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Dichloro(6-aminoethylaminopurine)platinum(II) and its Hydroxy Analogues: Synthesis and Preliminary Evaluation

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Abstract

The synthesis, chemical characterization and functional evaluation are reported for dichloro(6-aminoethylaminopurine)platinum(II) and dichloro(6-hydroxyethylaminopurine)platinum(II) and dichloro(6-hydroxyethylmethylaminopurine)platinum(II) (*i.e.* Pt(6-AEAP), Pt(6-HEAP) and Pt(6-MHEAP) new complexes of platinum(II). Certain reaction conditions favored the formation of the tripurine platinum complex, but the monopurine complex could be obtained either by hydrolysis of the tripurine or by reacting at reduced temperature and concentration. Although neither compound was as effective as *cis*-diamminedichloroplatinum(II) (*i.e.* DDP) at reducing tumor cell viability or proliferation, both were associated with much less renal toxicity than DDP in the mouse kidney (*i.e.* Pt(6-AEAP):~20 X less; Pt(6-MHEAP): ~100 X less).

Introduction

Binding of the transition metals to nucleic acids has been of great interest for many years [1, 2]. Studies on the interaction of nucleosides with platinum(II) indicate that the metal ion is coordinated to the N7 nitrogen atom of adenosine [3] and guanosine [4], while both the oxygen (C6 position) and nitrogen atom(N7) in inosine can be binding sites depending on pH [5]. Both nitrogens (N1 and N7) of adenosine are simultaneously coordinated to two different platinum ions, while the N7 atom is significantly favored as a binding site in the case of 6-methylaminopurine riboside [6].

A limited number of reports exist concerning the reaction of monosubstituted purines with potassium tetrachloroplatinate, and most of these have mainly been related to 6-mercaptopurine [7, 8].

Our studies on the interaction of some 6-substituted purines with platinum(II) are reported herein. These studies are part of a continuing effort to characterize the chemical coordination of platinum(II) with monosubstituted purines. Ultimately these studies are related to the rational design and synthesis of new platinum-containing agents with anticancer activity. In certain instances the complexing of a purine possessing antitumor activity with Pt(II) produces an agent with more activity than that of the ligand [9]. In the present work we have chosen 6-aminoethylaminopurine as one of the carrier ligands because the combination of a purine with 1,2-diaminoethane can be expected to have structural features which, when complexed with platinum(II), may result in enhanced anticancer activity. Moreover, this newly synthesized purine–platinum(II) complex can be meaningfully compared to *cis*-dichloroethylenediamine platinum(II), a commercially available compound which contains a similar substitution.

Results

6-Aminoethylaminopurine (6-AEAP) was synthesized according to the general procedure for the preparation of N-substituted 6-aminopurines [10]. The reaction of K_2PtCl_4 with 6-AEAP in water at 80 °C yielded a yellow product with a composition that was independent of the molar ratio of starting materials, as indicated by platinum and nitrogen content. The composition of this product corresponded to the empirical formula: $Pt_2(6\text{-AEAP})_3$. Formation of this adduct was rapid and nearly quantitative. The pH of the reaction mixture (6-AEAP alone pH 5.42; K_2PtCl_4 alone pH 4.11) decreased after mixing to pH 2.35. The filtrate separated after reaction completion had pH 1.26; thus several protons were released from 6-AEAP. The analysis of $Pt_2(6\text{-AEP})_3$ was limited by its very low solubility and attempts to obtain a proton NMR spectrum were unsatisfactory. The $Pt_2(6\text{-AEAP})_3$ adduct was only soluble in boiling DMSO; however, these conditions permit ligand exchange [11] and can provide an explanation for the unrecognizably broad NMR signals. Alternatively, the oligomeric nature of the

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TABLE I. The NMR Chemical Shifts of 'Pt(AEAP)' Complexes in ppm (δ).

Compound	HC-8	HC-2	H_2CNH	H_2CNH_2
(6-AEAP)	8.37(s)	8.53(s)	4.08(t)	3.34(t)
6-AEAP + K_2PtCl_4[a]	8.73(s)[b]	8.71(s)	4.23(t)	3.44(t)
6-AEAP + K_2PtCl_4[c]	(A) 8.74(s)[d]	(B) 8.80(s)[d]		
	(C) 8.87(s)[d]	(D) 8.92(s)[d]	4.39(t)[e]	3.48(t)
	(E) 9.10(s)[d]	(F) 9.48(s)[d,i]		
[Pt_2(6-AEAP)$_3$]	9.70 to 8.50[g]		4.62; 4.44	3.52(broad t)[h]
Pt(6-AEAP)[f]	8.78(bs)	8.50(s)	4.21(t)	3.47(t)

[a]5 min after mixing of reagents in D_2O/DCl/KCl at 80 °C. [b]Width of the base of the peak 30 Hz. [c]15 min after mixing of reagents in D_2O/DCl/KCl at 80 °C. [d]Several species present in the mixtures; the ratio A:B:C:D:E:F 2:2:1.5:5:10:0.7. [e]Partly overlapped with 4.23 ppm(t). [f]In DMSO-d_6/D_2O. [g]Six distinguishable singlets, broad, overlapping. [h]The ratio of the peaks 1:2:3. [i]Assigned to HC-8 of AEAP coordinated to two Pt(II) ions.

complex may account for the broadened NMR signals.

