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Novel Bis-platinum Complexes Endowed with an Improved Pharmacological Profile

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Abstract: Multinuclear platinum complexes are characterized by a peculiar DNA binding mode and higher cytotoxic potency than the mononuclear complexes, and efficacy against a wide range of preclinical tumor models. To reduce the high irreversible plasma protein binding and improve the chemical and metabolic drug stability, novel bis-platinum complexes were designed starting from the parent compound CT-3610. The novel second-generation bis-platinum complexes utilize alkylcarboxylate as leaving groups to improve their pharmacokinetic and pharmacodynamic profiles, thus overcoming the limitations of the previously developed multinuclear compounds. The selected compounds [CT-47518 and CT-47463, respectively (bis-capronate) platinum and (bis-butyrate) platinum], have similar *in vitro* degradation kinetics in human and murine plasma and, above all, an increased stability when compared to CT-3610, particularly in human plasma. In addition, both compounds exhibited a marked cytotoxic potency as compared with cisplatin and oxaliplatin. Interestingly, they were capable of overcoming resistance mediated by DNA mismatch repair defects in different cellular models. The complexes showed marked antitumor efficacy in Pt-refractory tumor xenografts, with remarkable activity in terms of tumor growth inhibition and tumor growth delay. The improved stability profile in human plasma compared to early bis- and triplatinum complexes together with the marked activity in cellular systems as well as in *in vivo* models, make CT-47518 and CT-47463 attractive candidates for further development.

Keywords: Bis-platinum complexes; antitumor efficacy; plasma binding; drug development

Introduction

Platinum (Pt)-based compounds are widely used in antitumor therapy of solid tumors and represent the cornerstone for treatment of testicular and ovarian tumors, colorectal and lung carcinomas and various squamous cell carcinomas. In particular cisplatin, its analogue carboplatin and the diaminocyclohexane-containing compound oxaliplatin are fundamental components of standard chemotherapy regimens.[1] In spite of the therapeutic benefit of Pt-based treatment regimens, the efficacy of Pt compounds is still limited by relatively low therapeutic ratios, the frequent acquisition of drug resistance and cumulative neural and renal toxicity.[2]

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An innovative approach in the development of Pt drugs has been represented by the synthesis of multinuclear (dinuclear and trinuclear) Pt compounds.[3] Multinuclear Pt complexes contain reactive Pt centers linked by alkanediammine chains of variable length and were originally designed to form long-distance cross-links with DNA (1,4-intrastrand and 1,5-interstrand cross-links).[3,4] The first multiplatinum compound to enter human clinical trials was the trinuclear Pt complex BBR3464, a 4^+ cationic complex which has two monofunctional *trans*-$PtCl(NH_3)_2$ units bridged by the Pt tetraamine unit *trans*-$Pt(NH_3)_2[NH_2(C_6H_{12})NH_2]_2^{2+}$. The compound displayed cytotoxicity against cisplatin-resistant cell lines and efficacy against p53 mutant tumor cells.[5,6] Although BBR3464 produced objective responses in phase I trials and phase II ovarian cancer and non-small cell lung cancer, unconvincing efficacy results and the low therapeutic index, likely related to extensive metabolism and irreversible protein binding in human plasma, did not support further evaluation.[7−11] The second-generation agent CT-3610 ({bis[*trans*(diamino)(chloro)platinum(II)]-μ-(1,16-diamino-7,10-diazahexadecane-N1,N16)}tetranitrate, CT3610) contains two Pt coordination units and was designed by replacing the central Pt unit of BBR3464 with an amine, while maintaining the same overall length between the Pt−chloride bonds responsible for DNA platination and the same total charge at physiological pH.[8] This structural modification increased cytotoxicity, but had no impact on the pharmacokinetic profile of the compound.

As part of an ongoing program aimed at reducing the high irreversible plasma protein binding and improving the chemical and metabolic stability of multinuclear complexes, a series of bis-(carboxylateplatinum) compounds were prepared starting from CT-3610, by replacement of the chloride ligands with alkylcarboxylates. The selected novel complexes belonging to second-generation bis-platinum compounds (CT-47518 and CT-47463), utilize butyrate or capronate moieties to improve their pharmacokinetic and pharmacodynamic profiles, in an attempt to overcome the limitations of previously developed multinuclear compounds.

