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Synthesis and characterization of Sodium *cis*-dichlorochenodeoxycholylglycinato(*O,N)* platinum(II) – Cytostatic activity

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Abstract

With a view to using bile acids as shuttles for delivering platinum-related cytostatic drugs to liver tumors, a chenodeoxycholylglycinato(CDCG)-derivative of platinum(II) has been synthesized. The complex - named Bamet-M2- was chemically characterized by elemental analysis, FT-IR, NMR, FAB-MS, and UV spectroscopy. The results indicate the following composition: $C_{26}H_{42}N_2O_5Cl_2NaPt$(II), the metal Pt(II) being bound to two Cl^- and one bidentate CDCG moiety, i.e., Na[Pt CDCG*(N,O)* Cl_2]. The compound is highly soluble (up to 20 mM) in water and (up to 35 mM) in ethanol, methanol and DMSO. Hydrolysis was investigated because this is assumed to be an important step in intracellular activation and interaction with DNA of this type of compounds. The reaction kinetics of this complex in aqueous solution show unusual behaviour; the substitution process with the displacement of two Cl^- was almost instantaneous, and the resulting species were found to be very stable. Kinetic studies carried out in the presence of different NaCl concentrations (up to 500 mM) revealed similar fast and nonreversible aquations of Bamet-M2. This contrasts with the slow aquation of cisplatin in extracellular-line solutions (i.e., at high NaCl concentrations) as compared with fast hydrolysis in cells. This difference may partly account for the low cytostatic activity observed here for Bamet-M2 against several tumor cell-lines.

Abbreviations: CDCG–chenodeoxycholylglycinato ligand; MTS–3-(4,5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PMS–Phenazine methasulfate; cis-DDP–cis-dichlorodiammine platinum(II), CMC, critical micellar concentration, NBA–3-nitrobenzyl alcohol; EA–elemental analysis; IC50–inhibitory concentration 50

Introduction

The continued interest in platinum-based anti-tumor compounds has been stimulated by the fact that certain tumors are resistant to the clinically used drug cisplatin. Furthermore, the toxic side effects of cisplatin pose severe limitations to its use in clinical settings and considerable efforts have been made to find analogs of lower toxicity and with improved effectiveness (Davies *et al.* 1998).

Several strategies have been envisaged to either circumvent resistance to such drugs (Canon *et al.* 1990) or to improve their vectoriality towards tumors (Konno 1992). In this sense, the marked organotropism of bile acids towards the hepatobiliary system has been proposed as an interesting feature in the use of these endogenous compounds or their analogs as shuttles for drugs towards the liver (Kramer *et al.* 1992, 1994).

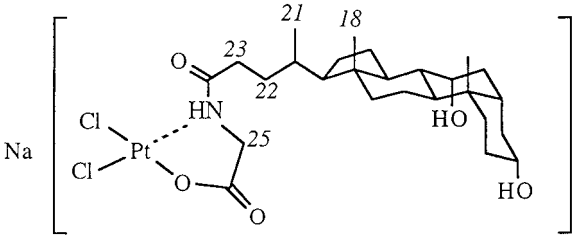

Figure 1. Schematic representation of proposed structure for the Na[Pt CDCG(*N,O*) Cl_2] complex named Bamet-M2.

With this aim in mind, our group has synthesized and characterized (Criado *et al.* 1997a, b) several bile acid derivatives which have in common the presence of a platinum(II) atom as the DNA-reactive part of the resulting molecule (Marin *et al.* 1998a, b). These compounds have been termed Bamet-'Ba' standing for bile acid and 'met' for metal. The present work reports the results of the chemical characterization and investigation of the *in vitro* cytostatic activity of a new compound of this family (Bamet-M2) obtained by binding an atom of platinum(II) to the carboxyl and amide groups of a 3α,7α-dihydroxy-5β-cholanoyl glycinato moiety.

