]. The proposed mechanism of action of these antibacterial and anticancer compounds is linked to the inhibition of the iron-requiring enzyme ribonucleotide reductase (RR) [5,8], the rate determining enzyme supplying deoxyribonucleotides for DNA synthesis [16]. The mammalian enzyme consists of two subunits: R1 harbors the catalytically active center; R2 contains a tyrosyl radical and a diiron center [17]. Compounds targeting RR include antimetabolites such as gemcitabine, scavengers of the tyrosyl radical (hydroxyurea), gallium(III) complexes and various chelators. Triapine, a tridentate α-*N*-pyridyl thiosemicarbazone is a potent RR inhibitor currently undergoing phase I and II clinical trials [18–20]. Unfortunately, Triapine is subject to multidrug resistance (MDR), as it is recognized by the ATP-binding cassette (ABC) transporter P-glycoprotein (Pgp/ABCB1) [21]. MDR is a major obstacle in the treatment of cancer. Hence, there is an urgent need for new strategies aiming to overcome MDR [22]. Numerous metal complexes have been developed with the aim of bypassing or even targeting MDR, while minimizing the side effects of clinically used platinum complexes [23].

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Arylhydrazones represent a promising chelator class characterized by antibacterial, antimycobacterial, analgesic and anticancer activities [12,13,24,25]. Several pyridyl- and pyrimidinylhydrazones were reported to be effective against multidrug resistant strains of leprosy and tuberculosis [13,26] and cancer cell lines [14]. Compared to closely related derivatives, pyrimidinylhydrazones with 4′-methoxy- and 5-ethyl-substituents have been suggested to show a higher and broader activity against Mycobacteria [15]. In the case of structurally related aroylhydrazones the introduction of an additional pyridine ring at the imine carbon of pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) led to increased iron chelation and enhanced antitumor activity [11]. Here we have investigated the antitumor activity of arylhydrazones from the pyrimidinyl type. Since the α-position to the hydrazine moiety was shown to influence toxicity, we synthesized a derivative with methylation at the imine carbon. The ligands were tested for their in vitro antiproliferative activity in six human cancer cell lines. We show that three donor nitrogen atoms enable the formation of stable and redox-active complexes with iron(III) and copper(II). UV–visible (UV–Vis) spectrophotometric titrations were applied to investigate speciation in aqueous solution, and cyclic voltammetry was used to characterize the redox activity of the complexes. The impact of metal ions on the antiproliferative activity and the formation of ROS were also investigated.

2. Materials and methods

2.1. Chemicals

Chemicals used for synthesis were procured from Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Sigma-Aldrich (Schnelldorf, Germany) or TCI (Eschborn, Germany) and used without further purification. Column chromatography was performed using Silica gel 60 (40–63 μm, Merck, Darmstadt, Germany) as stationary phase. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC) and 2′,7′-dichlorofluorescin diacetate (DCFDA) were purchased from ABCR Chemicals (Karlsruhe, Germany), TCI (Eschborn, Germany) and Sigma-Aldrich (Schnelldorf, Germany), respectively. The Pgp inhibitor WK-X-24 (XR9577) was used from prior synthesized stocks (Laboratory of Prof. M. Wiese, Bonn university, Germany) [27], Tariquidar was a kind gift from Dr. Susan Bates (NCI). Solid KOH, and tetrabutylammonium chloride (TBACl), ferrocene, uracil, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), ascorbic acid (ASC) and glutathione (GSH) were purchased from Sigma-Aldrich and HCl, KCl, $CuCl_2$, $FeCl_3$ were Reanal products (Budapest, Hungary). Fe(III) and Cu(II) stock solutions were prepared by dissolving the appropriate amount of the metal chlorides in known amounts of HCl. Their concentrations were determined by complexometry via the EDTA complexes. Accurate strong acid content of the metal stock solutions were determined by pH-potentiometric titrations. Ligand **3** was dissolved in pure DMSO to obtain the stock solution (0.01 M).

2.2. Synthesis and physical measurements

The ligands were prepared following the synthetic route suggested by Seydel et al. with minor modifications [15]. ^{1}H NMR and ^{13}C NMR spectra were obtained on either a Bruker Advance 300 or 500 spectrometer, respectively. DMSO-d_6 or $CDCl_3$ were used as solvents. Standard pulse programs were applied. Chemical shifts are expressed in ppm values using the residual solvent peaks as internal standards (DMSO-d_6 2.50; 39.52 ppm or $CDCl_3$ 7.26; 77.16 ppm) [28]. ^{13}C NMR signals were assigned with the aid of attached proton test (APT) spectra. Elemental analyses of the final products were performed on a Vario EL elemental analyzer (Hanau, Germany). The values for carbon, nitrogen and hydrogen are given in percentage. Electrospray ionization-mass spectrometry (ESI-MS) measurements for the characterization of the final products were carried out with a Waters Q-TOF Premier instrument (Waters Kft., Hungary) operating in positive ion mode; the samples were dissolved in methanol.

