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Effects on erythroid differentiation of platinum(II) complexes of synthetic bile acid derivatives

Ilaria Lampronti,[a] Nicoletta Bianchi,[a] Cristina Zuccato,[a] Alessandro Medici,[b] Paola Bergamini[c] and Roberto Gambari[a,d,*]

[a]*ER-GenTech, Department of Biochemistry and Molecular Biology, Molecular Biology Section, University of Ferrara, Ferrara, Italy*
[b]*Department of Natural and Cultural Resources, University of Ferrara, Ferrara, Italy*
[c]*Department of Chemistry, University of Ferrara, Ferrara, Italy*
[d]*GenTech-for-Thal, Laboratory for the Development of Pharmacological and Pharmacogenomic Therapy of Thalassemia, Biotechnology Center, University of Ferrara, Ferrara, Italy*

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Abstract—In this study, we compared some bile acid derivatives and their platinum(II) complexes with respect to their ability to induce erythroid differentiation of human leukemic K562 cells. The complexes analyzed were *cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)] (compound **1**) and *cis*-[di(dehydrocholanoate)-bis(triphenylphosphine)-platinum(II)] (compound **3**), together with their free ligands, respectively, 3-dehydrocholanoyliden-L-tartaric acid (compound **2**) and dehydrocholanoic acid (**4**), and their parent compounds, respectively, cisplatin and *cis*-[dichloride-bis(triphenylphosphine)-platinum(II)] (**5**). We found that compound **1** stimulates erythroid differentiation of K562 cells and an increase of fetal hemoglobin (HbF) production in erythroid precursor cells isolated from peripheral blood of human subjects. This increase is similar to that obtained by hydroxyurea, a potent inducer of HbF production both in vitro and in vivo. Another important conclusion of this study is related to the evaluation of the effects of compound **1** on production of γ-globin mRNA in human erythroid precursors grown in the two-stage liquid culture system. We demonstrated that compound **1** induces preferential accumulation of γ-globin mRNA. The results presented in this manuscript could have practical impact, since it is well known that an increase in HbF production could ameliorate the clinical status of patients with β-thalassemia and sickle cell anemia.

1. Introduction

The search of potential therapeutic agents for β-thalassemia and sickle cell anemia focuses on the pharmacologically mediated regulation of the expression of human γ-globin genes.[1–8] It is well established that an increase of production of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) leads to a significant improvement of the clinical status of the patients.[4,5,7] Therefore, many recently published experiments were designed to find hormones, cytotoxic agents, hemopoietic cytokines, and short fatty acids as agents able to increase HbF levels in humans.[1–3,9,10]

In this respect, platinum complexes are of great interest. We demonstrated and published that cisplatin is a potent inducer of HbF production in human erythroid cells.[11] These data have been recently supported by the results obtained after in vitro treatment with these compounds of cells harboring a GFP construct under the control of the human γ-globin promoter.[12]

However, cisplatin and related compounds exhibit a high level of cytoxicity in vitro and in vivo. Therefore, they might be proposed for 'acute' treatments (such as in the case of patients affected by tumors), being of low priority in the chronic treatment of disorders, such as thalassemia, for which recurrent administrations are expected to be required for several years. Therefore,

Abbreviations: Hb, hemoglobin; HbF, fetal hemoglobin; EPO, erythropoietin; FBS, fetal bovine serum; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; GAPDH, glyceraldehyde phosphate dehydrogenase; Ara-C, cytosine arabinoside; HU, hydroxyurea; RT, reverse transcription; PCR, polymerase chain reaction.

Keywords: Bile acid derivatives; Platinum complexes; Erythroid differentiation; γ-Globin; Fetal hemoglobin; β-Thalassemia.

* Corresponding author. Tel.: +39 0 532 424443; fax: +39 0 532 424500; e-mail: gam@unife.it

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ligands for platinum complexes able to alter the pharmacokinetics might be of interest. These should maintain however the cisplatin-mediated effect inside the cell.