Materials and methods

Chemicals

Sodium tetrachloroplatinate(II) tetrahydrate, $Na_2PtCl_4 \cdot 4H_2O$, was purchased from Fluka Quimica (Madrid, Spain). Glycochenodeoxycholic acid sodium salt (NaCDCG, >97% pure by TLC) were obtained from Sigma Quimica (Madrid, Spain), MTS and PMS was from (Promega, Boehringer Mannheim, Barcelona, España). All other reagents were of high purity and were used as purchased without further purification.

Synthesis

The platinum complex named Bamet-M2 Na[Pt CDCG*(N,O)*Cl_2], with a molecular formula of $C_{26}H_{42}Cl_2NO_5PtNa$, was obtained by the following procedure: 100 ml of $Na_2PtCl_4 \cdot 4H_2O$ (5 mM, previously prepared and filtered onto paper at room temperature) were added dropwise to 100 ml (5 mM) of an NaCDCG solution in water at 50 °C. To prevent physicochemical effects due to the presence of bile acid micelles in the reaction mixture, attempts were made to keep the concentrations of free NaCDCG in the reaction mixture below the critical micellar concentration (CMC) for this bile acid. Once the addition of sodium tetrachloroplatinate(II) had been completed, the reaction was maintained in the dark at 50 °C for 10 h with continuous stirring. The initial pH = 5.2 did not change during this time, but the color of the reaction moved from intense red- brown at the beginning to reddish grey at the end. The final solution was then allowed to evaporate to dryness in a desiccator containing P_4O_{10}, to afford a grey solid as crude reaction product.

Purification

Reaction products were separated from the excess of unreacted platinum by solid-liquid extraction in octadecylsilane cartridges (C18, Sep-Pak, Waters Cromatografia SA, Madrid, Spain) following a classic procedure (Setchell & Worthington 1982). The retained compounds were recovered from the cartridges in methanol. The extract was then concentrated before carrying out thin layer chromatography (TLC) on silica gel plates (60 F254, Merck, Darmstadt, Germany) using butyl acetate-methanol 65/35 (v/v) as eluent. Two major bands, one of unreacted NaCDCG (Rf = 0.35) and the other of the complex named Bamet-M2 (Rf = 0.75), were obtained. The yield of the whole process was approximately 10%.

Analytical methods

Chemical analyses for C, H and N were performed on a Perkin-Elmer 2400 elemental analyzer (Perkin-Elmer, Hispania SA, Madrid, Spain). Platinum was determined by atomic absorption on a flameless graphite furnace spectrophotometer (Z-8100 Hitachi, Tokyo, Japan) set at a wavelength of 265.9 nm and using the following temperature program: 90 °C (20 s), 100 °C (20 s), 800 °C (20 s), 1600 °C (30 s), 2800 °C (5 s) and 3000 °C (4 s). Infrared (IR) spectra were recorded in the 4000–200 cm^{-1} range on a Perkin-Elmer FT-IR 17 300 instrument coupled to a Perkin-Elmer 3600 Data Station. KBr pellets and spectrophotometric grade Nujol (Fluka Quimica) or polyethylene (Aldrich, Madrid, Spain) disks were respectively used to record spectra above and below 400 cm^{-1}. Mass spectrometry studies were carried out on a VG-Autospec (Mass Spectrometry Service, Universidad Autonoma, Madrid, Spain), using L-SIMS ionization in the FAB^+ mode (Cs ion emission) and m-NBA as matrix. Electrical conductivity in solution was measured using a CDM2e conductimeter (Radiometer,

Copenhagen, Denmark) with a CDC104 immersion cell. Temperature was controlled with a Unitherm water bath (Selecta, Barcelona, Spain) with a precision of ±0.01 °C. Electronic spectra were recorded on a Shimadzu UV-2101S (Izasa SA, Barcelona, Spain) coupled to a CPS temperature controller. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (102.6 MHz) spectra were obtained in CD_3OD on a Bruker DX400 instrument (Karlsruhe, Germany). The ^{195}Pt NMR spectrum was obtained in CD_3OD (64.5 MHz) using 0.1 M of K_2PtCl_6 as external standard (200 000 scans with a 2 s relaxation delay were acquired).

