Research in Pharmaceutical Sciences, October 2011; 6(2): 117-122
Received: Mar 2011
Accepted: Aug 2011

School of Pharmacy & Pharmaceutical Sciences
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Original Article

Synthesis and biological evaluation of bidentate 3-hydroxypyridin-4-ones iron chelating agents

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Abstract

A series of 3-hydroxypyridin-4-one derivatives (HPOs) were synthesized and their partition coefficient values (K_{part}) were determined. The cytotoxic effects of these iron chelators against HeLa cancer cells were also evaluated. The IC_{50} of HPOs was determined using MTT assay. Among these ligands, compound **4e** (K_{part}=5.02) with an IC_{50} of 30 μM and **4f** (K_{part}=0.1) with an IC_{50} of 700 μM showed the lowest and highest IC_{50}s, respectively. In conclusion, the introduction of a more hydrophobic functional group (such as butyl in compound **4e**) on the nitrogen of pyridinone ring resulted in higher cytotoxic activity of ligands.

***Keywords*:** 3-Hydroxypyridin-4-one derivatives; Iron chelators; Partition coefficient; Cytotoxicity; HeLa cells

INTRODUCTION

Although iron is essential for the proper functioning of all living cells, it is toxic when present in excess. In the presence of oxygen and under appropriate conditions, iron is able to generate free radicals through the Fenton reaction (1). Free radicals such as hydroxyl radicals are highly reactive species that attack all biological molecules including proteins, lipids, nucleic acids and sugars; resulting in oxidative damage to cells and tissues (2).

Under normal physiological conditions, iron levels are under tight control and there is little opportunity for iron to catalyze free radical generating reactions. However, in some abnormal conditions the iron status can change, either locally as in ischaemic tissue, or systematically as with genetic haemochromatosis or transfusion-induced iron overload. In such circumstances, the high levels of iron may lead to tissue/organ damage and eventual death (3). Although excess iron can be removed by phlebotomy where there is enough erythropoietic reserve (for example haemochromatosis) iron chelation therapy is the only useful way to remove toxic iron in transfusion-dependent β–thalassaemia patients, hypoproteinemia or cardiac disease. Desferrio-xamine B (DFO), a natural siderophore (Fig. 1), has been used for the treatment of iron overload for over 40 years, and currently is the only really clinically useful drug for this purpose. However, desferrioxamine is orally inactive and only causes sufficient iron excretion to keep pace with the transfusion regimes when given either subcutaneously or intravenously over 8-12 h several times per week (4) and consequently there is an urgent need for an orally active iron chelating agent.

3-Hydroxypyridin-4-one derivatives (Fig. 1) are good candidates for development as orally active iron chelators replacements for DFO since they possess many of the desirable molecular features deemed advantageous for effective iron chelation *in vivo* (5). To date some of them have been widely investigated for iron chelation, both in iron overloaded animal models and in thalassaemia patients. Most results have shown that excretion of iron can be enhanced via both the urinary and biliary routes, and some compounds have potential as clinically useful chelators (6,7).

In addition to the potential treatment of iron overload in thalassaemic patients, hydroxy pyridin-4-ones may well find other clinical

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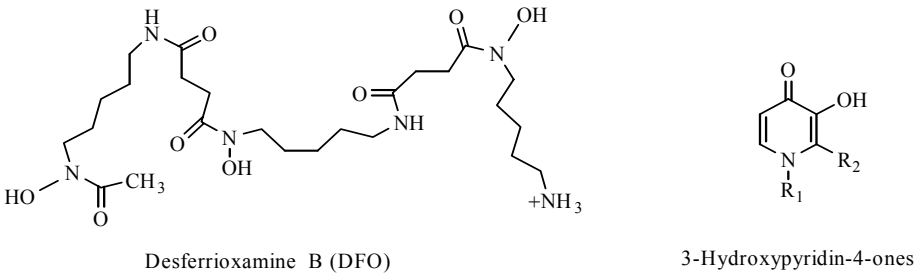

Fig. 1. Structures of two iron (III) chelators

applications centered on iron removal. The hydroxypyridones are being investigated for the treatment of malaria (8), antimicrobial activity (9) and aluminum removal especially aluminum mobilization in renal dialysis patients (10), localized iron overload (11) and inhibition of iron-containing enzymes (12).