Looking for anticancer platinum complexes with alternative biodistribution to cisplatin, we have recently screened the antiproliferative activity of a large group of Pt-complexes bearing bile acids or bile acid derivatives as carrier ligands with special properties like amphiphilicity and organotropism towards the hepatobiliary system.[13,14] We found that the most active species were the amino–Pt complex **1** and the triphenylphosphine–Pt complex **3**.[15] While amino–platinum complexes (cisplatin and its various analogues) have been considered and largely used as anticancer drugs, the use of lipophilic triphenylphosphine as ligand for platinum is still controversial, although it appears in some metal-complexes with diagnostic or therapeutic applications.[16]

In the present study, we report the biological characterization of **1** and **3** (in parallel with their carrier ligands and their parent compounds) with respect to: (a) induction of differentiation of human erythroleukemia cells and (b) possible induction of HbF in erythroid precursor cells.

These compounds were therefore investigated for potential erythro-differentiation ability, in order to verify whether some of them could be able to increase γ-globin gene expression. First, we analyzed erythroid differentiation by benzidine/H_2O_2 assay using as cellular model the human leukemia K562 cell line.[17,18] Second, we analyzed γ-globin mRNA and HbF production after in vitro treatment of erythroid precursor cells using the two-phase liquid culture procedure.[19,20] This methodology can be used to identify compounds stimulating HbF accumulation in erythroid progenitors obtained from normal donors as well as subjects with hematological disorders.[21]

2. Biological results

2.1. Evaluation of erythroid differentiation and antiproliferative effects on K562 cell line after treatment with platinum(II) bonded forms of synthetic bile acid derivatives

Figure 1 shows the molecular structures of the employed platinum complexes *cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)] (compound **1**) and *cis*-

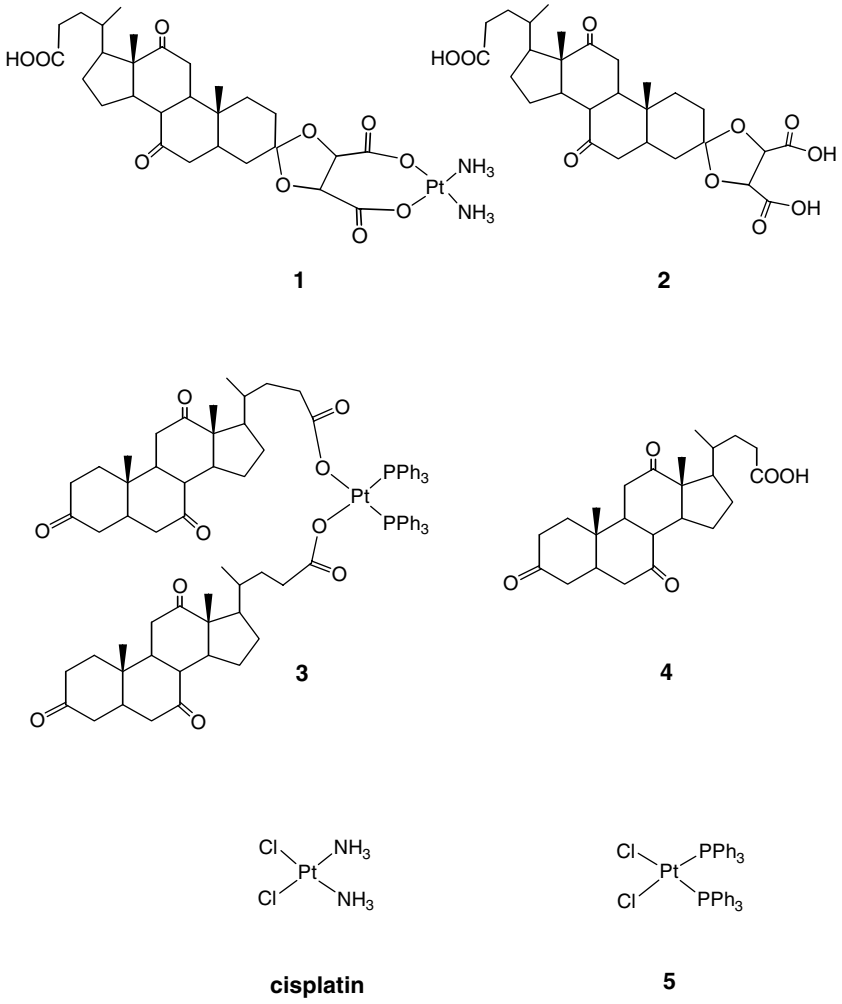

Figure 1. Chemical structure of the molecules employed in this study.