In vitro *cytostatic studies*

The complex was evaluated for *in vitro* cytostatic activity against human hepatoma (Hep G2), human colon adenocarcinoma (LS 174T), mouse hepatoma (Hepa 1-6), rat hepatoma (McA RH-7777), mouse leukemia (L-1210) and mouse sarcoma (S-180II) cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in appropriate medium as recommended by the supplier in a CO_2:air (5%:95%) atmosphere at 37 °C.

Cells were seeded at a density of 10×10^3 per well and cultured for 24 h. After this time, the medium was replaced by a fresh one containing the desired amount of drug to be tested and cell were exposed to it for 48 h of culture. Studies comparing the cytostatic activity of cisplatin and Bamet-M2 were carried out using six different concentrations (from 2 to 100 μM). The number of living cells at the end of the incubation time was determined by the capacity to biotransform MTS into formazan.

Results and discussion

Chemical characterization

The pure complex named Bamet-M2 is a solid showing a melting point with decomposition between 135–137 °C. The compound was found to be soluble (up to 20 mM) in water and was highly soluble (more than 35 mM) in ethanol, methanol and DMSO.

Bamet-M2 elemental analysis (EA) revealed a 1:1 ratio between the chenodeoxycholylglycinato ligand and platinum in the complex, in agreement with the molecular formula $C_{26}H_{42}NO_5Cl_2NaPt$ (Calculated: C 42.35; H 5.74; N 2.01; Pt 26.4. Found by EA: C 42.38; H 5.75; N 1.96; Pt 26.4), which also precludes other combinations containing higher or lower number of nitrogen atoms and chenodeoxycholylglycinato residues (Figure 1).

The complex was characterized by a combination of spectroscopic methods, which allowed us to propose its structure in the absence of X-ray diffraction studies. The latter were not carried out because it was not possible to crystallize the compound under any of the large list of solvents and conditions assayed (data not shown).

In the IR spectrum, no large differences were seen between the chenodeoxycholylglycinato $[CDCG]^-$ ligand and the complex. Both showed the stretching vibrational modes of ν(OH+NH) at 3414 cm^{-1} and 3444 cm^{-1}, respectively (Altman *et al.* 1991; Paul *et al.* 1993; Khokhar & Lumeta 1992). The main differences with the $[CDCG]^-$ ligand lie in the 'glycinato' moiety, since this contains the carboxylato and amide donor groups. In the free ligand, a broad distorted band centered at 1599 cm^{-1} with two shoulders (1638 and 1546 cm^{-1}) was observed whereas in the complex there was a strong and well defined band at 1636 cm^{-1}and a smaller band at 1544 cm^{-1}(Lee *et al.* 1995, 1996; Minghetti *et al.* 1995). These absorptions were assigned to the υ CONH-amide-I, υ_{as} COO- and υ CONH-amide-II stretching vibrations (Paul *et al.* 1993). The absorption peaks (1400–600 cm^{-1})appeared without changes in the complex because they are related to unmodified skeletal vibrations, with a sharp peak at 611 cm^{-1}, characteristic of bile acids. The stretching vibration modes for Pt-N and Pt-O appeared in the 600–200 cm^{-1}range. Two absorptions can be assigned to ν(Pt-N) at 529 cm^{-1} and to ν(Pt-O) at 454 cm^{-1}, corresponding to a bidentate arrangement. These values are close to those described in the literature (Kieft & Nakamoto 1967; Arpalahti *et al.* 1996; Talman *et al.* 1997) for this type of complex but this assignment is difficult and not definitive for structural elucidation purposes. The two typical stretching vibrational modes of the cis- Pt-Cl bonds at 322 and 353 cm^{-1} were also observed (Talman *et al.* 1997; Lee *et al.* 1995; Cornia *et al.* 1997).