To clarify the structural features of the Pt_2(6-AEAP)$_3$ adduct, proton NMR spectra were obtained after DCl and KCl were included in the reaction. Since low temperature ^{1}H NMR [12] studies have indicated that protonation of 6-aminopurines occurs at annular nitrogen atoms which do not already bear a hydrogen, the rate of complexation should be slowed in the presence of DCl if the annular nitrogen atoms participate as binding sites. The positions of the aromatic protons were assigned after deuteration. The C-8 position of 6-AEAP was completely deuterated after heating *in vacuo* in a sealed NMR tube for five days. After three days, 50% deuteration was observed and the signal at 8.37 ppm was assigned to the proton at the C-8 position, while the downfield resonance at 8.53 ppm was assigned to the C-2 proton (Table I). The interactions of K_2-$PtCl_4$ with (6-AEAP) were followed for both HC-8 and CD-8 compounds in the presence of DCl with KCl added to further slow the reaction progress. The HC-8 proton signal shifted substantially (0.36 ppm) in the first stage of complexation due to Pt–N7 bond formation. The HC-8 resonance was wide at the base but no clear coupling was observed. The remaining HC-2 proton also shifted (0.18 ppm); but, to a lesser extent than HC-8 (Table I). A similar shift was also observed for both triplets of the ethylene group in the side chain. Thus, the N-7 nitrogen atom was a primary target for platinum attack in the first phase of the reaction. The minor shifts of both triplets and of the HC-2 proton singlet are probably due to changes in the π-electron distribution in the purine ring. Attempts to separate this intermediate failed, because workup of the reaction mixture caused formation of the previously described Pt_2(6-AEAP)$_3$. When the mixture was allowed to react further in the presence of DCl and KCl, new peaks appeared in the ^{1}H NMR spectrum (Table I). A triplet at 4.39 ppm, in the downfield position [*i.e.* relative to the free ligand (4.08 ppm)], was observed. This shift of the methylene group signal was accompanied by the appearance of several new peaks in the aromatic region, which were shifted downfield with respect to the position of the free ligand resonances (*i.e.* the peak in the lowest field was positioned at 9.48 ppm). The appearance of a triplet at 4.39 ppm indicated that the platinum ion was in very close proximity to one of the CH_2 groups, and may be coordinated through the amino group at the C-6 position. Further reaction produced a yellow precipitate that was identical to [Pt_2(6-AEAP)$_3$]. (^{1}H NMR spectrum signals are listed in Table I). Complex aromatic region signals were observed so that the unambiguous assignment of the peaks was impossible. Deuteration of the C-8 position did not result in simplification of the spectrum sufficient for its interpretation. Nevertheless, the order of complexation for two binding sites was established. Clearly, the initial attack of platinum(II) occurred at the N7 nitrogen atom, followed by chelation through the amino group at the C-6 position. Indirectly, the ^{1}H NMR studies showed that the terminal amino group in the side chain was not engaged in the complexation. These conclusions were further supported by studies involving the interactions of K_2PtCl_4 with 6-HEAP and 6-(2′-hydroxyethyl)methylaminopurine (*i.e.* 6-MHEAP).

Substitution of the amino group with a hydroxyl group eliminated one coordination site in the side chain. The reaction of 6-HEAP with K_2PtCl_4 was monitored with ^{1}H NMR and UV spectroscopy. At high concentrations (0.10 M) of both reagents the complexation was rapid but the appearance of the precipitate was observed only after 12 h at room temperature or after 1 h at 80 °C. The ^{1}H NMR studies indicated that a monomeric complex was

TABLE II. The Chemical Shifts of Pt(6-HEAP) in ppm (δ).