Iron chelating agents such as DFO and HPOs are known to facilitate cell synchronization and may, therefore, have relevance to the chemotherapy of some tumors (13). Cancer cells divide rapidly and therefore they have a higher requirement for iron than normal cells. For this reason they are more sensitive to iron depletion. Hence, iron ligands could be used in cancer chemotherapy (14). In this regard, cytotoxic activity of DFO has been studied against various cancer cells (15). For instance, neuroblastoma cells treated with DFO, showed a 10-fold higher sensitivity to iron-depleted cells than normal bone marrow cells (16). Herein we evaluated partition coefficient and *in vitro* cytotoxic activity of recently synthesized 3-hydroxypyridin-4-ones as novel iron chelators. We found that compounds with higher K_{part} have more cytotoxic activity against HeLa cell line.

MATERIALS AND METHODS

Materials

2-methyl-3-hydroxypyran-4-one (maltol), benzyl chloride, 3-aminobenzoic acid, 4-amino benzoic acid, methylamine, propylamine, butylamine, N,N-dimethylethylamine and palladium on activated carbon 5% were obtained from Aldrich (Gillingham, UK).

Instrumentation

Melting points were determined using an Electrothermal IA 9100 digital melting point.

IR spectra were recorded on a Perkin-Elmer 1420. Proton NMR spectra were determined with EM-390 (80 MHz). Mass spectra were taken using a vacuum generator 16F (35eV). Elemental analyses (Leco CHNCl-932) were performed by micro analytical laboratories, University of Manchester, Manchester M13 9PL, UK.

General procedure for preparation of 3-hydroxypyridin-4-ones

The general methodology (17) which has been adopted for the synthesis of 1-substituted-3-hydroxypyridin-4-ones is summarized in Fig. 2 The commercially available maltol **1** was benzylated to give compound **2**. Reaction of **2** with arylamines (or alkylamines) produced the benzylated pyridinones **3a-f**, which were subsequently subjected to catalytic hydrogenation under acidic condition to remove the protecting group, yielding the corresponding bidentate 1-substituted -3-hydroxypyridin-4-ones **4a-f**. Compounds **4c-f**, were synthesized at normal reflux conditions as previously described (5,8). The purity of ligands was confirmed by spectroscopic and elemental analysis.

Cell line

HeLa (Human cervix carcinoma) cell line was purchased from Pasture Institute (Tehran, Iran). They were grown in RPMI-1640 [each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum, 5 ml of penicillin/ streptomycin (50 IU/ml and 50 μg/ml, respectively), 5 ml of sodium pyruvate (1 mM), $NaHCO_3$ (1 g) and 5 ml of L-glutamine (2 mM)]. Completed media were sterilized by 0.22 μm microbiological filter after prepara-tion and kept at 4°C before using.

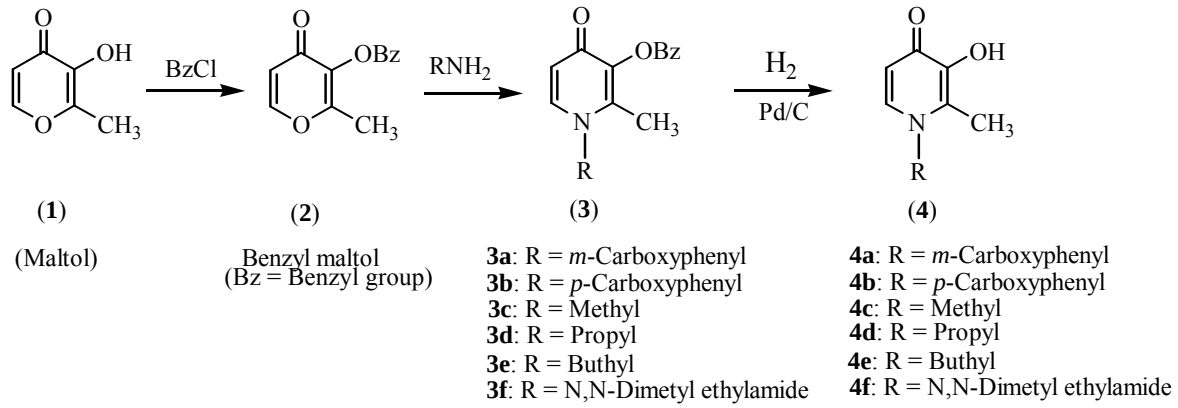

Fig. 2. Synthesis scheme of 1-substituted-3-hydroxypyridin-4-ones derivatives

Preparation of stock solutions

Stock solution (10 µM) of each compound was prepared in 1 ml of DMSO and 9 ml of PBS. The final solutions (1, 10 and 100 µM) were then obtained by diluting these solutions with PBS or culture media and stored at -20°C before using.