[di(dehydrocholanoate)-bis(triphenylphosphine)-platinum(II)] (compound **3**), their carrier ligands 3-dehydrocholanoyliden-L-tartaric acid (compound **2**) and dehydrocholanoic acid (**4**), and their parent compounds, cisplatin and *cis*-[dichloride-bis(triphenylphosphine)-platinum(II)] (**5**).

The antiproliferative effects of compounds **1**–**5** and cisplatin are shown in Figure 2 and summarized in Table 1. It should be noted that all the Pt-derivatives exhibit a high antiproliferative activity on K562 cells, even if at different concentrations; *cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)] (compound **1**) is the most effective analogue, characterized by an IC_{50} value of 4.03 ± 0.83 μM, whereas the diphosphodidehydro-platinum(II) derivative **3** inhibits the cell growth with an IC_{50} value of 38.10 ± 7.01 μM. On the contrary, the free ligands 3-dehydrocholanoyliden-L-tartaric acid (compound **2**) and dehydrocholanoic acid (**4**)

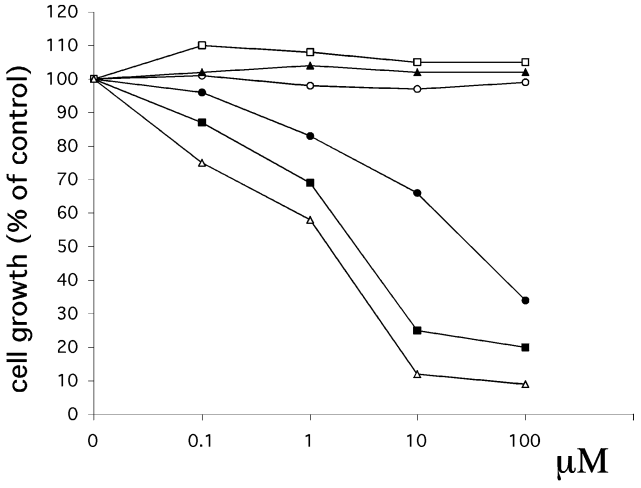

Figure 2. Effects of Pt-complexes on cell proliferation of K562 cells. K562 cells were seeded at the initial cell concentration of 30,000 cells/mL and then cultured for 7 days in the presence of the indicated amounts of compound **1** (black squares), **2** (white circles), **3** (black circles), **4** (white squares), **5** (black triangles), and cisplatin (white triangles).

Table 1. Effects of *cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)] (**1**), 3-dehydrocolanoyliden-L-tartaric acid (**2**), *cis*-[di(dehydrocholanoate)-bis(triphenylphosphine)-platinum(II)] (**3**), dehydrocholanoic acid (**4**), *cis*-[dichloride-bis(triphenylphosphine)-platinum(II)] (**5**), and cisplatin on erythroid differentiation of human erythroleukemic K562 cells

Compound	Antiproliferative effect (IC_{50}, μM)	Erythroid differentiation (% of benzidine-positive cells ± SD)
1	4.03 ± 0.83	84 ± 10.2 (50 M) 68 ± 7.6 (5 μM)
2	>200	31 ± 5.2 (50 μM) 25 ± 5.0 (5 μM)
3	38.10 ± 7.01	2.3 ± 0.4 (50 μM) 3.5 ± 0.7 (5 μM)
4	>200	5.8 ± 0.8 (6 μM)
5	>200	7.1 ± 1.9 (2 μM)
Cisplatin	2.10 ± 0.95	85.0 ± 5.2 (6 μM)

Table 2. Effects of compound **1** on erythroid differentiation of human erythroleukemic K562 cells compared to known inducers

Inducer	Erythroid differentiation (% of benzidine-positive cells ± SD)
Compound **1**	82.7 ± 8.3 (50 μM)
Cytosine arabinoside	75.6 ± 5.8 (0.25 μM)
Mithramycin	72.9 ± 1.9 (10 nM)
Butyric acid	36.7 ± 6.5 (1.50 mM)
Hydroxyurea	41.2 ± 3.7 (120 μM)

were found to be unable to inhibit K562 cell growth. Concerning the parent compounds, the well-known antiproliferative activity of cisplatin was confirmed in these experiments, while *cis*-[dichloride-bis(triphenylphosphine)-platinum(II)] (**5**) was found inactive.