The mass spectrum (Figure 2A) of a freshly prepared sample of the complex showed the base peak at $m/z = 472.2$, which corresponds to $[NaCDCG+H]^+$ (Eckers *et al.* 1991), and the highest mass/charge value peak at $m/z = 760.1$ (20% relative abundance). The latter can be assigned to the $[M+H+Na]^+$, taking into account the usual addition of Na^+ in the FAB^+ mode and the presence of a platinum atom, and is accompanied by another peak at $m/z = 738.2$ (10% relative

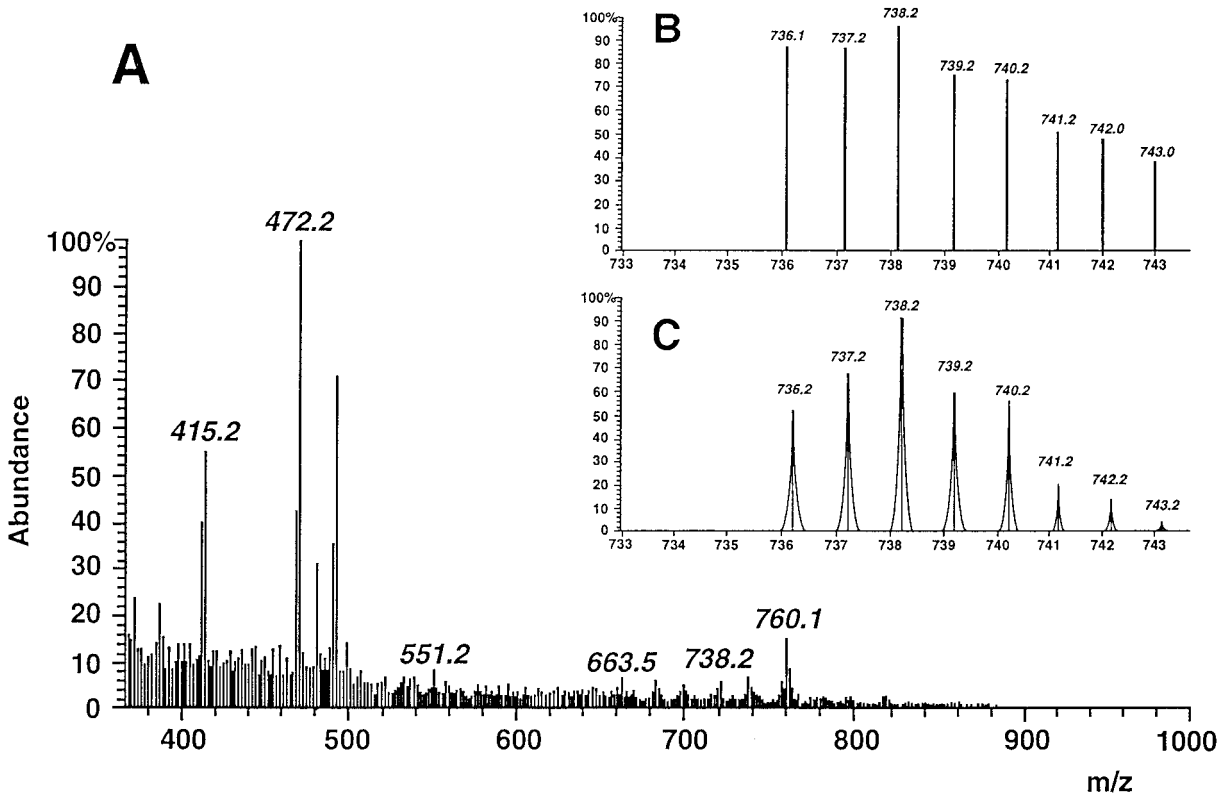

Figure 2. MS/FAB$^+$ spectrum of a freshly prepare sample of Bamet-M2 (A). Zoom of this spectrum in the m/z region corresponding to the $[M+1]^+$ ion (B). Calculated isotopic mass distribution for the $[M+H]^+$ ion (C).

abundance), corresponding to the $[M+H]^+$. It was possible to recognize the presence of the metal in the peaks of the MS because they appeared as a set of peaks, mainly owing to the existence of six different isotopic masses for the metal (190, 192, 194, 195, 196 and 198). As shown for the $[M+H]^+$in the inset of Figure 2A, the observed set of peaks (Figure 2B) matched those calculated (Figure 2C) for $C_{26}H_{43}Cl_2NO_5NaPt$ perfectly. This was also the case for other sets of peaks observed in the spectrum (data not shown).