Compound	HC-8	HC-2	$NHCH_2CH_2OH$
(6-HEAP)[a]	7.67(s)	7.70(s)[b]	3.26(s)
(6-HEAP)[c]	8.30(s)	8.53(s)	3.66(s)
(6-HEAP) + K_2PtCl_4[d]	8.76(s)	8.54(s)	4.07(s); 4.17(s)[e]
(6-HEAP) + K_2PtCl_4[f]	9.00(s)	8.53(s)	4.10(s); 4.17(s) 4.23(s)[g]
Pt(6-HEAP)[h]	8.06(b)	7.65(s)	3.90 to 2.90 (broad)

[a]In DMF-d_7. [b]Positions assigned arbitrarily. [c]In DCl/D_2O. [d]1 h after mixing of reagents in D_2O/DCl. [e]This signal was assigned to the complexed (HEAP) while peak at 4.07 ppm to free (HEAP). [f]5 h after mixing of reagents. [g]This signal was assigned to CH_2NH complexed with Pt(II). [h]Precipitate of Pt(6-HEAP) redissolved in DMF-d_7/D_2O.

formed (Table II). The N7 nitrogen atom was again the primary binding site and the amino group metallation was slower than for 6-AEAP. The HC-2 singlet shift was smaller (less than 0.1 ppm) relative to the amino analog. UV spectra indicated that the adduct formed had a 1:1 platinum(II) to purine ratio (confirmation by elemental analysis). At three wavelengths used, the excess absorption *vs.* K_2PtCl_4 molar fraction plots showed the extremum at $X_{k_2PtCl_4} = 0.5$ (data not shown).

The ^1H NMR spectrum indicated that there was a downfield shift in the HC-8 proton signal during the first stage of complexation, but it was not accompanied by a significant change in pH. The substantial change of pH was in concert with the appearance of a new triplet at 4.23 ppm, a further downfield shift of HC-8 resonance and a small shift of HC-2 signal in the ^1H NMR spectrum. There was no clear coupling of H—^{195}Pt but the base of the signal had a width of about 35 Hz. Increased temperatures or prolonged reaction time brought about reprotonation and the eventual precipitation of the neutral complex (Pt-(6-HEAP)).

Analogous product, dichloro(6-aminoethylaminopurine)platinum(II), (*i.e.* Pt-6-AEAP) was isolated from the reaction of the $Pt_2(6\text{-AEAP})_3$ with excess HCl. An identical product was formed in the reaction of 6-AEAP with K_2PtCl_4 at low concentration and temperature. The ^1H NMR signals of Pt(6-AEAP) are listed in Table I. The IR spectrum of this compound showed a band at 324 cm^{-1} which was absent from the spectra of 6-AEAP, $Pt_2(6\text{-AEAP})_2$ and $Pt_2(6\text{-AEAP})_3$, attributed to Pt—Cl. The reaction of $Pt_2(6\text{-AEAP})_3$ with excess 6-AEAP yielded $Pt(6\text{-AEAP})_2$. Changes in pH have already been noted during the formation of $Pt(6\text{-AEAP})_3$ (see above); however, which protons were released have not here-to-fore been established. The overall interactions of 6-aminoethylaminopurine with platinum-(II) are given in Fig. 1.

It was apparent that the NH_2 group in the side chain promotes the formation of $Pt_2(6\text{-AEAP})_3$. The mechanism of the amino group participation had been established by kinetic studies of the reaction

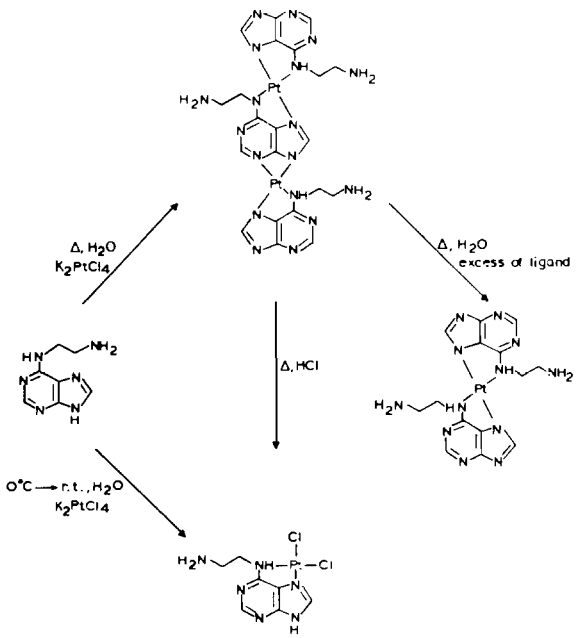

Fig. 1. Proposed reaction scheme.