In order to determine the effects of these molecules on erythroid differentiation, K562 cells were treated with increasing concentrations of **1**–**5** derivatives and cisplatin and erythroid differentiation was determined after 5–7 days of cell culture by counting the proportion of benzidine-positive cells.[17] The results obtained and reported in Table 1 demonstrate that compound **1** displayed the highest effects on induction of erythroid differentiation of K562 cells. All the other tested compounds were either unable or only slightly effective in inducing differentiation.

In a second set of experiments, the activity of compound **1** to induce erythroid differentiation on human leukemic K562 cells was compared to those of other known differentiation agents. As clearly appreciable from the data shown in Table 2, compound **1** is very active, and the level of differentiation approaches those of the most powerful erythroid inducers, such as cytosine arabinoside (Ara-C) (0.25 μM), mithramycin (10 nM), and cisplatin (6 μM) (Table 1); interestingly, compound **1** is more active than butyric acid (1.5 mM) and hydroxyurea (HU) (120 μM) to increase the proportion of benzidine-positive K562 cells.

2.2. Compound 1 induces preferential accumulation of γ-globin mRNA and production of HbF in erythroid precursor cells from normal donors

To study the effects of compound **1** on differentiation of human erythroid progenitor cells, we employed the two-phase liquid culture system as described elsewhere.[19–22] In this procedure, early erythroid committed progenitors (BFUe) derived from the peripheral blood proliferate and differentiate during phase I (in the absence of EPO) into late progenitors (CFUe). In phase II, in the presence of EPO, the latter cells continue their proliferation and mature into Hb-containing orthochromatic normoblasts. Compound **1** at 10 and 25 μM was added on day 4–5 of phase II for 7 days. As positive control, we used cultures treated with HU (100 μM), cisplatin (50 μM) and mithramycin (20 nM).

The results of quantitative RT-PCR analysis are shown in Figure 3 and suggest that concentrations as low as 10 μM of compound **1** are able to stimulate an increase

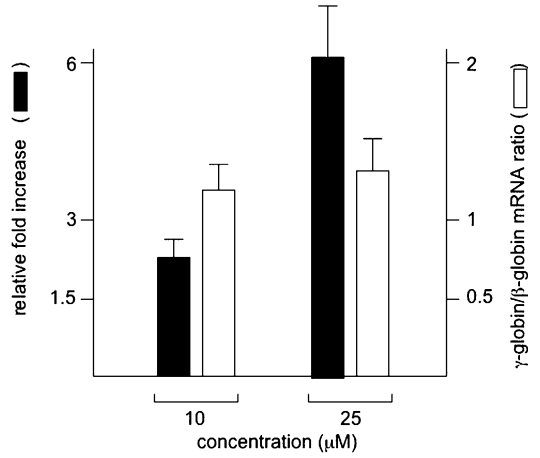

Figure 3. Effects of compound **1** on fold increase of γ-globin (black boxes) mRNAs in erythroid progenitor cells using GAPDH mRNA as reference molecule. White boxes indicate the γ-globin/β-globin mRNA ratios.

of γ-globin mRNA using GAPDH as reference mRNA. Interestingly, compound **1** treatment leads to a preferential accumulation of γ-globin mRNA with respect to β-globin mRNA (Fig. 3).

Accordingly with the data shown in Figure 3, the effects of treatment with 10 μM compound **1** were determined on human erythroid precursors harvested on day 12. These cells were lysed and their hemoglobins were analyzed by HPLC. The results obtained demonstrated that compound **1** induced a 5.2-fold increase in the percentage of HbF as compared with that for untreated cells. Parallel experiments conducted with cisplatin demonstrated a 2.5-fold increase of HbF production (data not shown).