The 1:1 ratio between chenodeoxycholylglycinato and Pt and the MS support a structure with two chlorine atoms and a bidentate chenodeoxycholylglycinato bonded to the metal, together with a sodium atom as a counter ion, as depicted in Figure 1.

In order to confirm the above structural assignment and to obtain new spectroscopic data, the ^{1}H and ^{13}C-NMR spectra of the complex and the free ligand, as well as the ^{195}Pt spectrum of the complex were obtained. The ^{1}H and ^{13}C-NMR spectroscopic data obtained in methanol-d_4 and DMSO-d_6 are shown in Table 1. These spectra show that the chenodeoxycholylglycinato moiety is maintained in the complex with no major structural changes because the H-3, H-7 and H-18-H-21 and the C-1-C-24 signals are observed to be almost identical in both solvents for the free ligand and the complex. Unfortunately, the H-25 and the C-24, 25 signals, corresponding to the part of the ligand closest to the metal, are not observed in the NMR spectra of the complex; only the H-N appears slightly shifted in the DMSO-d_6 spectrum. Nonetheless, in previously characterized compounds namely, related bidentate complexes obtained from cholylglycinato (Kortes *et al.* 1996; Nagao *et al.* 1997) this part of the molecule, which was very useful for its structural assignment, could be observed.

The ^{195}Pt-RMN spectrum of the complex shows a signal at $\delta = -1496$ ppm, in agreement with a square planar environment. This value is close to those previously described for other *cis*-dichloro bidentate (*O, N*) platinum complexes ($\delta = -1602$ ppm, Pregrosin 1986, $\delta = -1634$ ppm, Sandman *et al.* 1998, $\delta = -1650$ ppm, Watanabe *et al.* 1995).

Table 1. ^{1}H and ^{13}C assignments of ligand and complex Bamet-M2 in CD_3OD and DMSO-d4

C	Bamet-M2 DMSO	Bamet-M2 CD_3OD	NaCDCG DMSO	NaCDCG CD_3OD
1	35.3#	36.8	35.2#	36.6
2	30.5	31.4	30.9	31.4
3	70.2	72.9	70.3	72.9
4	39–40	40.5	39–40	40.5
5	41.4	43.2	41.4	43.2
6	34.7#	35.9	34.7#	35.9
7	66.1	69.0	66.1	69.1
8	39-40	40.8	39–40	40.8
9	32.2	34.1	32.2	34.0
10	35.2	36.2	35.1	36.2
11	20.2	21.8	20.2	21.8
12	39–40	41.1	39–40	41.1
13	41.8	43.7	41.8	43.7
14	49.9	51.5	49.9	51.5
15	23.1	24.6	23.1	24.6
16	27.7	29.2	27.8	29.2
17	55.6	57.4	55.6	57.4
18	11.6	12.2	11.6	12.2
19	22.7	23.4	22.7	23.4
20	34.8	37.0	34.8	37.0
21	18.3	18.9	18.3	18.9
22	31.5	33.1	31.7	33.1
23	32.2	34.1	32.5	34.2
24	171.6	177.1	170.6	176.3
25	n.o.	n.o.	44.1	44.5
26	n.o.	n.o.	171.3	176.4
H				
18	0.58 *s*	0.68 *s*	0.59 *s*	0.68 *s*
19	0.82 *s*	0.92 *s*	0.83 *s*	0.92 *s*
21	0.87 *d*	0.97 *d*	0.87 *d*	0.97 *d*
3	3.16 *m*	3.36 *m*	3.17 *m*	3.36 *m*
7	3.61 *bs*	3.78 *bs*	3.62 *bs*	3.78 *bs*
25			3.47 *s*	3.78 *s*
OH	4.09 *bs*		4.09 *bs*	
OH	4.30 *bs*		4.29 *bs*	
NH	7.17 *t*		7.01 *t*	

n.o;: not observed
#: interchangeable
s: singlet
d: doublet
t: triplet
m: multiple
b.s.: broad signal