of thiourea with Pt(6-AEAP) and Pt(6-HEAP). The observed pseudo-first order rate constant for the reaction of Pt(6-AEAP) with thiourea in dimethylformamide ($(9.5 \pm 0.1) \times 10^{-5}$ s^{-1}) was 6 times greater than the rate constant observed for Pt(6-HEAP), [$(1.7 \pm 0.1) \times 10^{-5}$ s^{-1}] (Fig. 2). The formation of $[Pt(tu)_4]Cl_2$ (*i.e.* tu = thiourea) was verified by obtaining the IR spectrum on the yellow crystalline reaction product. Spectra and the molar conductivity of this product were identical with $[Pt(tu)_4]Cl_2 \times DMF$ independently synthesized from K_2PtCl_4 and thiourea. The molar conductivities of the final reaction mixtures were 127 ohm^{-1} mol^{-1} cm^2 (Pt(6-AEAP)) and 126.5 ohm^{-1} mol^{-1} cm^2 (Pt(6-HEAP)). (Note: the molar conductivity of $[Pt(tu)_4]Cl_2$ in DMF is 124 ohm^{-1} mol^{-1} cm^2). Clearly, the observed rate constants, k_{obs}, indicated the assistance of the terminal amino group in the ligand exchange reaction.

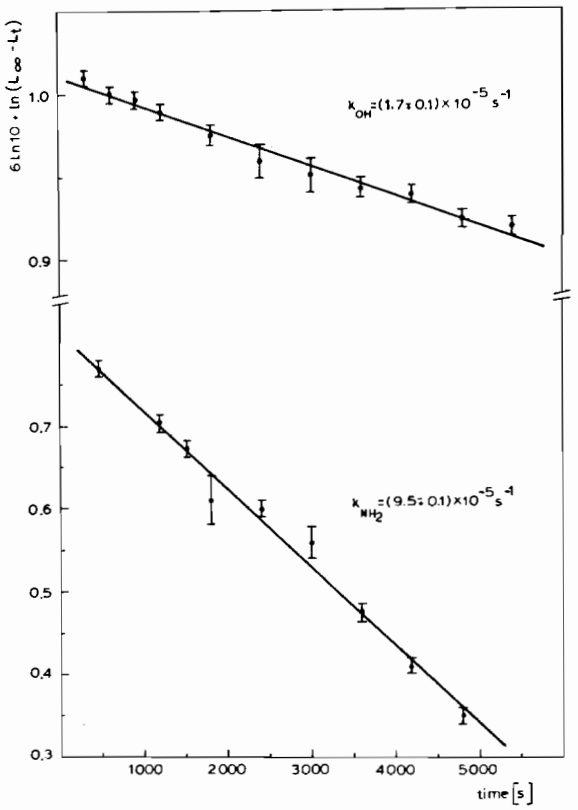

Fig. 2. Difference of conductance for the reaction of thiourea with Pt(AEAP) and Pt(HEAP). The observed rate constant for Pt(AEAP) is designated k_{NH_2}, and for Pt(HEAP) is designated k_{OH}. Means and standard deviations are shown on experiments repeated three times.

The close proximity of the C(2')H_2NH_2 group to the central metal ion facilitates the removal of the chlorine ligand from the coordination sphere by formation of a penta-coordinate transition state/intermediate which is reacting faster with thiourea than Pt(6-HEAP) in the absence of similar assistance from the hydroxyl substituent. Alternatively, it is plausible that the chlorine is displaced by the terminal NH_2 and this highly reactive intermediate (e.g., ring strain) undergoes fast reaction with thiourea. In either case, the neighbouring group effect of NH_2 enhances the rate of the reaction. To the presence of this effect in 6-aminoethylaminopurine complex can also be attributed the formation of Pt_2(6-AEAP)$_3$. The intermediate (transition state) bears at least a partial positive charge, remains in the aqueous solution and is capable of reacting with the N7 nitrogen atom of the second molecule of 6-AEAP which as a softer base should form strong Pt(II)–N bonds. 6-Hydroxyethylaminopurine complex (Pt(6-HEAP)), once formed, precipitates from the reaction mixture and cannot undergo further reaction.

Both Pt(6-AEAP) and Pt(6-MHEAP) were evaluated for their ability to inhibit the *in vitro* proliferation of human bladder tumor cells (Table III). Neither agent reduced proliferation or viability as well as DDP over the concentration tested.

Significant renal toxicity can complicate the clinical course of DDP therapy. DDP has been shown to bring about the release of renal gamma-glutamyl transpeptidase into urine, probably as a result of cellular damage [13]. As a consequence, one indication of platinum compound related renal toxicity is increased urinary gamma-glutamyl transpeptidase [13]. Mice receiving either Pt(6-AEAP) or Pt(6-MHEAP) demonstrated remarkably lower urinary gamma-glutamyl transpeptidase activity than mice which received DDP (Table IV). Moreover, Pt(6-MHEAP) contributed less to elevated urinary transpeptidase activity than Pt(6-AEAP). These observations indicate that Pt(6-AEAP) and Pt(6-MHEAP) are much more moderate mediators of the elevated

