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Short communication

Synthesis, characterization and preliminary cytotoxicity assays of poly(ethylene glycol)–malonato–Pt–DACH conjugates

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Abstract

Oxalate 1,2-diaminocyclohexane platinum (oxaliplatin®), a successfully employed platinum compound belonging to the family of Pt–DACH complexes, has been conjugated to different molecular weight poly(ethylene glycols) (PEG) by means of peptide spacers and a malonic acid bidentate residue. Tri- and tetrapeptidic substrates of lysosomal enzymes were used in order to increase the release of Pt–DACH complex inside the cell following endocytosis and enzymatic degradation of the peptide spacer. Other aminoacids (e.g. norleucine) have been also employed. ^{1}H-NMR of some conjugates was performed as characterisation of the product, while ^{195}Pt-NMR analysis was carried out to detect the rearrangement of the platinum complex from the Pt(O,O) to the Pt(O,N) form. The compound PEG(5000)–Nle–malonato–Pt–DACH (**4**) has been tested against L1210-implanted mice and showed and appreciable increase in cytotoxicity as compared to the reference standard Cl_2PtDACH.

Keywords: Platinum; Poly(ethylene glycol); Anticancer drug; Oxaliplatin; Pt–DACH

1. Introduction

Cisplatin is one of the most potent antitumor agents currently available to clinicians for the treatment of testicular, ovarian, head and neck, cervical and bladder cancers [1] Its antitumor activity results from the ability of the diacqua species to form cross-linking bridges between N7 guanine residues of DNA, leading to intrastrand reticulation. The drug is, therefore, especially active against proliferating cells that enter the G1 phase of the cell cycle [2]. The main reasons for its limited use in therapy are the narrow antitumor activity spectrum and the severe dose-related toxicity such as nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting, and mielosuppression [3,4]. The synthesis of cisplatin derivatives with lower toxicity, better antitumor activity and water solubility directed the researchers' efforts at developing molecules such as carboplatin, which is in clinical use today. It was also found that platinum complexes of 1,2-diaminocyclohexane (1,2-DACH) are active on cisplatin-resistant tumors and oxalato, 1,2-*trans*-L-diaminocyclohexane platinum (oxaliplatin, **1**, Fig. 1) [5], is now used for the treatment of advanced colorectal cancer.

The preparation of polymer bound platinum complexes is attractive as a means of increasing solubility, reduce systemic toxicity and localise more drug molecules in the tumor through the enhanced permeability and retention (EPR) effect [6]. On these premises, a number of polymer-bound antitumor agents have been developed so far and some are currently under preliminary clinical trials: taxol [7], doxorubicin [8], camp-

Abbreviations: DACH, diaminocyclohexane; DCCI, dicyclohexyl carbodiimide; DMF, dimethylformamide; HOSu, N-hydroxy succinimide; EPR, enhanced permeability and retention; Np, p-nitro phenol; PEG, poly(ethylene glycol); mPEG, methoxy–poly(ethylene glycol).

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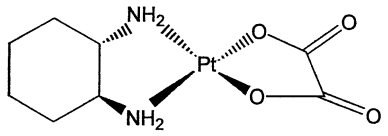

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Fig. 1. Oxalato 1,2-*trans*-L-diaminocyclohexane platinum (oxaliplatin®).

tothecin [9], Ara-C [10] and *cis*-platinum [11] are just a few examples of the molecules that have been conjugated to polymers such as *N*-2-(hydroxypropyl) methacrilamide (HPMA), dextranes, poly (glutamates) and poly (ethylene glycols). Poly(ethylene glycol) (PEG) has been extensively used as carrier of proteins and small molecules being a fully biocompatible molecule: it is non-toxic, non-immunogenic and non-antigenic, and it is able to solubilize and vehiculate large, anphipatic molecules into the bloodstream, while HPMA was successfully used for small drugs conjugation only. Important results were obtained in the conjugation of antracyclines (some derivatives are in advanced clinical evaluation), and, more recently, of *cis*-platinum. A general, yet discussed, characteristic of large molecular weight biocompatible polymers (and PEG among them) is to concentrate in the tumor site due to the increased extravasation at the highly fenestrated capillaries and the reduced lymphatic drainage (EPR effect) [6]. Moreover, the higher concentration of the polymer-bound drug induced at the tumor site would also minimise systemic toxicity.

The use of PEG as carrier of Pt derivatives with the aim of increasing water solubility, prolonging circulation half life, localising and concentrating the active drug at the tumor site and releasing a platinum species having antitumor activity were all investigated here together with a suitable chemistry for the platinate binding. The PEG chain residue used as carrier may be linear or branched, with one functionalizable hydroxyl group and a methoxy group at the end of the chain (mPEG), or two hydroxyl groups at both extremes of the chain (PEG). The branched PEG is designed in such a way that two mPEG chains are bound to the two amino groups of lysine, leaving the carboxylic group of the amino acid free for the conjugation (PEG-2). The PEG, mPEG, or PEG-2 carriers can span a variety of molecular weights, usually from about 5000 to about 40000, corresponding from about 100 to about 1000 –(CH_2–CH_2–O)– units.

The advantages of using this type of polymer for the derivatization of drugs, and platinate drugs in particular, is the variety of polymeric forms that may be devised and that are currently available on market. Further advantage of PEGs is its narrow polydispersivity, which makes it unique among other synthetic polymers. PEG is also known to be non-toxic, non-immunogenic, non-antigenic and provides conjugation products with more precise chemistry and stoichiometry with respect to other synthetic polymers. The polyfunctionality of other synthetic polymers (dextrans, polyamides, poly (glutamic acid) etc.) may create problems in the preparation of pharmaceutical compositions, because an exact composition is of paramount importance for a precise dosage of potentially toxic drugs. The analytical problems connected to the characterisation of the successfully used poly (hydroxyethyl methacrylates), recently employed for Pt conjugation, are also well known [11]. To develop new, water soluble and tumor-targeted forms of oxaliplatin, we devised a prodrug system consisting of a carrier polymer (PEG), a peptide linker and the drug ballast (Fig. 2).

In the general formula of the polymeric platinate conjugate, a special peptide spacer has been optionally linked between the PEG residue and a platinate complex. Said peptide linker has been chosen for its property of being stable towards proteases present in blood, but sensitive to those present in the intracellular lysosomal compartment. The sequences –Gly–Leu–Phe–Gly–, –Gly–Phe–Leu–Gly– and –Gly–Leu–Gly– are all well-known substrates for cathepsin and other enzymes of the lysosomal enzymatic pool [12]. This peptide spacer may provide an easier release of the platinate complex in cancer cells only, where the polymer is accumulated by endocytosis stimulated by the polymer moiety itself. Another research group successfully synthesized a HPMA platinum conjugate with a peptidic linker sensitive to cathepsin, and their results (although the final release of platinum in buffer solution is only the 14% of the total payload) in terms of antitumor activity are encouraging and comparable to ours.