These bis-platinum complexes have been examined for (i) *in vitro* protein binding in murine and human plasma, (ii) tumor cell sensitivity *in vitro*, cellular accumulation and DNA platination in different human tumor cell lines sensitive and resistant to cisplatin and (iii) *in vivo* antitumor efficacy in xenograft tumor models. The improved protein-binding kinetic profile in human plasma together with the marked activity in cellular studies as well as in *in vivo* experiments, makes CT-47518 and CT-47463 attractive candidates for further development.

Experimental Section

Drugs. Cisplatin (Bristol-Myers Squibb/Teva Pharma, Utrecht, The Netherlands) was diluted in saline. Oxaliplatin (Eloxatin, Sanofi-Synthelabo) was reconstituted in water and diluted in saline. CT-47518 and CT-47463 were prepared as tetranitrate salts (Cell Therapeutics Inc., Bresso, Italy). Powder was stored at −20 °C and dissolved in water immediately before use. The compounds are stable at 5 °C in aqueous solution. A slow increase in the monoaquo form is observed at a higher temperature. Prior to the optimization of the preparation procedure of the tetranitrate salts, some experiments were performed on the corresponding dodecylsulfate salts. For the sake of simplicity, these compounds have been identified in this paper as CT-47518 DS salt and CT-47463 DS salt. The solutions of these lipophilic salts were prepared by dissolving the powder in EtOH (e.g.: 5 mg in 50 μL), adding a 1:1 warm mixture of propylenglycol/Solutol HS15 (1 g), then diluting to final volume with water.

Synthesis of {μ-(1,8,11,18-Tetraazaoctadecane-N^1, N^{18})bis[*trans*-diamino(capronato-*O*)platinum(II)]} Tetra(dodecylsulfate) (CT-47518 DS Salt). The reactions were carried out shielded from light and in Milli-Q H_2O. A solution of CT-3610 (0.2 g, 0.193 mmol) in H_2O (18 mL) was added with $AgNO_3$ (0.072 g, 0.424 mmol), and the resulting suspension was kept under stirring for 24 h. The solid was removed filtering the mixture twice on a double microfiber filter, and the filter was washed with 1 mL of H_2O which was pooled with the filtrate. The filtrate was

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added drop by drop and under stirring with a sodium capronate solution (0.16 g, 1.158 mmol) in MeOH (19 mL); the resulting solution was maintained under stirring at room temperature for 24 h, then concentrated to half volume (35 °C) under reduced pressure and added drop by drop and under stirring with a sodium dodecylsulfate solution (0.222 g, 0.77 mmol) in H_2O (15 mL). After stirring for 15 min at room temperature, the precipitated solid was collected on a buchner filter, washed with a few drops of water and dried under vacuum at 35 °C to give 0.301 g (78% yield) of the title product. Elemental analysis for $C_{26}H_{70}N_8O_4Pt_2 \cdot 4C_{12}H_{25}O_4S$, % found (calculated): C 43.53 (44.21), H 8.42 (8.52), N 5.45 (5.57), Pt 19.02 (19.40), S 6.80 (6.38). MS: 1159.0, $[MH + C_3F_7COOH - 4C_{12}H_{25}OSO_3H]^+$. ^1H NMR (CD_3OD): δ 4.17 (12H, bs); 4.00 (8H, t, $J = 6.58$ Hz); 3.20 (4H, s); 2.98 (4H, t, $J = 7.5$); 2.65 (4H, m); 2.18 (4H, t, $J = 8.24$); 1.76−1.21 (108H, m); 0.90 (18H, m).