TABLE III. Antiproliferative Activity and Lethality of Pt(II) Complexes.[a,e]

Complex	Cell line															
	Colo232		TCCSUP		639V		T-24		647V		253J		SW1710		J-82	
	P[b]	V[c]	P	V	P	V	P	V	P	V	P	V	P	V	P	V
DDP[d]	<10	20	<10	20	30+	50	35	60	40	70	40	90	50	50	65	85
Pt(6-AEAP)	60	80	70	100	100	75	40	95	75	100	85	75	80	80	70	100
Pt(6-MHEAP)	80	100					90	90	20	70	80	70			70	80

[a]Cell lines are arranged in the order of decreasing sensitivity to the antiproliferative activity of *cis*-[$Pt(NH_3)_2Cl_2$] with Colo232 being the most sensitive cell line. [b]Proliferation estimate (%, 100% = cell number growing in the absence of drug). Drug continuously present in culture for 7–10 days. [c]Viability estimate (%, 100% viable = all cells are green due to esterase hydrolysis of fluorescein diacetate and exclusion of nucleophilic propidium iodide; 0% viable = all cells are red due to penetration of red propidium iodide with esterases turning over and disappearing). [d]Exposures: DDP, 0 - 20 μg/ml; Pt(6-AEAP) and Pt(6-MHEAP), 0 - 40 μg/ml. [e]At least 12 different concentrations spanned the exposure range. All determinations were made in duplicate. Values for DDP are expressed at 5 μg/ml, while values for Pt(6-AEAP) and Pt(6-MHEAP) are for the greatest inhibition achieved over the complete concentration range.

TABLE IV. Urinary Gamma-glutamyl Transpeptidase Activity After Exposure to Platinum(II) Complexes.[a]

Complex	Dosage (μg/mouse)									
	0	10	50	100	200	300	400	500	Mean	Std. Dev.
DDP	0	100	100	100	100	100	100	100	100	–
Pt(6-AEAP)	0	10.01	5.58	2.30	14.17	2.73	2.09	1.85	5.53	4.45
Pt(6-MHEAP)	0	1.12	0.00	0.00	5.56	0.00	0.00	0.27	0.99	1.90

[a]Female CF-1 mice (5 mice per dosage) received each compound, dissolved in saline, as a single intraperitoneal injection (0.1 cc/mouse). Results are expressed relative to gamma-glutamyl transpeptidase activity in the urine of mice receiving different dosages of *cis*-$[Pt(NH_3)_2Cl_2]$, ('DDP').

urinary transpeptidase activity that is associated with DDP administration. Based upon these criteria, Pt(6-AEAP) and Pt(6-MHEAP) appear to be much less nephrotoxic than DDP.

Discussion

Dichloro(6-aminoethylaminopurine)platinum(II): Pt(6-AEAP), dichloro(6-hydroxyethylaminopurine)-platinum(II):Pt(6-HEAP) and dichloro(6-hydroxy-ethylmethylaminopurine)platinum(II):Pt(6-MHEAP) have been synthesized. When K_2PtCl_4 was reacted with 6-AEAP at high temperature the formation of tripurine platinum complex was favored. Monopurine dichloroplatinum complex could be obtained either by HCl hydrolysis of the tripurine complex or by running the reaction under conditions of reduced temperature and concentrations. The anchimeric assistance of the terminal amino group was established.

Neither Pt(6-AEAP) nor Pt(6-MHEAP) was as effective as DDP in reducing the viability or inhibiting the *in vitro* proliferation of several human bladder cancer cell lines. However, it must be realized that assignment of DDP sensitivity to a cell line is made when significant reductions have been observed at or below the concentration of DDP that is clinically achievable in patient serum during well-tolerated therapy (*i.e.* 5 μg/ml [14]). At present, no information exists relating Pt(6-AEAP) or Pt(6-MHEAP) to clinical pharmacokinetic correlates. The striking characteristic of both Pt(6-AEAP) and Pt(6-MHEAP) was the moderated extent to which they elevated urinary gamma-glutamyltranspeptidase activity. Admittedly, considerations of drug toxicities need to be subjected to multifocal evaluation. However, at this preliminary level of evaluation, the evidence for reduced renal toxicity indicates some promise for these compounds. Moreover, since antitumor activity evaluations were performed up to the aqueous limits of agents solubility, it is possible that elimination of the present solubility limitation may unmask an antitumor activity at high dosages which is not accompanied by the renal damage associated with high dose DDP.