The linkage between the peptide spacer arm and the malonic residue is an amide, or a carbamate linkage where PEGs are directly linked to the aminomalonate moiety. In the chosen platinum complex malonato–Pt–DACH, the platinum atom is surrounded by two bidentate ligands, i.e. the malonate and the DACH residues (Fig. 3). Given the presence of an amide moiety five atoms apart from Pt atom, the coordination of the resulting polymeric amidomalonato moiety to the platinum can be achieved in two different ways: a Pt(O,O) coordination via the carboxylic groups, or a Pt(N,O) coordination via one carboxylic group and the nitrogen of the amide moiety connecting the last aminoacid and the aminomalonate. From Pt-NMR experiments we could confirm that the initial Pt(O,O) chelate undergoes

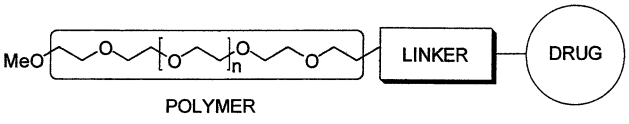
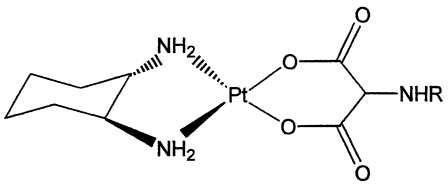
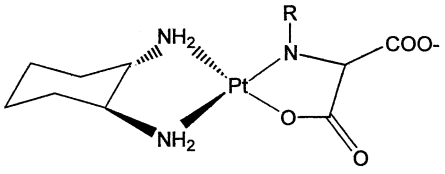


Fig. 2. Generic scheme of polymeric prodrug conjugate system.



DACH-Pt-malonate (Pt O-O coordination, 6-membered ring)



DACH-Pt-malonate (Pt O-N coordination, 5-membered ring)

Fig. 3. (O–O) and (O–N) coordination forms of DACH–Pt–malonate.

a partial rearrangement into the thermodynamically favoured Pt(N,O) chelate, in agreement with literature evidences [13,14].

Lacking literature data on the relative stability of such species inside the cellular compartment, we can not at present exclude that, although in aqueous solution the Pt(N,O) isomer is stable and do not release Pt, it could be degraded in particular conditions encountered in the cytoplasmic environment.

2. Chemistry

2.1. General procedure for compounds 2–8

The conjugation of small drug-like molecules to PEG is still a young technique, derived from the widely used bioconjugation of polymers to proteins and peptides, although the synthetic chemist armoury is rapidly being adapted to polymer–drug coupling. In the platinum complexes, the stability of the platinate is paramount, and great care must be taken to avoid reaction conditions that may cause the complex to react with nucleophiles, or degrade (e.g. water hydrolysis). For this reason we decided to synthesize the polymer–aminomalonate ester, or polymer–peptide–amidomalonate ester first, and to hydrolyse them to afford the free acids for the final coupling to Pt–DACH. A synthetic route involving the reaction of the polymer to the preformed Pt–DACH–aminomalonate intermediate was in fact found inconvenient, as the Pt complex easily dissociate under the synthetic conditions reducing the final yield.

The polymeric platinate conjugates **2**–**8** (Fig. 4) have been synthesized and characterized.

mPEG active esters **9**, **10** and **11**, readily available from the corresponding commercial carboxylic acids (Scheme 1), were directly coupled with diethyl aminomalonate to afford the intermediate PEG–amidomalonate esters **12**–**14**. mPEG–OH activated as *p*-nitrophenyl carbonate (**15**, Np = *p*-nitrophenol) and $mPEG_2Lys$–OSu (**16**, OSu = succinimidyl ester) were coupled with the suitable aminoacid or peptide to afford the PEG–peptide intermediates **17**–**20** (Scheme 2). The carboxylic acid functions were then activated as *N*-hydroxysuccinimidyl esters (**21**–**24**) with DCCI and H–OSu, and coupled with diethyl aminomalonate to afford the intermediate PEG–amidomalonate esters **25**–**28**.

The amidomalonates **12**–**14** and **25**–**28** were finally hydrolysed by means of barium hydroxide and provided the PEG–malonic barium salts.

The Pt complex *cis*-[DACHPtCl_2] was converted into [DACHPtSO_4] by treatment with equimolar amount of Ag_2SO_4. The reaction of PEG–malonic barium salts and [DACHPtSO_4] was driven by precipitation of the insoluble salt $BaSO_4$. For all compounds, filtration of the barium sulphate, freeze-drying and gel-filtration column chromatography afforded the desired products, that were then characterized.

2.2. Purification and analysis of PEG conjugates

Gel-filtration chromatography is the elective purification technique to separate high molecular weight polymers from low mass compounds. Usually these separations are carried out in aqueous media, but in our case this was impracticable due to the platinum complex reactivity. To overcome this limitation we therefore used a Pharmacia LH-20 gel-filtration resin suitable for organic solvents, and DMF as mobile phase. Fractions were collected and checked for PEG content using the iodine assay [15], while platinum content was evaluated via atomic absorption. Superimposition of the iodine assay and atomic absorption diagrams indicated the fractions containing the PEGylated platinates. All the PEG conjugates were tested for Pt loading either by atomic absorption measurements or by ICP-MS. The Pt content was expressed as w/w percentage [(mol. wt. Pt–DACH/mol.wt. PEG–Pt complex) × 100]. The final products were also analysed by MALDI-TOF mass spectrometry. Despite the typical polydispersivity of poly(ethylene glycol), which gives broad peaks centred at the average molecular weight, all results are in agreement with the theoretical values. ^{1}H-NMR measurements could only be performed on compounds **2** and **4**, confirming the expected structures. For the other products, the complexity of the peptide linker and the broad signal from the PEG chain did not allow a clear attribution of the aliphatic signals.

For an exact attribution of the ^{1}H signals, the model compound acetylaminomalonato–Pt–DACH was also synthesized in our laboratories following literature procedures [16]. Summary of analytical data for the products are given in Table 1.