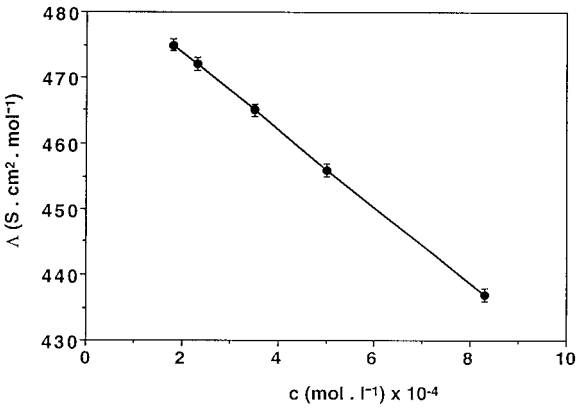

Figure 3. Equivalent molar conductivity showing a instantaneous dissociation process.

Table 2. Cell growth inhibition induced by cisplatin and Bamet-M2

$IC_{50}(\mu M)$	Cisplatin	Bamet-M2
Human hepatoma	16.4	69.1
Rat hepatoma	15.1	90.9
Mouse hepatoma	6.4	>100
Human colon adenocarcinoma	9.5	>100
Mouse leukemia	12.7	>100
Mouse sarcoma	15.8	>100

IC_{50} was defined as the drug concentration (in μM) in the culture medium able to reduce cell viability by 50 %. The number of living cells in culture dishes at the end of the incubation time (72 h) was measured by the MTT-test and expressed as percentage of control cells. IC_{50} was calculated from mean values obtained in 3 different cultures carried out in triplicate.

Kinetic study

The behaviour of the complex in aqueous solution is characterized by a high equivalent molar conductivity (Figure 3) $\Lambda_M = 423$ S cm^2 mol^{-1}, which corresponds to a dissociation process of the type

$$Na[Pt(CDCG)Cl_2] \rightarrow Na^+ +$$

$$[Pt((CDCG)(H_2O)_2]^+ + 2Cl^- \text{(instantaneous)}$$

in agreement with the leaving character of the chloro ligands, which are immediately substituted by aquo ligands. This study was continued by recording the UV spectra of a freshly-prepared aqueous solution ($c = 9.03 \times 10^{-4}$M), which displayed a single maximum at 200 nm. After 24 h, 48 h and 72 h and 7 and 14 days, the recorded spectra of this solution did not show any significant variation, thus confirming the absence of changes in the coordination sphere after solution of the complex. When the behaviour of the complex in 4 mM (cytoplasmic), 150 mM (plasma) and 500 mM NaCl solutions was studied, the absorption band persisted with the same intensity as in the original spectrum (data not shown). It may thus be concluded that the process is non-reversible in the presence of chloride ions and that the complex formed is highly stable in this medium.

However, at present the following speculation can be advanced. The fact that Bamet-M2 was rapidly hydrolyzed both in the absence of NaCl and in the presence of low or high NaCl concentrations suggests that the cultured cells were exposed to the compound as a diaquo cationic complex, which would reduce its influx into the cells (Chen *et al.* 1998). Therefore, although the diaquo form is expected to be more reactive against DNA, it probably reached the nuclei of these cells in smaller amounts than cisplatin and several Bamet compounds (Marín *et al.* 1998a, b) previously studied.

Cytostatic activity

Bamet-M2 was synthesized with the hope of obtaining a more effective compound than the previously described Bamet-H2 and Bamet-R2. This was expected because Bamet-M2 is smaller than both Bamet-H2 and R2 and maintains a negative charge, similarly to what happens in the case of natural bile acids. Moreover this complex, like cisplatin, contains two leaving chloride atoms which presumably increase its reactivity toward DNA as compared to Bamet-H2 and Bamet-R2. However our results did not confirm these expectations (Figure 4). Bamet-M2 showed only moderate activity against human and rat hepatoma cells, while its cytostatic effect on mouse hepatoma sarcoma and leukemia cells as well as on human colon carcinoma cells was very mild, with IC50 values probably much higher than 100 μM (Table 2). The reason for this unexpected result is unknown. Bamet-R2 has been shown to be taken up by hepatocytes following sodium-independent pathways (Marín *et al.* 1998). Whether Bamet-M2 is taken up by a less efficient mechanism or whether its DNA-reactivity is lower than expected are issues currently under investigation by our group.