Supplementary Material

Potassium tetrachloroplatinate was prepared according to the procedure described by Livingstone [15]. 6-Chloropurine was purchased from Aldrich Chemical Company and was used without further purification. 1,2-Diaminoethane and 2-aminoethanol were purchased from Eastman Kodak Company and used freshly distilled. Proton NMR spectra were recorded on a Varian EM-390 90 MHz spectrometer in either DMSO-d_6 or D_2O solution using TMS or DSS, respectively, as internal reference standards. The chemical shifts were expressed in parts per million downfield from TMS or DSS. The concentration of ligand solutions for proton NMR studies were from 0.05 M to 0.10 M in D_2O, pH 4 adjusted with DCl. The saturated aqueous solution of potassium tetrachloroplatinate was added in small portions to the solution of ligand. In those cases where a large excess of K_2PtCl_4 was used, K_2PtCl_4 was added directly into an NMR tube which contained ligand solutions; no correction was made for changes in volume. UV spectra were obtained on a Beckman model 26 spectrophotometer. Water for UV studies was distilled twice and degassed. Concentrations of both ligahd and K_2PtCl_4 were 1–10 μM. The pH of ligand solutions varied from 7.50 to 5.45 depending on the concentration and the quality of water used. The solutions for UV, containing excess KCl, were stored in dark bottles to prevent exposure to light. IR spectra were recorded on a Beckman IR-8 spectrophotometer in KBr pellets or Nujol mulls. Mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6E mass spectrometer. Melting points were taken on a Fisher-Johns melting point apparatus and were uncorrected. Elemental analyses were performed in our laboratory and by Galbraith Laboratories, Knoxville, Tenn. The measurements of pH were performed on Corning pH meter model 125.

Kinetics

Reaction rates were determined at 20.0 ± 0.1 °C by monitoring the conductivity of DMF solutions

using a conductivity bridge, PM-70CB (Sybtron-Barnsted, cell G-1, k = 1.00). Pseudo first-order conditions were maintained by utilizing 100-fold excess of thiourea (concentrations of complexes 5×10^{-5} M, thiourea 5×10^{-3} M). Measurements were made at 5 or 10 min intervals for 2 h and the mixture was allowed to react until the conductivity of the solution corresponded to that of $[Pt(tu)_4]Cl_2$ in DMF (thiourea = tu). Rate constants were obtained for each complex on measurements made with three independent samples by averaging the values of all measurements. $[Pt(tu)_4]Cl_2$ was prepared as previously described [16].

A. 6-Aminoethylaminopurine, ('6-AEAP')

A mixture of 0.100 g (0.65 mmol) of 6-chloropurine, 0.100 g (1.67 mmol) of 1,2-diaminoethane and 0.50 ml of triethylamine in 25 ml of 1-butanol was refluxed for 3 h. The solvent was removed under reduced pressure and 10 ml of water was added to the residual solid. Adjustment to pH 8 was achieved with 2 M ammonium hydroxide. The solution was refrigerated for several hours and the resulting precipitate was filtered off, washed with a small amount of ice-cold water, and dried *in vacuo* for one day. The crude product was recrystallized from ethanol–water mixture and yielded 0.100 g (87%) of pure 6-aminoethylaminopurine (1), m.p. 290–291 °C (lit. [10] m.p. 289–290 °C). The product was homogenous on silica gel TLC in two solvent systems (water:ethanol:NH_4OH 2:30:3 v/v; 1-butanol saturated with 100 mM NH_4OH). Precise molecular weight: theoret., 178.09660; found, 178.09654. *Anal.* Calcd. for $C_7H_{11}ClN_6$: N, 39.20%. Found: N, 39.86%.

B. 6-Hydroxyethylaminopurine, ('6-HEAP')

To a solution of 0.500 g (3.20 mmol) of 6-chloropurine in 50 ml of 1-butanol and 0.50 ml of triethylamine was added 0.20 g (3.20 mmol) of 2-aminoethanol and the mixture was refluxed for 1 h. The colorless crystals deposited from the solution after cooling were collected by filtration and washed with cold 1-butanol. Recrystallization of crude product from water gave 6-HEAP in essentially a quantitative yield, 0.565 g (99.0%), m.p. 249–250 °C (lit. [17] 245–247 °C).

C. Deuterioderivatives

For 6-aminoethylamino-8-deuteriopurine and 6-hydroxyethylamino-8-deuteriopurine, 0.015 g of purine was dissolved in 1 ml of deuterium oxide, pH was adjusted with DCl to 5.5, and the solution was heated *in vacuo* in a sealed NMR tube for 3 to 5 days at 50 °C (at temperatures greater than 85 °C, 6-aminoethylaminopurine reacted to give 7,8-dihydro-9D-imidazo-8-deuterio[2,1,i]-purine). The solution was lyophylized and redissolved in D_2O. This was repeated twice to ensure complete deuteration of all exchangeable protons and to decrease the intensity of HDO NMR signal.