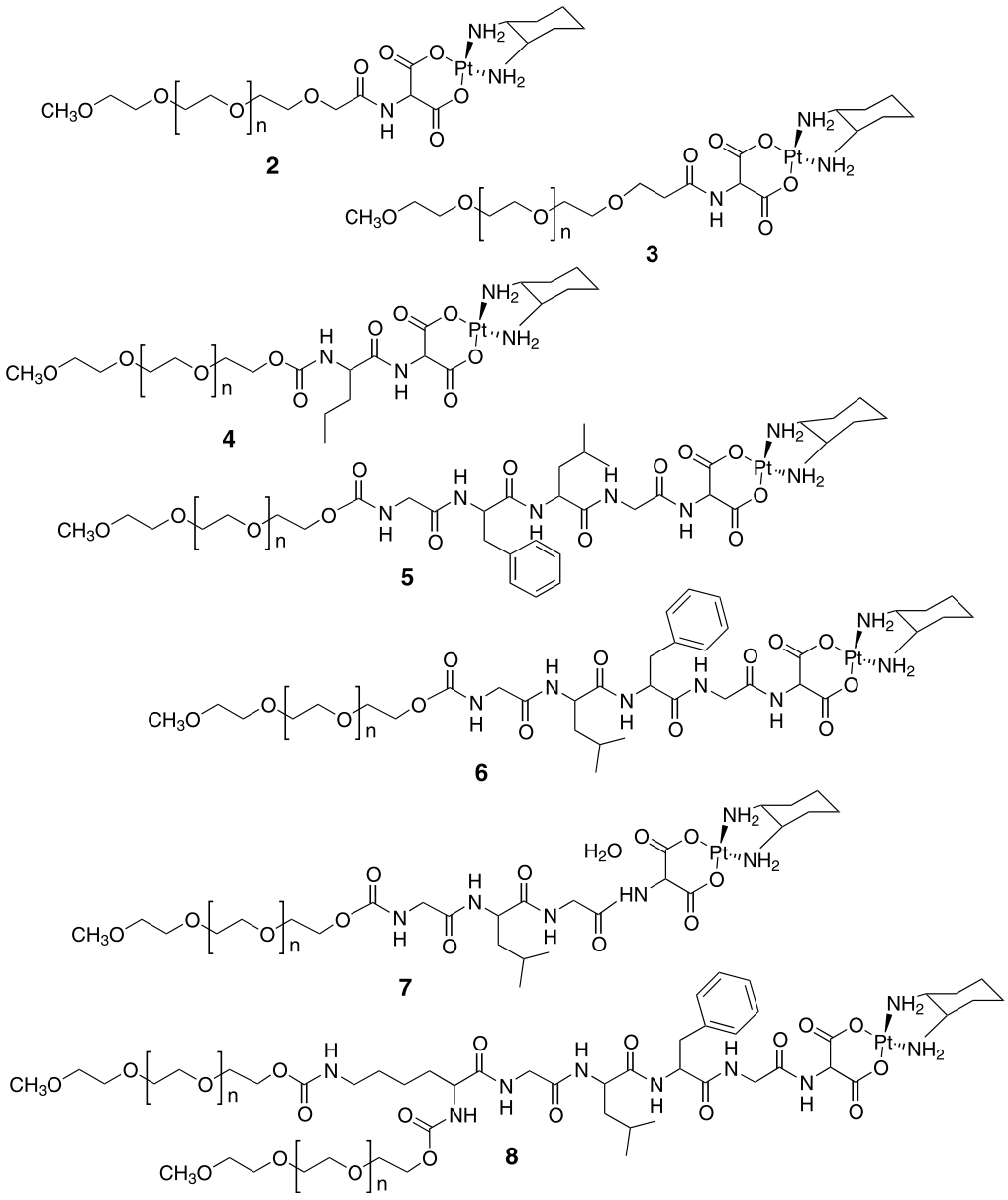


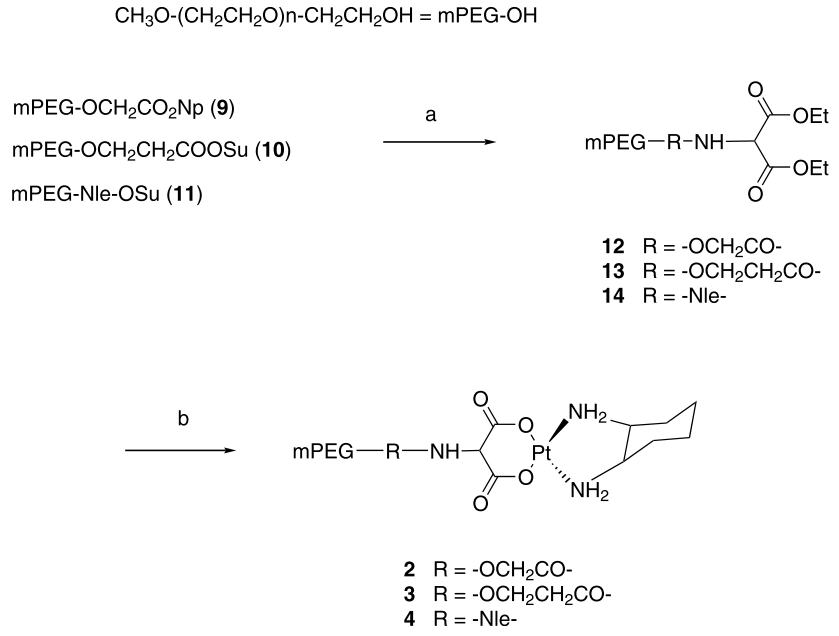
Fig. 4. Structures of synthesized PEG–Pt derivatives.

2.3. Release studies

The premature release of platinum from the polymer after submination is an undesired event, which can lead to toxic effects due to the presence of the free platinum complex in the bloodstream. The conjugates should ideally release the platinum payload only inside the cell, due to the lysosomal degradation of the peptide linker. So we synthesized compounds **2**, **3** and **4**, devoid of the cited linker, to test the release kinetic of Pt–DACH from the polymer in conditions that mimic the extracellular environment. The solution of **4** in PBS at pH 7.4 was filtered against an ultrafiltration membrane (Amicon YM1, cut-off 1000) and the liquid was collected and tested for Pt content (atomic absorption). The filtration was repeated at given intervals adding the same PBS amount in the filtration cell. The results are reported in Fig. 5.