Synthesis of {μ-(1,8,11,18-Tetraazaoctadecane-N^1,N^{18})-bis[*trans*-diamino(butyrato-O)platinum(II)]} Tetra(dodecylsulfate) (CT-47463 DS Salt). The reactions were carried out shielded from light and in Milli-Q H_2O. A solution of CT-3610 (0.2 g, 0.193 mmol) in H_2O (18 mL) was added with $AgNO_3$ (0.072 g, 0.424 mmol), and the resulting suspension was left under stirring at room temperature for 24 h. The solid was removed filtering the mixture twice on a double microfiber filter; the filter was washed with 1 mL of H_2O which was added to the filtrate. The filtrate was added with sodium butyrate (0.127 g, 1.154 mmol), and the resulting solution was kept under stirring at room temperature for 24 h, then added drop by drop and under stirring with a sodium dodecylsulfate solution (0.222 g, 0.77 mmol) in H_2O (10 mL). After stirring for 15 min at room temperature, the precipitated solid was collected on a buchner filter, washed with a few drops of H_2O and dried under vacuum at 35 °C to give 0.28 g (74% yield) of the title product. Elemental analysis for $C_{22}H_{62}N_8O_4Pt_2 \cdot 4C_{12}H_{25}O_4S$, % found (calculated): C 42.72 (43.02), H 8.46 (8.35), N 5.62 (5.73), Pt 19.66 (19.96), S 6.69 (6.56). MS: 1103.0, $[MH + C_3F_7COOH - 4C_{12}H_{25}OSO_3H]^+$. ^1H NMR (CD_3OD): δ 4.17 (12H, bs); 4.01 (8H, t, $J = 6.58$ Hz); 3.37 (4H, s); 3.10 (4H, t, $J = 6.95$); 2.66 (4H, m); 2.17 (4H, t, $J = 7.68$); 1.80−1.22 (100H, m); 0.90 (18H, m).

Synthesis of {μ-(1,8,11,18-Tetraazaoctadecane-N^1,N^{18})-bis[*trans*-diamino(capronato-*O*)platinum(II)]} Tetranitrate (CT-47518). The reactions were carried out shielded from light and in Milli-Q H_2O and MeOH Chromasolv. $AgNO_3$ (0.819 g, 4.821 mmol) was added to a solution of CT-3610 (2 g, 1.927 mmol) in H_2O (180 mL), and the resulting suspension was left under stirring at room temperature for 24 h. The solid was removed filtering the mixture twice on a double microfiber filter; the filter was washed with 10 mL of H_2O which was pooled with the filtrate. The filtrate was added dropwise and under stirring with a solution of sodium capronate (1.6 g, 11.582 mmol) in MeOH (190 mL); the resulting suspension was kept under stirring at room temperature for 24 h. The solid was removed filtering the mixture twice on a double microfiber filter, the filtrate was evaporated to dryness (35 °C) under reduced pressure, and the solid residue was dried under vacuum at 35 °C. The solid was suspended in H_2O (50 mL), and the insoluble material was discarded filtering the mixture twice on a double microfiber filter. The filtrate was added dropwise and under stirring with $NaNO_3$ 4 M (14 mL, 56 mmol); the resulting mixture was stirred at room temperature for 20 min. The precipitate was collected and dried under vacuum at 35 °C. A suspension of the solid in MeOH (150 mL) was left under stirring at room temperature for 1 h. The solid was collected, washed with MeOH and Et_2O, and dried under vacuum at 35 °C to give 1.197 g (52% yield) of a white powder. Elemental analysis for $C_{26}H_{70}N_8O_4Pt_2 \cdot NO_3$, % found (calculated): C 25.68 (26.09), H 5.91 (5.89), N 13.76 (14.04), Pt 32.14 (32.59). Mp: 164.8−168.6 °C. MS: 1159.1, $[MH + C_3F_7COOH - 4HNO_3]^+$. ^1H NMR (D_2O): δ 3.31 (4H, s); 3.03 (4H, t, $J = 7.68$ Hz); 2.62 (4H, m); 2.26 (4H, t, $J = 7.53$); 1.67 (8H, m); 1.52 (4H, m); 1.39 (8H, m); 1.26 (8H, m); 0.86 (6H, t, $J = 7.03$).