2.2.1. 4-Chloro-6-methoxypyrimidine

To a cooled solution (−10 °C) of 4,6-dichloropyrimidine (1193-21-1, 5 g, 33.56 mmol) in dry methanol, one equivalent of sodium hydride (titrated solution in dry methanol) was added, the solution was allowed to warm to room temperature and react for 48 h (depicted in *step a* of Scheme 1). The solvent was removed; the crude product was dissolved in brine, extracted with $CHCl_3$, and was used without further purification. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.68 (d, ^5J(H,H) = 0.8 Hz, 1H, *H-2*), 7.18 (d, ^5J(H,H) = 0.9 Hz, 1H, *H-5*), 3.96 (s, 3H, OCH_3).

2.2.2. 4-Hydrazinyl-6-methoxypyrimidine

To a solution of the 4-chloro-6-methoxypyrimidine, obtained as described in Section 2.2.1 (2.5 g crude product), in methanol an aqueous solution of hydrazine (80%, 6.1 mL) was added and refluxed (see *step b* of Scheme 1). After evaporation of the solvent, the aqueous solution of the crude product was alkalized with NaOH and extracted with methylene chloride to give the product as an ochre powder in 60% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.26 (s, 1H, *H-2*), 6.42 (bs, 1H, N*H*), 6.07 (s, *H-5*), 3.92 (s, 3H, OCH_3), 3.42 (bs, 2H, NH_2). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 170.72 (*C-6*), 167.17 (*C-4*), 157.68 (*C-2*), 84.90 (*C-5*), 53.87 (OCH_3).

2.2.3. 5-Ethyl-2-methyl-pyridine-N-oxide

35 mL of hydrogen peroxide (35% solution, 0.41 mol) was added to a solution of 5-ethyl-2-methyl-pyridine (104-90-5, 21.76 mL, 0.17 mol) in glacial acetic acid (200 mL) and refluxed for 16 h (depicted in *step c* of Scheme 1). After evaporation of the majority of the solvent, the reaction mixture was neutralized with NaOH and Na_2CO_3 and the starting material was retrieved by extraction with petroleum ether. 5-Ethyl-2-methyl-pyridine-*N*-oxide was extracted from the aqueous phase with $CHCl_3$, dried over Na_2SO_4 and after removal of the solvent obtained as a bright yellow liquid in 99% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.14–8.09 (m, *H-6*), 7.12 (d, ^3J(H,H) = 7.9 Hz, *H-3*), 7.00 (dd, ^4J(H,H) = 1.3 Hz, ^3J(H,H) = 7.9 Hz, *H-4*), 2.55 (q, ^3J(H,H) = 7.7 Hz, 2H, $C\underline{H_2}CH_3$), 2,48 (s, 3H, CH_3), 1.20 (t, ^3J(H,H) = 7.7 Hz, 3H, $CH_2\underline{CH_3}$).

2.2.4. 5-Ethylpyridin-2-yl-methyl acetate

As depicted in *step d* of Scheme 1, a solution of the 5-ethyl-2-methyl-pyridine-*N*-oxide, obtained in Section 2.2.5 (22.28 g, 0.16 mol), and acetic anhydride (28 mL, 0.23 mol) in glacial acetic acid (7 mL) was refluxed for 3 h, put on ice, neutralized, extracted with methylene chloride. After removal of the solvent, the extract was dissolved in diethyl ether, washed with a saturated solution of Na_2CO_3 and dried over Na_2SO_4 to give the product in 85% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.42 (d, ^4J(H,H) = 2.1 Hz, *H-6*), 7.50 (dd, ^4J(H,H) = 2.2 Hz, ^3J(H,H) = 7.9 Hz, *H-4*), 7.24 (d, ^3J(H,H) = 7.9 Hz, *H-3*), 5.16 (s, 2H, *H-7*), 2.63 (q, ^3J(H,H) = 7.6 Hz, 2H, CH_2CH_3), 2.11 (s, 3H, CO*CH3*), 1.22 (t, ^3J(H,H) = 7.6 Hz, 3H, CH_2CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 170.79 (*C*═O), 153.04 (*C-2*), 149.35 (*C-6*), 138.70 (*C-5*), 136.11 (*C-4*), 121.89 (*C-3*), 66.98 ($\underline{C}H_2$–OAc), 25.89 ($\underline{C}H_2CH_3$), 21.01 ($CO\underline{C}H_3$), 15.36 ($CH_2\underline{C}H_3$).

2.2.5. 5-Ethylpyridin-2-yl-methanol

As depicted in *step e* of Scheme 1, 5-ethylpyridin-2-yl-methyl acetate (24.29 g, 0.14 mol) was dissolved in tetrahydrofuran (THF) and refluxed with an aqueous solution of NaOH (8.1 g, 0.20 mol) for 5 h. The reaction mixture was reboiled on activated charcoal and was neutralized with glacial acetic acid and filtered. After concentration of the crude product under reduced pressure, it was dissolved in a saturated solution of $NaHCO_3$, extracted with diethyl ether and dried