3. Pharmacology

The antitumor activity of these new PEG–platinum conjugates was tested treating L1210-implanted mice with compound **4** following the protocol reported in the experimental section. Control mice (untreated) and Pt–DACH treated mice were also observed. Cell implant was followed by a first drug administration at 24 h. Three more administrations were given at alternate

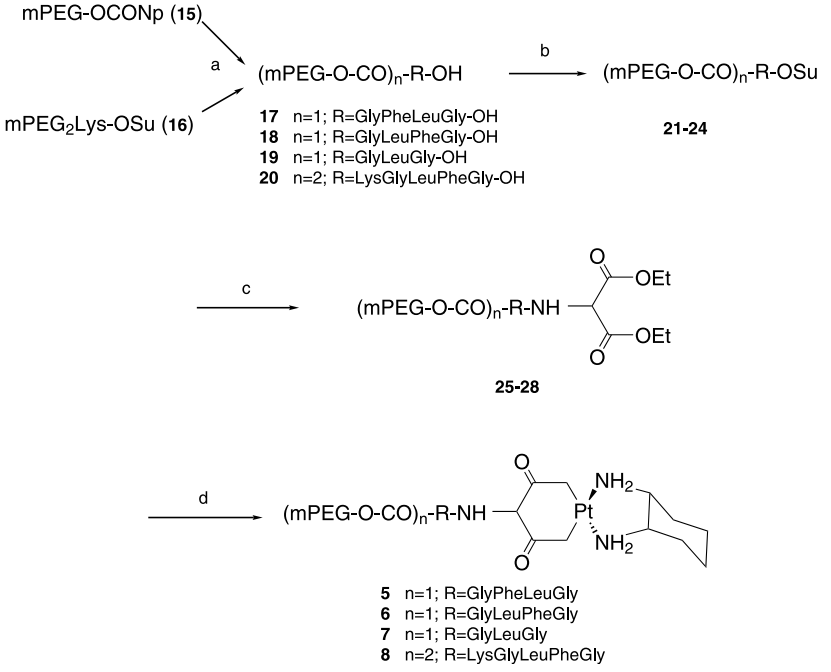


Scheme 1. Reagents: (a) diethyl aminomalonate, anhydrous DMF, Et_3N, r.t., 18 h; (b) 1. $Ba(OH)_2$ $8H_2O$, water, r.t., 8 days; 2. *cis*[$DACHPtCl_2$], Ag_2SO_4, water, r.t., 24 h, N_2 atm., in dark.

days, for a total of four doses. The results are summarised in Table 2.

4. Results and discussion

Eight original poly(ethylene glycol)–Pt conjugates were synthesized where the Pt–DACH complex was linked to the aminomalonate moiety. PEG was linked to aminomalonate either directly or through aminoacid spacers. This linkage however could not be carried out directly on the preformed aminomalonato Pt–DACH complex, since the conditions for amide formation were found incompatible with Pt–DACH integrity. Other authors [17] found in fact, after conjugation of the preformed malonate–Pt complex to a polymeric mole-



Scheme 2. Reagents: (a) peptide, Et_3N, 1:1 acetonitrile–water, r.t., 12 h; (b) CH_2Cl_2 anhydrous, *N*-hydroxy succinimide, DCC, 12 h, r.t.; (c) diethyl aminomalonate, anhydrous DMF, Et_3N, r.t., 18 h; (d) 1. $Ba(OH)_2$ $8H_2O$, water, r.t., 8 days; 2. *cis*[$DACHPtCl_2$], Ag_2SO_4, water, r.t., 24 h, N_2 atm., in dark.

Table 1
Analytical data for PEG–Pt conjugates

Compound	Yield [a] (%)	Mol. weight (theor.) [b]	Pt content % (theor.)
2	45	5420 (5466)	2.9 (3)
3	50	5500 (5480)	2.9 (3)
4	48	5500 (5551)	2.9 (3)
5	50	10 800 (10826)	1.7 (1.8)
6	54	10 800 (10826)	1.7 (1.8)
7	55	10 600 (10697)	1.6 (1.8)
8	48	11 000 (10982)	1.7 (1.8)

[a] Yield of Pt–DACH coupling step.
[b] Molecular weight calculated from MALDI MS; values correspond to center of Gaussian distribution.

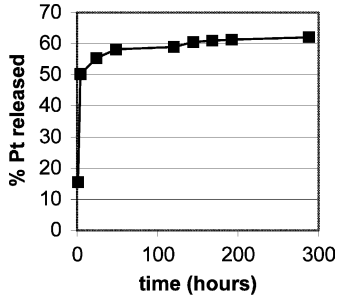


Fig. 5. Pt release kinetic at pH 7.4 (PBS buffer) for compound **4**, measured by Pt atomic absorption.

Table 2
Antitumor activity of PEG–Nle–aminomalonate–Pt–DACH (**4**)

Compound	Survival time (days ± SD)	T/C	LTS [a]
Saline solution	12.4 ± 0.9	1.00	0/6
Pt–DACH	15.6 ± 0.8	1.25	1/6
4	18.0 ± 2	1.45	4/6

[a] LTS, long-term survival after 30 days.

cule (HPMA), that only a fraction (14%) of the total platinum content was released in PBS saline buffer conditions, probably due to the formation of the stable species Pt(N,O). For this reason the following synthetic strategy was devised: assembling of the PEG-linker, followed by aminomalonate ethyl ester conjugation, conversion of the ester to the barium salt and final coordination with Pt–DACH complex by the barium sulphate precipitation strategy.

PEG was linked to the aminomalonate–Pt–DACH either directly (**2**, **3**) or by means of one aminoacid only (norleucine, **4**) or peptide spacers known to be specifically hydrolysed by the lysosomal enzymatic pool present inside the cells (**5**–**8**). The tetrapeptides Gly–Phe–Leu–Gly (**5**) and Gly–Leu–Phe–Gly (**6**, **8**), with the second and third aminoacid position inverted, were used, as well as the tripeptide Gly–Leu–Gly (**7**). The branched poly(ethylene glycol) PEG_2LysOH was employed for compound **8**. In every derivative PEG was coupled to the aminoacids through a carbamate moiety. Gel filtration purification was employed to remove any trace of unreacted Pt–DACH from the polymer conjugate, but to avoid decomposition of the Pt complex organic solvent was used in the chromatographic step.

The compounds were characterized for the PEG and platinum content by iodine reaction and atomic absorption or ICPS-MS, respectively, while NMR could be employed only in the simpler products **2**, **3** and **4**. For these products the spectrum was interpreted in comparison with that of acetyl aminomalonate Pt–DACH that was synthesized as close reference structure.

When we tested the stability of our PEG–Pt conjugates in aqueous physiological conditions, we found that the active drug Pt–DACH was released with an initial fast kinetic even from conjugates lacking of the peptide spacer. In the model compound **4**, as an example, more than 50% of Pt–DACH was released within 2 h, while more Pt (up to a 60% total release) was depleted with a much slower kinetic (see Fig. 5). We can therefore consider that, since a construct of 5000–10 000 Da the circulation $t_{1/2}$ is between 20 and 60 min [18], the observed Pt release from the conjugate ($t_{1/2} > 60$ min) ensures that, while the fraction of polymer captated by the tumor (via EPR effect) concentrates and releases the active drug, the circulating fraction is eliminated before the payload can be released, reducing systemic toxicity.