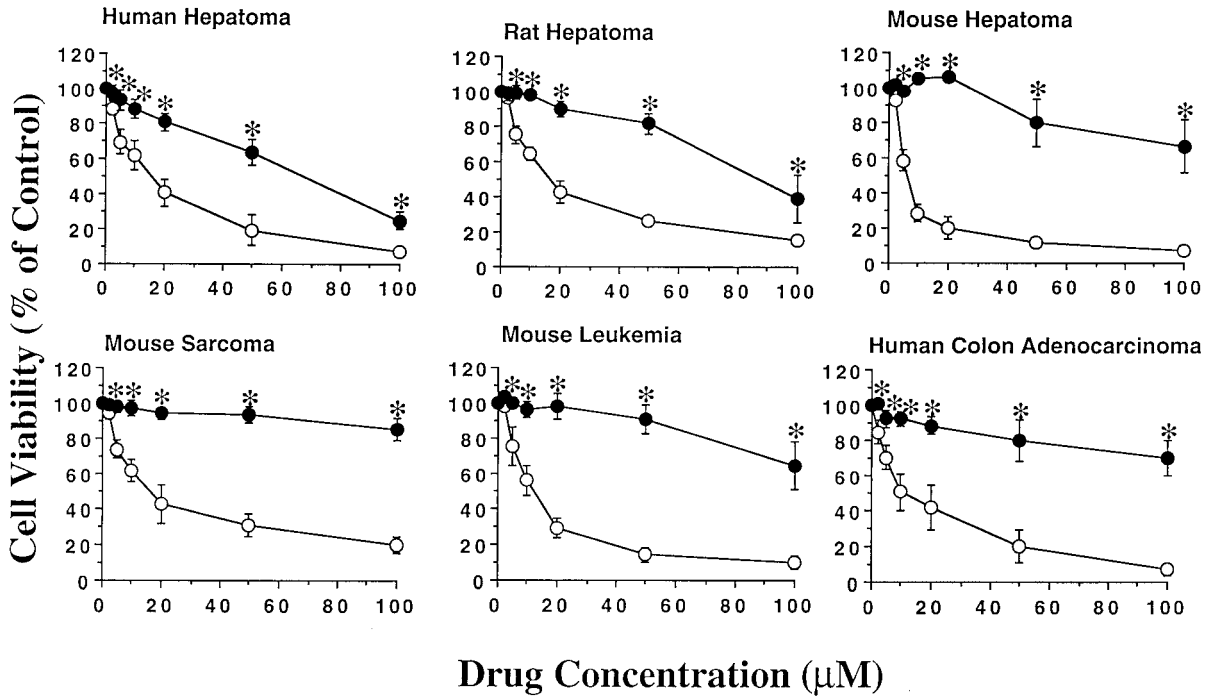

Figure 4. Dose-effect relationship of Bamet-M2 (closed circles) and cisplatin (open circles) on human hepatoma cells (Hep G2), human colon adenocarcinoma (LS 174T), mouse hepatoma (Hepa 1-6), rat hepatoma (McA RH-7777), mouse leukemia (L-1210) and mouse sarcoma (S-180II) cells in culture. The indicated compound was added at 24 h of culture. The number of living cells in culture dishes at the end of the incubation time (72 h) was measured by the MTS/PMS-test and expressed as percentage of control cells. Values are means ± SE from 3 different cultures carried out in triplicate. $*$, $p < 0.01$ as compared Bamet-M2 with the same concentration of cisplatin by the Student t-test.

Acknowledgements

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