3. Discussion

HbF inducers could be of great interest for therapy of β-thalassemia and sickle cell anemia,[1–8] since an increase of HbF is able to ameliorate the symptoms of these diseases.

We report in this paper that a platinum(II) bonded form of a synthetic bile acid derivative (compound **1**) is a potent inducer of erythroid differentiation of K562 cells.

The K562 cell line has been proposed as a useful in vitro model for studying the molecular mechanism(s) regulating the expression of embryonic and fetal human globin genes,[23] as well as for screening of potential new differentiation-inducing compounds.[21,25–27] This cell line, isolated and characterized by Lozzio and Lozzio[17] from a patient with chronic myelogenous leukemia in blast crisis, exhibits a low proportion of Hb-synthesizing cells under standard culture conditions, but it is capable of undergoing erythroid differentiation when treated with a variety of compounds, including hemin,[24] cytosine arabinoside (Ara-C),[27] 5-azacytidine,[17] chromomycin and mithramycin,[26] tallimustine,[25,27] cisplatin, and cisplatin analogues.[11] Following erythroid induction of K562 cells, Hb Portland ($\zeta_2\gamma_2$) and Hb Gower 1 ($\zeta_2\varepsilon_2$) accumulate, due to increase in the expression of human ζ-, ε-, and γ-globin genes.[17] In vitro studies demonstrated that known inducers of erythroid differentiation of K562 cells, such as HU, butyrates, and 5-azacytidine, are also capable of inducing HbF production when administered singularly or in combination to normal erythroid cells.[1] Butyric acid, HU and 5-azacytidine have been the subject of reports on the treatment of β-thalassemia patients.[2,3,28,29]

Another important conclusion of this study is related to the evaluation of the effects of compound **1** on production of γ-globin mRNA in human erythroid precursors grown in the two-stage liquid culture system. Accordingly, we demonstrated that compound **1**, (*cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)]), stimulates also an increase of HbF production. This increase is similar to that obtained by HU, a potent inducer of HbF production both in vitro and in vivo.[1,30,31]

In addition, we would like to underline that compound **1** exhibits the ability to stimulate HbF production when administered to erythroid precursor at 10 μM concentration, which is in the range of physiological serum concentrations of bile acids, reported to vary from 5 to 20 μM in healthy subjects.[32,33] However, in vivo experiments should be considered, since higher concentrations of bile acids cause cholestasis and associated in vivo toxic effects. In any case, the possibility to lower the concentration of compound **1** in combination with sub-optimal concentration of other HbF inducers should be analyzed in the future. It is generally accepted that combined treatment using sub-optimal concentrations of inducers with different mechanisms of action increases their effects, limiting the toxicities of single compounds.

This study does not clarify several points, the major of which is the mechanism of action of compound **1** on erythroid cells, which lacks the most studied bile acids transporters, whose expression is restricted to hepatocytes, cholangiocytes, and enterocytes.[32,33] However, our data are in agreement with the observations that these compounds exhibit high levels of antiproliferative activity on tumor cells.

On the other hand, the results presented in this manuscript could have practical impact, since it is well known that an increase in HbF production could ameliorate the clinical status of patients with β-thalassemia and sickle cell anemia.[4,5,7] The interest in identifying novel HbF inducers is related to the observation that induction of HbF is highly variable from patient to patient. Alternative HbF inducers are therefore of relevance for a personalized therapy of thalassemia and sickle cell anemia.