The antitumor activity of the potential prodrug **4** was evaluated in mice implanted with L 1210 tumor cells. Although this compound lacked the peptidic spacer involved in the enzymatic intracellular degradation, the conjugate was active, probably due to a different Pt–DACH release mechanism involving aquation of the conjugate. The observed activity, higher than that of Pt–DACH alone, and matching that of similar compounds (see Ref. [17]), was interpreted on the basis of the accumulation of the conjugate at the tumor site and the displacement of platinum from the carboxylate ligands by physiological nucleophiles, proving that the presence of an intracellular enzymatic release is not mandatory for this class of Pt derivatives.

More intriguing is the presence of a two-step kinetic for the Pt–DACH release in water (see Fig. 4). We suggest that this behaviour is related to the presence of the two platinate isomers, Pt(O,O) and Pt(O,N), as revealed by ^{195}Pt-NMR (Fig. 6): the latter, thermodynamically stabler complex is released much slowlier than the former, which is fully released within few hours.

These results confirm the literature data [13,14] regarding the stability of the Pt(O,N) species in aqueous environments, (although to date we could not find in vivo experiments reported for such species) and prompted us to devise new compounds, currently under evaluation for antitumor activity, where such Pt(O,N) isomerisation is prevented.

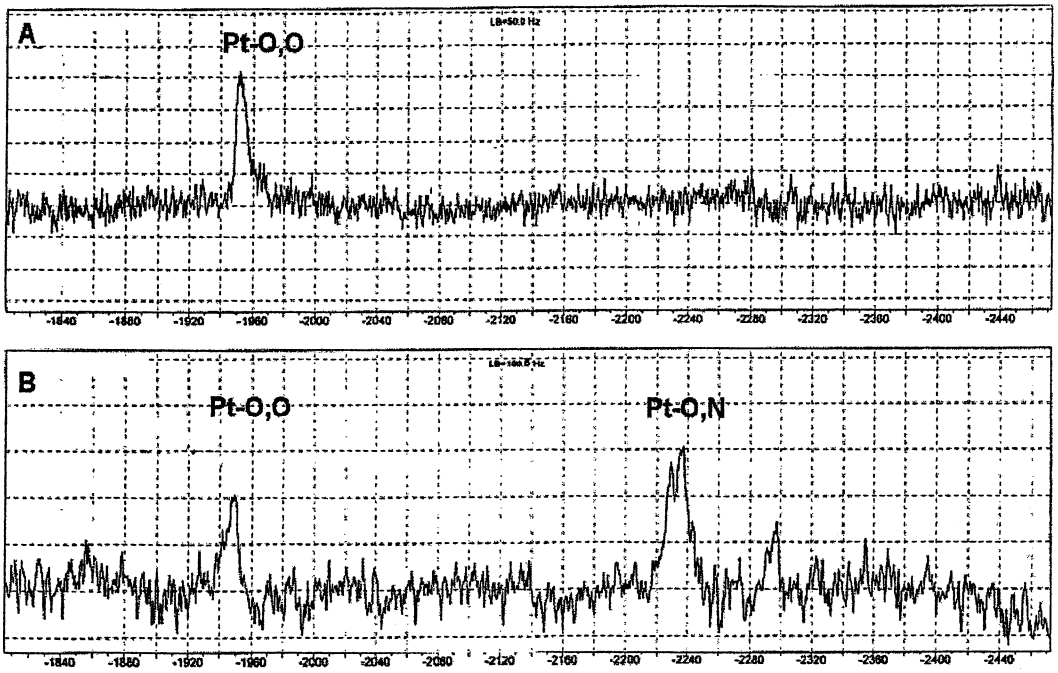


Fig. 6. ^{195}Pt-NMR experiments on: (A) acetamidomalonate–Pt–DACH; and (B) PEG–Nle–aminomalonate–Pt–DACH (**4**). The signal at −1950 ppm is related to the Pt(O,O) conjugate species (see Fig. 3), the signal at −2240 is related to the Pt(O,N) species (cf. Ref. [13]). The conversion of the Pt(O,O) species into the Pt(O,N) for compound **4** is evident. Longer acquisition times (3 days) result in loss of the Pt(O,O) signal and total conversion to the Pt(O,N) form (data not shown).

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods

Solvents were purified according to the procedure reported in W.L.F. Armarego, D.D. Perrin 'Purification of Laboratory Chemicals', IV ed. (1998), Ed. BH, Oxford UK.

^{1}H-NMR spectra were recordered by Bruker AC-200 Spectrometer. ^{195}Pt-NMR spectra were recordered by Bruker Avance DRX500, using Na_2PtCl_6 in DMSO-d_6 as the external reference. Pt content has been used for most final products, in combination with HPLC analysis, to determine the compound purity. Where possible, ^{1}H- and ^{195}Pt-NMR spectra were registered and evaluated for qualitative purposes only, considering the low sensitivity due to PEG signals superimpositions and high viscosity. The Pt content was measured by Atomic Absorption using flame Perkin–Elmer 380 instrument, calibrated with aqueous solutions of Na_2PtCl_4.

UV determinations were obtained by UV–Vis Perkin–Elmer lambda 5 spectrophotometer.

PEG content was evaluated by Iodine assay according to literature [15]. The gel filtration chromatography was carried out by a 2.5 × 55 cm column, loaded with LH-20 gel filtration resin, with a Pharmacia Biotech pump P-1.

Ft-IR spectra were recordered by an Avatar 320 E.S.P. spectrometer with Nicolet's EZ OMNIC software.

5.1.2. Synthesis of [$mPEG_{5000}$–CO–NH–$CH(COO)_2$]Pt–DACH (**2**)

*5.1.2.1. mPEG–malonate diethyl ester (**12**).* Diethylaminomalonate hydrochloride (30 mg, 0.14 mmol) was dissolved in 10 ml of anhydrous DMF; 500 mg (0.1 mmol) of mPEG-Np (**9**, *p*-nitrophenyl ester of methoxypoly ethylene glycol acid), M_W 5000 Da, were added to the solution under magnetic stirring. After a few minutes 40 μl (0.3 mmol) of Et_3N were added and the solution was stirred for 18 h. After concentration under vacuum, the compound was precipitated in 400 ml of diethyl ether. Yield: 0.47 g (94%)

^{1}H-NMR ($CDCl_3$): δ = 1.27 (t, 6H, –OCH_2CH_3); 3.35 (s, 3H, PEG –OCH_3); 3.61 (m, PEG –CH_2–CH_2O– signals); 4.25 (q, 4H, –OCH_2CH_3); 5.12 (d, 1H, –CH–); 7.37 (d, 1H, NH).