MTT-based cytotoxicity assay

The cytotoxic effects of synthesized compounds against human tumor cell line were determined by a rapid colorimetric assay, using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) which compared with untreated controls (18).

This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolution in DMSO. Briefly, 200 µl of cells (5×10^4 cells/ml) were seeded in 96 well microplates and incubated for 24 h (37°C, 5% CO_2 air humidified). Then 20 µl of final concentration of each compound (1, 10 and 100 µM) was added and incubated for another 72 h in the same condition. Taxol (2.3 µM) was used as a positive control. HeLa cells (5×10^4 cells/ml) were considered as negative control with 100% viability. Cell survival was determined as previously described (18). To determie IC_{50}, concentration-response curves were generated relative to negative control (curves are not shown). IC_{50} values were calculated from the linear portion of the curves (19). The K_{part} values of the synthesized compounds were also determined in MOPS buffer (pH 7.4)/octanol system using an automated continuous flow method as previously described (20).

Statistical analysis

All results are expressed as the mean ± SD of at least three experiments. *P* values for significance were determined using the two-tailed Student′s *t* test. Significance was considered at 5% level.

RESULTS

Details of preparation procedures and chemistry of synthesized compounds

2- methyl -3- benzyloxypran -4- one (Benzyl maltol) (2)

To a solution of 2-methyl-3-hydroxypyran-4-one (**1**) (12 g, 0.1 mol) in methanol (100 ml) was added 10 ml of sodium hydroxide solution (4.4 g, 0.11 mol) followed by addition of benzyl chloride (13.9 g, 0.11 mol). The mixture was then refluxed for 6 h. After removal of solvent by rotary evaporation, the residue was mixed with water (50 ml) and extracted into dichloromethane (3 × 50 ml). The combined extracts were washed with 5% sodium hydroxide (3 × 150 ml) and then with water (2 × 150 ml). The organic fraction was dried over anhydrous sodium sulphate, filtered and rotary evaporated to yield an oily orange residue which became solid on cooling. Recrystallization from diethyl ether gave the pure product as colorless needles. 17.7 g (82%). mp 52-53°C, ^{1}H NMR (DMSO-d_6): δ 2.10 (s, 3H, 2-CH_3), 5.10 (s, 2H, O-CH_2-Ph), 6.39 (d, 1H, 5-H), 7.23-7.48 (m, 5H, Ph), 7.94 (d, 1H, 6-H): MS (EI): m/z = 216 (M), IR (KBr): 1640 (C=O) cm^{-1}. Anal. Calcd. For

$C_{13}H_{12}O_3$: C, 72.21; H, 5.59%. Found: C, 72.31; H, 5.65%.

1- (3-carboxyphenyl) -2-methyl-3-benzyloxy pyridin-4-one (3a)

To a solution of benzyl maltol (**2**) (2.16 g, 0.01 mol) in ethanol (20 ml)/water (20 ml) was added 3-aminobenzoic acid (2.74 g, 0.02 mol). The mixture was heated in a thick-walled sealed glass tube at 150-160^{o}C for 20 h. After removal of solvent by rotary evaporation, the residue was mixed with water (40 ml) and extracted into dichloromethane (3 × 40 ml). The organic layers were then dried over anhydrous sodium sulphate, filtered and rotary evaporated to yield a brown compound. The resulting compound was purified by column chromatography on silica gel (eluent=10% methanol/chloroform, Rf=0.28) to give a yellow pure product (1.71 g, 51%). ^{1}H NMR (DMSO-d_6): δ 2.3 (s, 3H, 2-CH_3), 5.15 (s, 2H, O-CH_2-Ph), 6.36 (d, 1H, 5-H (pyridinone ring)), 7.25-7.9 (m, 10H, O-CH_2-Ph, N-C_6H_4-COOH & 6-H (pyridinone ring).