D. Di-Platinum Complex with 6-Aminoethylaminopurine, ['Pt_2(6-AEAP)$_3$']

Hot aqueous solutions of K_2PtCl_4 and 6-AEAP were mixed in 4:1, 2:1 and 1:1 molar ratios. The reaction mixture was kept at 80 °C for a few minutes. The solution turned light yellow and a voluminous yellow precipitate was formed. The complex was removed by filtration and washed with water. The pH of filtrate was 1.25. The collected precipitate was dried *in vacuo* at room temperature for 3 days. Yields based on the amount of ligand used were in all cases nearly quantitative, m.p. >320 °C. *Anal.* Calcd. for $C_{21}H_{26}N_{18}Pt_2$: Pt, 42.39%; N, 27.39%. Found: Pt, 42.70%; N, 28.00%.

E. Monomeric Platinum Complex with 6-Aminoethylaminopurine, ['Pt(6-AEAP)']

The solution of 6-AEAP in 2 N HCl and the solution of K_2PtCl_4 in water were cooled to 0 °C. The cold solutions were mixed in 6:1 molar ratio of K_2PtCl_4 to 6-AEAP and allowed to warm up to room temperature. A light yellow precipitate was formed slowly even at 0 °C. This was collected by filtration, washed with 2 N HCl and water, and dried *in vacuo* at room temperature for 3 days, m.p. >300 °C (begins to darken at 270 °C). *Anal.* Calcd. for $C_7H_{10}Cl_2N_6Pt$: Pt, 43.92%. Found: Pt, 44.09%.

A similar monomeric complex was produced upon refluxing of the trimer with the excess of hydrochloric acid. The suspension of 0.200 g of Pt_2(6-AEAP)$_3$ was refluxed in 50 ml of 5 N HCl. After 2 days the solution became dark brown and a small amount (0.012 g) of orange precipitate was collected by filtration. This was washed several times with hydrochloric acid and water until a yellow color appeared, and dried *in vacuo* at 50 °C for 2 days, (yield of Pt(6-AEAP) 12.4%, m.p. >300 °C. *Anal.* Calcd. for monomer: Pt, 43.92%. Found: Pt, 44.20%).

F. Di-(6-aminoethylaminopurine)platinum(II), ['Pt-(6-AEAP)$_2$']

An aqueous suspension of Pt_2(6-AEAP)$_3$ (0.100 g) was refluxed with 6-fold excess of 6-AEAP (0.120 g). The appearance of the mixture did not change even after 7 days at 100 °C, but the TLC analyses run periodically indicated a decrease of the free 6-AEAP concentration. The reflux was stopped after 3 days and the amount of platinum in the collected precipitate was determined (Pt 38.73%). Since the platinum content decreased as compared with the starting trimer (Pt_2(6-AEAP)$_3$), but was still too high for the dimer, an additional two-fold excess of 6-AEAP was added and the reflux continued for 4 more days.

After one week the precipitate was collected by filtration, washed several times with hot water and dried *in vacuo*, yield of 0.04 g (67%), m.p. >300 °C. A TLC of the filtrate revealed several components; attempts to separate them failed (*Anal.* Calcd. for $C_{14}H_{18}N_{12}Pt$: Pt, 35.52%. Found: Pt, 35.65%).

G. Platinum Complexes of 6-Hydroxyethylaminopurine and 6-(2'-Hydroxyethyl)methylaminopurine, 'Pt(6-HEAP)' and 'Pt(6-MHEAP)'

Aqueous solutions of 6-HEAP or 6-MHEAP and K_2PtCl_4 were mixed in 1:1 molar ratio and allowed to react at approximately 40 °C for 24 h. The slightly yellowish precipitate that was collected by filtration was dried under reduced pressure at 50 °C; the products were recovered in nearly quantitative yield, m.p. >300 °C (with some decomposition beginning at 290 °C). *Anal.* Calcd. for $C_7H_9Cl_2N_5OPt$: Pt, 43.82%; N, 15.73%. Found: Pt, 43.11%; N, 15.82%; for 'Pt-(HEAP)'. 'Pt(6-MHEAP)' was recovered as a monohydrate. *Anal.* Calcd. for $C_{18}H_{11}Cl_2N_5OPt \cdot xH_2O$: N, 14.67; C, 20.12; H, 2.72; Pt 40.89%. Found: N, 14.07; C, 20.15; H, 2.70; Pt,41.05%.

Agent Evaluation

Cell lines

Dr. Jorgen Fogh (Memorial-Sloan Kettering, Rye, N.Y.) provided cell lines originating from transitional cell carcinomas of the urinary bladder (253J, T24, SW-1710, TCCSUP, 639V, J82 and 647V). Dr. George Moore (Denver General Hospital) provided a transitional cell carcinoma cell line (COL232).