*5.1.2.2. [$mPEG_{5000}$–CO–NH–$CH(COO)_2$]Pt–DACH (**2**).* $Ba(OH)_2 \cdot 8H_2O$ (18.6 mg, 0.06 mmol) was added to a solution of 307 mg (0.06 mmol) of mPEG–CO–NH–$CH(COOEt)_2$ (**12**) in 10 ml of distilled water. The solution was stirred for 7 days (or the shortest time necessary to obtain a pH 7 solution).

A suspension of *cis*-[DACHPtCl_2] (68 mg; 0.18 mmol) in 20 ml of distilled water, containing an equivalent amount of Ag_2SO_4 (56 mg; 0.18 mmol) was stirred for about 24 h at room temperature (r.t.), under N_2 in the dark. The AgCl precipitated was separated by filtration

and the filtrate was transferred into the solution of the previously prepared barium salt of mPEG aminomalonate. The reaction mixture was stirred for about 18 h and $BaSO_4$ was removed by filtration.

The reaction product was freeze-dried and the purification was carried out by gel-filtration on a Sephadex LH-20 column (DMF, elution rate 0.5 ml min^{-1}; 5 ml fractions collected). The fractions collected were tested by I_2 assay for the PEG elution and Atomic Absorption for Pt. The peak fractions containing the complex were collected, concentrated under vacuum to about 2 ml and precipitated with 400 ml of ether, to afford 147 mg (45% yield) of a white, crystalline solid.

% Pt found (atomic absorption): 2.9 (calc. % Pt = 3%).

^{1}H-NMR ($CDCl_3$): ppm (δ) = 0.5–2.6 (m, 10H, DACH signals); 3.35 (s, 3H, PEG $-OCH_3$); 3.61 (m, PEG $-CH_2CH_2O-$ signals); 5.82 (bd, 1H, –CH–); 6.87 (bs, 1H, NH).

5.1.3. Synthesis of [$mPEG_{5000}$–CH_2–CH_2–CO–NH–CH(COO)$_2$]Pt–DACH (**3**)

*5.1.3.1. $mPEG_{5000}$ malonate diethyl ester (**13**).* Diethylaminomalonate hydrochloride (61 mg, 0.3 mmol) was dissolved in 10 ml of anhydrous DMF; 1 g (0.2 mmol) mPEG–OCH_2CH_2OSu (**10**, *N*-hydroxysuccinimidyl ester of methoxypolyethylene glycol propionic acid, M_W = 5266 Da) was added to the solution under magnetic stirring. After a few minutes 80 μl (0.6 mmol) of Et_3N were added and the solution was stirred for 18 h. After concentration under vacuum the compound was precipitated in 800 ml of ether. Yield: 0.98 g (99%)

^{1}H-NMR ($CDCl_3$): δ = 1.26 (t, 6H, $-OCH_2CH_3$); 3.36 (s, 3H, PEG $-OCH_3$); 3.60 (m, PEG $-CH_2-CH_2O-$ signals); 4.24 (q, 4H, $-OCH_2CH_3$); 5.14 (d, 1H, –CH–); 7.38 (d, 1H, NH).

*5.1.3.2. [$mPEG_{5000}$–CH_2–CH_2–CO–NH–CH(COO)$_2$]Pt–DACH (**3**).* One equivalent of $Ba(OH)_2 \cdot 8H_2O$ (30 mg; 0.09 mmol) was added to a solution of 500 mg (0.09 mmol) of **13** in 15 ml of distilled water. The solution was stirred for 8 days (time necessary to obtain a pH 7 solution). A suspension of *cis*-[$DACHPtCl_2$] (107 mg, 0.28 mmol) in 20 ml of distilled water containing an equivalent amount of Ag_2SO_4 (88 mg, 0.28 mmol) was stirred for about 24 h at r.t., under N_2 in the dark. The AgCl precipitated was separated by filtration and the filtrate was transferred into the solution of mPEG aminomalonate barium salt. The reaction product was freeze-dried and the purification was carried out by gel-filtration on a Sephadex LH-20 column. (elution rate 0.5 ml min^{-1}; 5 ml fractions collected). The fractions collected were tested by I_2 assay for the PEG elution and Atomic Absorption for Pt. The peak fractions containing the complex were collected, concentrated under vacuum to about 2 ml and precipitated with 400 ml of ether, to afford 250 mg (50% yield) of a white, crystalline solid. % Pt found (atomic absorption): 2.9 (calc. % Pt = 3%).

^{1}H-NMR ($CDCl_3$): ppm (δ) = 0.7–2.5 (m, 10H, DACH signals); 3.34 (s, 3H, PEG $-OCH_3$); 3.63 (m, PEG $-CH_2CH_2O-$ signals); 5.79 (bd, 1H, –CH–); 6.88 (bs, 1H, NH).

5.1.4. Synthesis of [$mPEG_{5000}$–Nle–NH–CH(COO)$_2$]Pt–DACH (**4**)

*5.1.4.1. $mPEG_{5000}$–Nle–malonate diethyl ester (**14**).* $H_2NCH(COOEt)_2 \cdot HCl$ (30 mg, 0.14 mmol) was dissolved in 6 ml of anhydrous DMF. Five hundred milligrams (0.095 mmol) of mPEG–Nle–OSu (**11**, M_W = 5256 Da) was added to the stirring solution. After a few minutes 40 μl (0.28 mmol) of Et_3N were added and the solution was stirred at r.t. for 18 h. After concentration under vacuum the compound was precipitated in 400 ml of ether. Yield: 0.4 g (80%).

^{1}H-NMR ($CDCl_3$): δ = 1.12 (t, 3H, Nle Hδ); 1.26 (t, 6H, $-OCH_2CH_3$); 1.5–1.6 (m, 4H, Nle Hβ & γ); 3.36 (s, 3H, PEG $-OCH_3$); 3.60 (m, PEG $-CH_2-CH_2O-$ signals); 4.24 (q, 4H, $-OCH_2CH_3$); 4.35 (m, 1H, Nle Hα); 5.14 (d, 1H, –CH–); 7.38 (d, 1H, NH).