Finally, the analysis of possible effects of these compounds on in vivo model systems mimicking thalassemia would be of great interest.[34] In this context, thalassemia mice exhibiting minor (wt/Th3)[35] and major (Th3/Th3)[34] forms of thalassemia-like phenotype have been described (reviewed by Breda and Rivella).[36]

4. Experimental

4.1. Chemistry

All the solvents were distilled according to standard methods and other chemicals were used as purchased (reagent grade). Dehydrocholanoic acid (**4**) was supplied by I.C.E., cisplatin and *cis*-[dichloride-bis(triphenylphosphine)-platinum(II)] were prepared following literature procedures.[37,38] Elemental analyses (C, H, and N) were performed using a Carlo Erba instrument model EA1110. FT-IR spectra were recorded on a Nicolet 510 P FT-IR instrument (4000–200 cm^{-1}) using KBr. NMR spectra were recorded on a Bruker AM spectrometer 200 MHz (^1H NMR), 81.15 MHz (^{31}P) or on a Varian Gemini 300 spectrometer (^1H NMR), 75.43 MHz (^{13}C NMR), 121.44 MHz (^{31}P). Peak positions are relative to tetramethylsilane and were calibrated against the residual solvent resonance (^1H) or the deuterated solvent multiplet (^{13}C), and measured relative to external 85% H_3PO_4 with downfield values taken as positive (^{31}P).

4.1.1. Synthesis and characterization of 3-dehydrocholanoyliden-L-tartaric acid (2). The tricarboxylic acid **2** was prepared in the following way: L(+)-tartaric acid (33 g, 220 mmol) and *p*-toluenesulfonic acid (1 g, 5.8 mmol) were added to a solution of dehydrocholic acid (10 g, 24.8 mmol) in 60 mL of ethanol and the mixture was refluxed under stirring for 10 h.

The solvent was then evaporated under reduced pressure and the crude residue treated with 100 mL of toluene. In a flask equipped with a Dean–Stark apparatus, the mixture was refluxed for further 10 h. The solvent was then removed under vacuum leaving a brown oil, which was treated with a solution containing 20 g of NaOH in 150 mL of 1:1 MeOH and water. The saponification occurred promptly with formation of a white precipitate (sodium tartrate). MeOH (100 mL) was then added to the mixture and the solid residue was eliminated after filtration and washing with MeOH. The filtrate was reduced to half volume and water (150 mL) was added. After a pH adjustment to 5 with HCl (20% in water), the solution was extracted with ethylacetate (2× 100 mL) to remove the excess of dehydrocholic acid.

The aqueous layer was re-adjusted at pH 3 with HCl (20% in water) and re-extracted with ethyl acetate (2× 100 mL). The organic phase, containing the tricarboxylic acid **2**, was stirred with Na_2SO_4, filtered, and finally taken to dryness to give the product as a white solid (6 g, 45%).

Found: C, 62.81; H, 7.64; O, 29.55. $C_{28}H_{38}O_{10}$ requires: C, 62.90; H, 7.16; O, 29.94.

^1H NMR (pyridine-d_5 + D_2O): 1.06–1.13 (6H, d, s 21/18-CH_3); 1.36 (3H, s, 19-CH_3); 1.4–2.6 (m, aliphatic CH and CH_2); 2.8 (1H, t, 11-CH); 2.98–3.1 (2H, m, 6/8-CH); 5.4 (2H, m, 25/26-CH).

^{13}C NMR (pyridine-d_5 + D_2O): 141.1 (C-1); 140.9 (C-8); 132.0 (C-4); 81.9 (C-7); 62.9 (C-14); 55.14 (C-15); 52.43 (C-16); 22.16 (C-17); 21.23 (C-18).

IR (cm^{-1}): 2980–2900 (*v* CH); 1750–1770 (*v* C═O); 1715–1700 (*v* C═O); 1400 (*v* CH); 1250–1300 (*v* C–O).

4.1.2. Synthesis of *cis*-[(3-dehydrocholanoyliden-L-tartrate)-diammineplatinum(II)] (complex 1). A solution containing 4.0 g (23 mmol) of $AgNO_3$ in 50 mL of water was added to a suspension of *cis*-[$(NH_3)_2PtI_2$] (5.0 g, 10.3 mmol)[39] in 100 mL of water. The reaction vessel was kept at 60 °C under stirring for 4 h, shielded from light. Suspended AgI was then eliminated by filtration. The obtained clear solution (solution A) contained the aquo-complex *cis*-[$(NH_3)_2Pt(H_2O)_2$]$(NO_3)_2$. Solution B was prepared treating 5.5 g (10.3 mmol) of **1** in water with 2 equiv of NaOH and was added dropwise to the above-described solution A at 60 °C.