Cell culture

Cells were grown as specified by the originating source in the required nutrient media (Dulbecco's MEM, McCoy's 5A, L-15, or RPMI(1640)) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin with bicarbonate, and 10 mM Hepes buffering. Where appropriate, additional supplementation consisted of insulin (0.01 U/ml), cortisone (10 μg/ml), and nonessential amino acids (obtained from Grand Island Biologicals Co. (1x)). Cells were subcultured by disrupting attachment through the use of Hank's balanced salt solution (HBSS) containing EDTA (2 mM, pH 7.4). Cells were grown in plastic tissue culture vessels after plating at $3-4 \times 10^3$ cells/cm^2 and were renourished with fresh liquid media every three days (0.4–0.6 ml media/cm^2). Tumor cell counting was performed on Coulter counters (Models ZBi and ZF, Coulter Electronics, Hialeah, Fla.).

Biochemicals

The Investigational Drug Branch of the National Cancer Institute provided pure *cis*-platinum (DDP), without buffer, extenders or stabilizers. All platinum complexes were dissolved in tissue culture medium (without serum) and sterilized by membrane filtration, as appropriate [18]. Fluorescein diacetate and propidium iodide were obtained from Sigma Chemical Co.

Viability Determinations based on Esterase Related Fluorescence and the Exclusion of Propidium Iodide

Filtered solutions of fluorescein diacetate dissolved in acetone (1 mg/ml) and propidium iodide dissolved in distilled water (1 mg/ml) were combined with cells suspended in HBSS (final concentration 25 μg/ml). With high cell concentrations ($>1 \times 10^6$ cells/ml), fluorescent probes were added to a final concentration of 100 μg/ml. Fluorescent staining of cells was maximal by 2–4 min at room temperature. For fluorescence microscopy, cells (5–10 ml) were centrifuged at 100 × g for 10 min, resuspended in 100–200 μl of HBSS and delivered to microscope slides (to which cover slips were applied). For flow cytometry, cells were resuspended in 5 ml of HBSS (Ca^{++} and Mg^{++} free).

Fluorescence microscopy and flow cytometry

A Leitz DiaLux 20EB fluorescence microscope was coupled to a Wild MPS45 Photoautomat camera and was used for microscopic examination of red and green fluorescence after excitation with a mercury arc lamp. Flow cytometry was performed with an Ortho System 10 flow cytometer (ICP-22A detector and 2103 distribution analyzer) fitted with a 100 watt mercury arc lamp. The system was configured with KG1, BG38, and FITC special barrier filters as well as a TK510 dichroic filter in the excitation filter assembly. The detection filter assembly for the photomultiplier measuring green signals contained a TK580 dichroic filter and a FITC Special barrier filter. The detection filter assembly for the photomultiplier tube measuring red signals contained a full reflecting mirror, as well as KV550 and LP610 barrier filters.

Urinary gamma-glutamyl transpeptidase activity

A modification of the method of Tate and Meister was used to measure urinary gamma-glutamyl transpeptidase. Substrate or gamma-glutamyl donor (L-gamma-glutamyl-p-nitroanilide) was prepared at 5 mM in Tris (0.5 M), NaCl (0.75 M) pH 8.0 by warming to 50 °C. The gamma-glutamyl acceptor (L-methionine) was prepared at 0.4 M in the same buffer. For the standard curves, gamma-glutamyl transpeptidase (Sigma Chemical) was prepared in buffer containing Tris-HCl (50 mM), NaCl (75 mM) and glycylglycine (20 mM), pH 8.0 and used at concentrations (0.005 U–0.4 U/ml) which yielded a linear change in absorbance measured at 410 nm with automated sampling at 1.5 min intervals over 10–15 min reaction times. All determinations were run at 20 °C.

Urinary 'unknowns' were run after dilution with distilled water to concentrations that were on the absorbance standard curve. The reaction mixture combined the following: (Tris HCl, 500 mM, NaCl, 750 mM, pH 8.0; 100 μl), (glycylglycine, 200 mM, pH 8.0; 100 μl), (substrate; 1000 μl), (acceptor; 1000 μl) and (up to 700 μl enzyme solution or urine with distilled water as the diluent when appropriate).

Agents dissolved in saline were injected intraperitoneally to female CF-1 mice (Harley Sprague-Dawley, Indianapolis, Ind.). Mice were housed in small metabolic cages (5 mice per cage). Individual mouse dosage was either 10, 50, 100, 200, 300, 400 or 500 μg of compound, while controls received only saline. Based upon injections with DDP, urinary volume and enzyme activity were maximal 24 h after a single injection of agent. Elevated urinary gamma-glutamyl transpeptidase activities following a single administration of DDP has previously been reported in rats [13].

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