*5.1.4.2. [$mPEG_{5000}$–Nle–NH–CH(COO)$_2$]Pt–DACH (**4**).* $Ba(OH)_2 \cdot 8H_2O$ (25 mg, 0.08 mmol) was added to a solution of mPEG–Nle–NH–$CH(COOEt)_2$ (**14**) (420 mg, 0.084 mmol) in 20 ml of distilled water. The solution was stirred for 7 days. The final pH of solution was checked for neutrality. A suspension of *cis*-[$DACHPtCl_2$] (91 mg, 0.24 mmol) in 15 ml of distilled water, containing an equivalent amount of Ag_2SO_4 (75 mg, 0.24 mmol) was stirred for about 24 h at r.t., under N_2 in the dark. The AgCl precipitated was separated by filtration and the filtrate was dropped into the solution of mPEG aminomalonate barium salt. The reaction mixture was stirred for about 18 h and $BaSO_4$ was removed by filtration. The reaction product was freeze-dried and the purification was carried out by gel filtration on a Sephadex LH-20 column according to the previously described procedure.

Yield: 226 mg (48%); % Pt found (by ICP-MS): 2.9 (calc. % Pt = 3%).

^{1}H-NMR ($CDCl_3$): δ = 0.7–2.5 (m, 17H, DACH signals + Nle Hδ, β and γ); 3.36 (s, 3H, PEG $-OCH_3$); 3.60 (m, PEG $-CH_2-CH_2O-$ signals); 4.35 (m, 1H, Nle Hα); 5.14 (d, 1H, –CH–); 7.38 (d, 1H, NH).

^{195}Pt-NMR (DMSO-d_6; ext. ref. Na_2PtCl_6): δ = −1949.24 ppm (bs, O,O co-ordination); δ = −2235.59 ppm (bs, N,O co-ordination)

*5.1.5. Synthesis of [mPEG$_{10000}$–Gly–Phe–Leu–Gly–NH–CH(COO)$_2$]Pt–DACH (**5**)*

*5.1.5.1. mPEG$_{10000}$–GlyPheLeuGly–OH (**17**).* The peptide H–GlyPheLeuGly–OH (five eq., 163 mg) was dissolved into 6 ml of a 1:1 H_2O–CH_3CN mixture. The solution was adjusted to pH 9 with Et_3N, then 770 mg (one eq.) of mPEG$_{10000}$–pNF (**15**, mPEG–OH activated as *p*-nitrophenyl chloroformate) were added portionwise.

The reaction mixture was stirred at r.t. for 12 h, then the pH was adjusted to 3 with 0.1 N HCl and the solution was concentrated at reduced pressure. The aqueous solution was extracted with diethyl ether (5 × 90 ml) and $CHCl_3$ (5 × 90 ml). The organic fractions were combined, dried with Na_2SO_4 and concentrated under reduced pressure to a volume of about 2 ml. This solution was added dropwise to 400 ml of diethyl ether. The precipitate which formed was filtered and dried under vacuum to afford 650 mg (85%) of a white crystalline solid.

*5.1.5.2. mPEG$_{10000}$–GlyPheLeuGly–OSu (**21**).* Compound **17** (564 mg) was dissolved in 6 ml of anhydrous dichloromethane. 19 mg (3 eq.) of *N*-hydroxy succinimide were added to the solution and the mixture was cooled in an ice bath. DCCI (34 mg, 3 eq.) were added and the reaction mixture was stirred at r.t. overnight.

At the end the mixture was concentrated in vacuo to 2–3 ml, filtered to remove DCU and poured dropwise into 400 ml of diethyl ether. The precipitate was collected and dried, affording 524 mg (92%) of the title compound which was used without further purification.

*5.1.5.3. mPEG$_{10000}$–GlyPheLeuGly–malonate diethyl ester ligand (**25**).* $H_2NCH(COOEt)_2 \cdot HCl$ (11.8 mg, 0.055 mmol) was dissolved in anhydrous DMF. 390 mg (0.037 mmol) of mPEG–Gly–Phe–Leu–Gly–OSu (**21**, $M_W = 10\,530$) were added to the solution under magnetic stirring. After a few minutes 8 μl (0.11 mmol) of Et_3N was added slowly and the reaction mixture was left stirring at r.t. for 18 h. After concentration under vacuum the compound was precipitated in 400 ml of ether yielding a white crystalline solid (341 mg, 87%).

*5.1.5.4. [mPEG$_{10000}$–Gly–Phe–Leu–Gly–NH–CH(COO)$_2$]–Pt–DACH (**5**).* $Ba(OH)_2 \cdot 8H_2O$ (10 mg, 0.03 mmol) was added to a solution of 340 mg (0.03 mmol) of mPEG–Gly–Phe–Leu–Gly–NH–$CH(COOEt)_2$ in 10 ml of distilled water. The solution was stirred for 8 days (time necessary to obtain a neutral solution).

A suspension of 34 mg (0.09 mmol) of *cis*-[DACHPtCl$_2$] in 20 ml of distilled water, containing an equivalent amount of Ag_2SO_4 (28 mg, 0.09 mmol) was stirred for about 24 h at r.t., under N_2 in the dark. The AgCl was separated by filtration and the filtrate was transferred dropwise into the solution of previously prepared barium salt of mPEG aminomalonate. The reaction mixture was stirred for about 18 h and $BaSO_4$ was removed by filtration. The reaction product was freeze-dried and the purification was carried out by gel filtration on a Sephadex LH-20 column according to the previously described procedure. 175 mg (50%) of white crystalline compound were obtained.

% Pt found (by atomic absorption): 1.7 (calc. % Pt = 1.8).

*5.1.6. Synthesis of [mPEG$_{10000}$–Gly–Leu–Phe–Gly–NH–CH(COO)$_2$]–Pt–DACH (**6**)*

*5.1.6.1. mPEG$_{10000}$–Gly–Leu–Phe–Gly–malonate diethyl ester ligand (**26**).* mPEG–Gly–Leu–Phe–Gly–OSu (**22**) was prepared according to the procedure described for **21**. 30 mg (0.14 mmol) of $H_2NCH(COOEt)_2 \cdot HCl$ were dissolved in 10 ml of anhydrous DMF, and 1 g (0.09 mmol) of mPEG–Gly–Leu–Phe–Gly–OSu (**22**, $M_W = 10\,530$) was added to the stirring solution. After few minutes 37 μl (0.27 mmol) of Et_3N was added and the solution stirred for 18 h. After concentration under vacuum the compound was precipitated in 400 ml of ether yielding a white crystalline solid (812 mg, 80%).