The mixture was kept under stirring at 60 °C for 10 h. During this time complex **1** precipitated as a white solid and was finally separated by centrifugation and dried under vacuum (7 g, 9.2 mmol, 89%).

Found: C, 41.26; H, 6.24; N, 4.00. $C_{28}H_{42}O_{10}PtN_2$ requires: C, 44.15; H, 5.52; N, 3.68.

^1H NMR (CD_3COOD + D_2O 2:1) 0.9 (3H, d, 21-CH_3); 1.19 (3H, s, 18-CH_3); 1. 45 (3H, s, 19-CH_3); 1.5–2.6 (m, aliphatic CH, CH_2); 2.9 (H, t, 11-CH); 3.1 (2H, m, 6/8-CH); 4.2 (6H, s, NH_3); 4.8 (2H, dd, 25/26-CH).

IR (cm^{-1}): 3285–3270 (*v* N–H); 2980–2900 (*v* CH); 1715–1700 (*v* C═O); 1650–1600 (*v* C═O); 1350–1380 (*v* COO).

4.1.3. Synthesis of *cis*-[di(dehydrocholanoate)-bis(triphenylphosphine)-platinum(II)] (complex 3). Ag_2O (1.6 g, 6.9 mmol) and dehydrocholic acid (6.4 g, 15.9 mmol) were suspended in 120 mL of water and refluxed for 6 h. The suspension, turned from black (silver oxide) to white (silver dehydrocholate), was left in an ice bath for 30 min and the solid silver salt was then separated by filtration, washed with water, acetone, and dried at 60 °C for 18 h (7 g, 13.7 mmol, 86%).

In the next step, silver dehydrocholate (6.4 g, 12.6 mmol) was suspended in a solution containing *cis*-[$PtCl_2(PPh_3)_2$], (5 g, 6.3 mmol) in 400 mL CH_2Cl_2. The suspension was kept under vigorous stirring in the dark for 18 h and then the suspended solid residue (AgCl) was eliminated by slow filtration across a Celite pad. The clear colorless solution was taken to dryness under reduced pressure, leaving the product as a white solid (6.7 g, 4.4 mmol, 70%).

Found: C, 66.01; H, 5.92. $C_{84}H_{96}O_{10}P_2Pt$ requires: C, 66.24; H, 6.34.

^{31}P NMR ($CDCl_3$, H_3PO_4 85% ext. ref): 6.6, s, (J_{PPt} 3813 Hz).

^{1}H NMR ($CDCl_3$): 0.68 (6H, d, 21-CH_3); 0.95 (6H, s, CH_3); 1.36 (6H, s, CH_3); 1.43–2.35 (48H, m, aliphatic CH, CH_2); 2.8 (6H, m, CH_2 vicinal to CO), 7–7.6 (60H, m, aromatic CH).

IR (cm^{-1}) 1771 (s, *v* CO); 1629 (m); 1435 (m); 1332 (m); 1098 (m); 693 (s); 529 (s).

4.2. Biological activities

4.2.1. General. Molecules used in the biological evaluation assays, including butyric acid, cytosine arabinoside, mithramycin, and cisplatin were purchased from Sigma/Aldrich (Milwaukee, WI, USA).

4.2.2. K562 cells and cell culture conditions. The human chronic myelogenous K562 cell line[17] was obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in vitro in RPMI 1640 medium (Sigma/Aldrich, Milwaukee, WI, USA), supplemented with 10% fetal bovine serum (FBS) (Celbio, Milano, Italy), 2 mM L-glutamine (Sigma/Aldrich, Milwaukee, WI, USA), and a solution of 50 U/mL penicillin and 50 μg/mL streptomycin (Sigma/Aldrich, Milwaukee, WI, USA) in 5% CO_2/air humidified atmosphere at 37 °C.[18] To determine the antiproliferative activity of these compounds, cells were seeded at an initial cell concentration of 30,000 cells/mL and continuously exposed to various concentrations of drugs for 72 h and the effects on cell growth analyzed by determining the cell number per milliliter using ZF Coulter Counter (Coulter Electronics, Hialeah, FL, USA).[11] Results were expressed as IC_{50} (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls). All experiments were repeated at least thrice. Solvent controls were run with each experiment. The length of treatment (72 h) was chosen because in these experimental conditions the control cells are in the log phase of cell growth.