1- (3-carboxyphenyl) -2-methyl-3-hydroxy pyridin-4-one hydrochloride (4a)

The solution of 1- (3-carboxyphenyl) -2-methyl-3-benzyloxypyridin-4-one (**3a**) (1.68 g, 0.005 mol) in ethanol (27 ml)/water (3 ml) was adjusted to pH 1 with hydrochloric acid prior to hydrogenolysis for 4 h in the presence of 5% Pd/C catalyst (0.3 g). Filtration which was followed by rotary evaporation gave a white solid. Recrystallization of resulting compound from ethanol/diethyl ether yielded a white powder (1.20 g, 85%); mp 270-271^{o}C. ^{1}HNMR (DMSO-d_6): δ 2.0 (s, 3H, 2-CH_3), 5.8 (bs, H, 3-OH), 6.3 (d, 1H, 5-H (pyridinone ring)), 7.6-8.3 (m, 5H, phenyl ring & 6-H (pyridinone ring)). MS (EI): m/z = 245 (M-HCl), 244 (M-H, HCl), 200 (M-COOH, HCl). IR (KBr): 3100 (OH), 1690 (C=0), 1580 (C=C), 1300 (C-N) cm$^{-1,}$ Anal. Calcd. for $C_{13}H_{11}NO_4$. HCl : C, 55.43; H,4.29; N,4.97; Cl,12.58%. Found: C, 55.31; H, 4.23; N,4.91 Cl, 12.69%. mp 270-271^{o}C.

1- (4-carboxyphenyl) -2-methyl-3-benzyloxy pyridin-4-one (3b)

Benzyl maltol (**2**) (2.16 g, 0.01 mol) and 4-aminobenzoic acid (2.74 g, 0.02 mol) were reacted as described for **2a** to afford a brown compound (**3b**). The resulting compound was purified by column chromatography on silicagel (eluent=10% methanol/chloroform, R_f=0.30) giving a yellow pure product (1.84 g, 55%).^{1}H NMR (DMSO-d_6): δ 2.35 (s, 3H, 2-CH_3), 5.20 (s, 2H, O-CH_2-Ph), 6.35 (d, 1H, 5-H (pyridinone ring)), 7.26-8.2 (m, 10H, O-CH_2-Ph, N-C_6H_4-COOH & 6-H (pyridinone ring)).

1- (4-carboxyphenyl) -2-methyl-3-hydroxy pyridin-4-one hydrochloride (4b)

An analogous hydrogenation procedure for preparation of ligand **3a** using compound **3b** (1.68 g, 0.005 mol) and 5% Pd/C catalyst (0.3 g) yielded 1.12 g of the above mentioned compound (80.0%) after recrystallization from ethanol/ diethyl ether, as a white powder; mp 305-306 ^{o}C. ^{1}H NMR (DMSO-d_6): δ 2.0 (s, 3H, 2-CH_3), 4.5 (bs, H, 3-OH), 6.3 (d, 1H, 5-H (pyridinone ring)), 7.6 (d, 3H, 6-H (pyridinone ring) & 2′-H and 6′-H (phenyl ring)), 8.1(d, 2H, 3′-H and 5′-H (phenyl ring)), MS (EI): m/z=245 (M-HCl), 244 (M-H, HCl), 200 (M-COOH, HCl). IR (KBr): 3100 (OH), 1630 (C=0), 1580 (C=C), 1300 (C-N) cm^{-1}Anal. Calcd. for $C_{13}H_{11}NO_4$. HCl : C, 55.43; H,4.29; N,4.97; Cl, 12.58%. Found: C, 55.30; H, 4.33; N, 5.05 Cl, 12.51%. mp 310-311^{o}C.

Partition coefficients

The K_{part} values of the synthesized compounds (Table 1) were also determined in an aqueous/octanol system using an automated continuous flow method as previously described (20).

Cytotoxic activity

Cytotoxic effects of synthesized compounds were primarily evaluated in logarithmic concentrations. At concentrations of 1 and 10 µM no cytotoxic effects were observed against HeLa cells (cell survival was more than 90%). As observed in Table 1, at higher concentrations, compounds **4d** and **4e** were cytotoxic and their IC_{50}s were 45 and 30 µM, respectively. Other compounds IC_{50}s were > 100 µM (Table 1). The orders of cytotoxic effects of compounds were as follows:
4e > **4d** > **4c** > **4b** = **4a** > **4f**.