*5.1.6.2. [mPEG$_{10000}$–Gly–Leu–Phe–Gly–NH–CH(COO)$_2$]–Pt–DACH (**6**).* $Ba(OH)_2 \cdot 8H_2O$ (15 mg, 0.047 mmol) was added to a solution of mPEG–Gly–Leu–Phe–Gly–NH–$CH(COOEt)_2$ (**26**) (500 mg, 0.047 mmol) in 25 ml of distilled water. The solution was stirred for 8 days (time necessary to obtain a neutral solution).

A suspension of 53 mg (0.14 mmol) of *cis*-[DACHPtCl$_2$] in 20 ml of distilled water, containing an equivalent amount of Ag_2SO_4 (44 mg, 0.14 mmol) was stirred for about 24 h at r.t., under N_2 in the dark. The AgCl was separated by filtration, and the filtrate was transferred into the solution of previously prepared barium salt of mPEG aminomalonate. The reaction mixture was stirred for about 18 h and $BaSO_4$ was removed by filtration. The reaction product was freeze-dried and the purification was carried out by gel filtration on a Sephadex LH-20 column according to the previously described procedure. 273 mg (54%) of white crystalline compound were obtained.

% Pt found (by atomic absorption): 1.7 (calc. % Pt = 1.8).

*5.1.7. Synthesis of [mPEG$_{10000}$–Gly–Leu–Gly–NH–CH(COO)$_2$]–Pt–DACH (**7**)*

*5.1.7.1. mPEG$_{10000}$–Gly–Leu–Gly–malonate diethyl ester ligand (**27**).* mPEG–Gly–Leu–Gly–OSu (**23**)

was prepared according to the procedure described for mPEG–Gly–Phe–Leu–Gly–OSu (**21**). 700 mg (0.07 mmol) of **23** (M_W about 10 450 Da) were added to a solution of 22 mg (0.1 mmol) of $H_2NCH(COOEt)_2 \cdot HCl$ in 10 ml of anhydrous DMF. After a few minutes 28 μl (0.2 mmol) of Et_3N were added, the mixture of reaction was stirred overnight. After partial concentration the residue was dropwise added to 700 ml of ether. The white precipitate was filtered and dried in vacuo (686 mg, 93%).

*5.1.7.2. [$mPEG_{10000}$–Gly–Leu–Gly–NH–CH(COO)$_2$]–Pt–DACH (**7**).* mPEG–Gly–Leu–Gly–NH–CH(COOEt)$_2$ (**27**, 302 mg, 0.03 mmol) was dissolved in 10 ml of distilled water. 9.5 mg (0.03 mmol) of $Ba(OH)_2 \cdot 8H_2O$ were added to this solution. The reaction mixture was stirred for 7 days (the time necessary to obtain a neutral solution).

Ag_2SO_4 (28 mg, 0.09 mmol) was added to a suspension of 34 mg (0.09 mmol) of *cis*-[DACHPtCl$_2$] in 10 ml of distilled water. The mixture was stirred for about 24 h at r.t., under N_2 in the dark. The AgCl was separated by filtration and the filtrate dropped into a solution of the previously prepared barium salt of PEG aminomalonate. The reaction mixture was stirred for 18 h and the $BaSO_4$ was removed by filtration with a Millipore filter (0.22 μm) and finally freeze-dried.

The purification of the crude product was carried out by gel filtration on a Sephadex LH-20 column according to the previously described procedure. 180 mg (55%) of white crystalline compound were obtained. % Pt found (by atomic absorption): 1.6 (calc. % Pt = 1.8)

*5.1.8. Synthesis of [$mPEG_2$Lys–Gly–Leu–Phe–Gly–NH–CH(COO)$_2$]– Pt–DACH (**8**)*

*5.1.8.1. $mPEG_2$Lys–Gly–Leu–Phe–Gly–malonate diethyl ester ligand (**28**).* mPEG$_2$–Gly–Leu–Phe–Gly–OSu (**24**) was prepared according to the procedure described for mPEG–Gly–Phe–Leu–Gly–OSu (**21**), starting from PEG_2LysOSu **16**. To a solution of 4.3 mg (0.02 mmol) of $H_2NCH(COOEt)_2 \cdot HCl$ in 7 ml of anhydrous DMF was added 144 mg (0.014 mmol) of **24** (M_W ca. 10 280 Da). After a few minutes 6 μl (0.04 mmol) of Et_3N were added, the mixture of reaction was stirred overnight, partially concentrated and added dropwise to 300 ml of ether. The white solid which was obtained was filtered and dried on vacuum. 135 mg (92%) of title compound were obtained.

*5.1.8.2. [$mPEG_2$–Gly–Leu–Phe–Gly–NH–CH(COO)$_2$]–Pt–DACH (**8**).* mPEG$_2$Lys–Gly–Leu–Phe–Gly–NH–CH(COOEt)$_2$ (**28**, 97 mg, 0.009 mmol) was dissolved in 10 ml of distilled water. At the solution were added 2.8 mg (0.009 mmol) of $Ba(OH)_2 \cdot 8H_2O$. The reaction mixture was stirred for 7 days (the time needed to obtain a neutral solution).

Ag_2SO_4 (8.4 mg, 0.0027 mmol) was added to a suspension of 10.4 mg (0.027 mmol) of *cis*-[DACHPtCl$_2$] in 10 ml of distilled water. The mixture was stirred for about 24 h at r.t., under N_2 in the dark. AgCl was separated by filtration and the filtrate added to the solution of PEG aminomalonate barium salt. The reaction mixture was stirred for 18 h and the $BaSO_4$ was removed by filtration with a Millipore filter (0.22 μm) and freeze-dried.

The purification of the crude product was carried out by gel filtration on a Sephadex LH-20 column according to the previously described procedure. 50 mg (48%) of white crystalline compound were obtained. % Pt found (by atomic absorption): 1.7 (calc. % Pt = 2).

5.2. Antitumor activity

All procedures involving animals and their care were conducted in conformity with the institutional guidelines, that are in compliance with the National and European Economic Community Council Directives.

L1210 leukemia was maintained in DBA/2 mice by weekly i.p. transplantation of 2×10^5 cells. The experiments were performed by implanting B_6DF1 mice (all of same sex, weighting 20–25 g) with 2×10^4 L1210 cells. Drug treatment began on day 1 after transplant and consisted of four i.p. injections every other day; the dose was 4.4 mg kg^{-1} as Pt–DACH or as PEG–malonato–PtDACH equivalent. Experiments were terminated on day 30 or when no mice remained alive. All drug tested and control saline groups consisted of six mice. The mice were observed daily and the results were expressed as the mean survival time and T/C values defined as the ratio between the mean survival times of the treated (T) and untreated control animals (C) at the 30th day.

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