In order to determine the effects of compounds on erythroid differentiation assays, cells were used after 5–7 days of cellular culture, when the cells are in the plateau phase of cell growth, by counting the proportion of benzidine-positive cells with a solution containing 0.2% benzidine in 0.5 M glacial acetic acid, 10% H_2O_2, as described elsewhere.[22]

4.2.3. Human erythroid cell cultures from normal donors. The two-phase liquid culture procedure was employed as previously described.[18–20] Mononuclear cells were isolated from peripheral blood samples of normal donors by Ficoll–Hypaque density gradient centrifugation and seeded in α-Minimal Essential Medium supplemented with 10% FBS (Celbio, Milano, Italy), 1 μg/mL cyclosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium from the 5637 bladder carcinoma cell line.[23] The cultures were incubated at 37 °C, under an atmosphere of 5% CO_2 in air, with extra humidity. After 7 days incubation in this phase I culture, the nonadherent cells were harvested, washed, and then cultured in fresh medium composed of α-medium, 30% FBS, 1% deionized bovine serum albumin (BSA), 10^{-5} M β-mercaptoethanol, 1.5 mM L-glutamine, 10^{-6} M dexamethasone, and 1 U/mL human recombinant erythropoietin (EPO) (Inalco, Milano, Italy). This part of the culture is referred to as phase II.[20] Compounds were added on day 4–5 of phase II. Cell samples were analyzed on days 12 or 13 of phase II. Hemoglobin (Hb)-containing erythroid precursor cells were counted following staining by the benzidine/H_2O_2 procedure.[18] The proportion of HbF out of the total Hb (%Hb) was determined by high-performance liquid chromatography (HPLC) as elsewhere described.[19,20]

4.2.4. Quantitative RT-PCR analysis. Quantitative real-time PCR assay of γ-globin, β-globin, and α-globin mRNAs was carried out using gene-specific double fluorescently labeled probes in a ABI Prism 7700 Sequence Detection System version 1.6.3 (Applied Biosystems, Warrington Cheshire, UK). The fluorescent reporter and the quencher were: 6-carboxyfluorescein (FAM) and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA), respectively. For quantification, the reference gene was human glyceraldehyde-3-phosphate dehydrogenase (GAPDH); this probe was fluorescent-labeled with VIC (Applied Biosystems, Monza, Italy). Sequences of primers and probes used to analyze the expression of globin genes by quantitative real-time RT-PCR were 5′-TGG CAA GAA GGT GCT GAC TTC-3′ (γ-globin forward primer), 5′-TCA CTC AGC TGG GCA AAG G-3′ (γ-globin reverse primer), 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′ (γ-globin probe), 5′-CAA GAA AGT GCT CGG TGC CT-3′ (β-globin forward primer), 5′-GCA AAG GTG CCC TTG AGG T-3′ (β-globin reverse primer), and 5′-FAM-TAGTGATGGCCTGGCTCACCTG GAC-TAMRA-3′ (β-globin probe).

4.2.5. High-performance liquid chromatography (HPLC) analysis. Cells were harvested on day 12, washed once with phosphate-buffered saline and the pellets were lysed in lysis buffer (sodium dodecyl sulfate 0.01%). After spinning for 1 min in a microcentrifuge, the supernatant was collected and stored at 4 °C. Hemoglobins in the hemolysates were separated by cation-exchange HPLC (Pharmacia LKB Gradient Pump 2249, VWM 2141), using Synchropak CM300 (250 × 4.6 mm) column (Eichrom Technologies, Inc. Darien, IL) and BisTris (30 mM) buffer. Standard HbA and HbF (Sigma/Aldrich, Milwaukee, WI, USA; Helena Laboratories, Beaumont, TX, USA) solutions were used for references.

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