Table 1. The partition coefficient values (K_{part}) and IC_{50} of ligands (**4a-f**) . K_{part} and IC_{50}s were determined as described in the method section. Data are shown as mean ± SD. (n=6). ND: not determined; * taxol K_{part} values obtained from literature (19).

Compounds	K_{part}	IC50 (μM)
4a	0.43 ± 0.03	600 ± 20
4b	0.52 ± 0.04	600 ± 16
4c	0.18 ± 0.02	250 ± 8
4d	1.52 ± 0.20	45 ± 2
4e	5.02 ± 0.02	30 ± 4
4f	0.10 ± 0.02	700 ± 32
+ve control (taxol)	8074*	<2 μM
-ve control	ND	Cell survival 100%

DISCUSSION

The conversion of pyran-4-one to pyridin-4-one involves an initial Michael reaction followed by ring-opening and ring closure. Mesomerisation of α,β-unsaturated carbonyl compound causes the β-carbon to be electron deficient and therefore susceptible to nucleophilic attack. When the nucleophile is a primary amine double attack at the α,β-unsaturated functions of the pyran-4-one leads to formation of pyridin-4-one with the loss of a water molecule (17).

As previously reported, conversion of maltol with aryl amines can be achieved without protection of the 3-hydroxyl group in acidic conditions. This, however, would result in low yield (21). In order to promote the yield, therefore, a three-step reaction was adopted (Fig. 2). The amination step with benzylated maltol was accomplished under two different conditions, either at normal reflux conditions or at elevated temperatures. A reaction period of 72 h at normal reflux resulted in low yields again (<20%). In contrast, the reaction of benzyl protected maltol with related aryl amines in a thick-walled sealed glass tube at 150-160^{o}C for 24 h afforded the desired benzylated 2-ethyl-3 benzyloxypyridin-4-ones **4a-b** in good yields (51-55%).

With regard to cytotoxic activity, as illustrated in Fig. 2, in **3** most cytotoxic compounds (**4e**, **4d**, and **4c**), R is substituted with an alkyl group. Bigger alkyl side chains resulted in greater cytotoxic activity. When R was replaced with carboxyphenyl or N,N-dimethylethyl amide, cytotoxic activities were reduced. Physicochemical properties such as membrane permeability and partition coefficient of 3-hydroxypyridin-4ones are important factors for their cytotoxicity (22,23). In general, hydroxypyridin-4ones skeleton which are neutral at physiologic pH, possess low molecular weight and high lipophilicity. Hence, they are considered as suitable candidates to have biological activity inside the cells. On the other side, compounds with carboxyphenyl or amide moiety (**4a**, **4b** and **4f**) possibly are ionized in culture media or form the hydrogen bond between the carboxyl or amide group with adjacent molecules which consequently will results a decrease in membrane penetration compared with N-alkylated counterparts (24).

Poor membrane permeability in compounds **4a**, **4b** and **4f** resulted in lower cytotoxic activities (higher IC_{50}s). Compound **4c** as N-methyl derivative had low K_{part} with moderate IC_{50} (250 μM) which could be explained by a balance between intermolecular hydrogen bond and low lipophilicity of methyl group compared to N-propyl and N-butyl groups in **4d** and **4e**. These results are in agreement with Gaboriau and co-workers findings (25) where they showed cell penetration and cytotoxicity of hydrophilic CP20 (**4c**) are less than CP411 with a K_{part} of 8-fold higher than CP20. The most cytotoxic compound (**4e**) had a K_{part} of 5.02 which is 50-fold greater than the least cytotoxic one (**4f** with a K_{part} of 0.1). Since iron has an essential role in cell processing (25,26), powerful iron chelating agents with higher lipophilicity and cellular penetration, might have great potentials as anticancer agents. Further studies are required to explore

the exact mechanisms of cytotoxicity of these compounds.

CONCLUSION

3-hydroxypyridin-4-ones with high partition coefficients were found to be more hydrophobic which renders them to enter the cells much easier and showed more cytotoxic activity. These bidentate iron chelators are potential cytotoxic agents against cancer cells.

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