Synthesis of {μ-(1,8,11,18-Tetraazaoctadecane-N^1,N^{18})-bis[*trans*-diamino(butyrato-*O*)platinum(II)]} Tetranitrate (CT-47463). The reactions were carried out shielded from light in Milli-Q H_2O or MeOH Chromasolv. $AgNO_3$ (0.819 g, 4.821 mmol) was added to a solution of CT-3610 (2 g, 1.927 mmol) in H_2O (180 mL), and the resulting suspension was left under stirring at room temperature for 24 h. The solid was removed filtering the mixture twice on a double microfiber filter; the filter was washed with 10 mL of H_2O which was pooled with the filtrate. The filtrate was added with sodium butyrate (6.366 g, 57.825 mmol) under stirring, and the resulting solution was kept under stirring at room temperature for 24 h. Traces of a black solid were removed filtering the mixture twice on a double microfiber filter, and then the clear filtrate was added with $NaNO_3$ (40 g, 470.644 mmol) under stirring. After stirring for 30 min at room temperature, the turbid solution was added with another 40 g (470.644 mmol) of $NaNO_3$ under stirring. The resulting mixture was kept under stirring for 1 h, and then the precipitate was collected and dried under vacuum at 35 °C. A suspension of the solid (2.1 g) in MeOH (140 mL) was left under stirring at room temperature for 1 h. The solid was collected, washed with MeOH and Et_2O, and dried under vacuum at 35 °C to give 1.368 g (62% yield) of a white solid. Elemental analysis for $C_{22}H_{62}N_8O_4Pt_2 \cdot 4NO_3$, % found (calculated): C 22.05 (23.16), H 5.31 (5.48), N 14.04 (14.73), Pt 32.45 (34.19). Mp: 107.5−118.3 °C. MS: 1103.0, $[MH + C_3F_7COOH - 4HNO_3]^+$. ^1H NMR (D_2O): δ 3.93 (2H, s); 3.33 (4H, s); 3.05 (4H, t, $J = 7.68$ Hz); 2.62 (4H, m); 2.24 (4H, t, $J = 7.40$); 1.68 (8H, m); 1.53 (4H, m); 1.40 (8H, m); 0.87 (6H, t, $J = 7.41$). ^{195}Pt NMR (D_2O): δ −2168.

***In Vitro* Plasma Protein Binding.** The *in vitro* protein binding of the selected compounds was evaluated in human and murine plasma. In the absence of an appropriate method to investigate the percentage of the free drug, the amount of the free plus noncovalent bound drug was measured. Plasma containing 37.5 μg/mL of either CT-47518 or CT-47463 DS

salts or 300 μg/mL of CT-3610 was incubated at 37 °C up to 8 h, and at least five selected time point aliquots were analyzed in duplicate by LC/MS, after acetonitrile protein precipitation. The disappearance of the compound over time was expressed as remaining percent compared to the initial concentration. Binding kinetics was characterized through the estimation of the half-life in replicates for each compound and species.

Cell Lines and Growth Conditions. Human cell lines of different tumor types were used, including two cisplatin (IGROV-1/Pt1) and oxaliplatin-resistant (IGROV-1/OHP) ovarian carcinoma sublines generated from the IGROV-1 parental cell line, ovarian carcinoma A2780 cell line and the BBR3464-resistant A2780/BBR3464 subline, squamous cell carcinoma A431 and the cisplatin-resistant variant A431/Pt. These cell lines were cultured in RPMI-1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (Invitrogen Italia, San Giuliano Milanese, Italy). The cisplatin-resistant osteosarcoma cell line U2-OS/Pt was selected from the U2-OS cell line. Both cell lines were grown in McCoy's 5A medium (BioWhittaker, Verviers, Belgium) containing 10% FCS. All the resistant sublines were selected by continuous exposure to increasing drug concentrations, and have been characterized in terms of alterations involved in the resistant phenotype, such as defense factors and alterations in cell response to drug.[12−15] The MLH1-deficient human colorectal adenocarcinoma cell line HCT116 and sublines complemented with chromosome 3 (HCT116/chr3) and chromosome 2 (HCT116/chr2) were kindly provided by Dr. R. Boland (San Diego, CA). The MSH2-deficient human endometrial carcinoma cell line HEC59 and subline complemented with chromosome 2 (HEC59/chr2) and the MSH6-deficient human colorectal adenocarcinoma cell line HCT15 and subline complemented with chromosome 2 (HCT15/chr2) were kindly provided by Dr. M. Koi (Dallas, TX). Cell lines were maintained in Iscove's modified Dulbecco's medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS. Chromosome-transferred cell lines were grown in the presence of 400 μg/mL Geneticin.

Cell Sensitivity Assays. The cell sensitivity to drugs was measured by using a growth-inhibition assay based on cell counting.[13] Exponentially growing cells were seeded in duplicate into six-well plates and, 24 h later, exposed to drugs for 1 or 72 h. At the end of treatment, cells were harvested and counted with a cell counter (Beckman Coulter, S.p.A., Milano, Italia). IC_{50} is defined as the drug concentration producing 50% inhibition of cell growth as compared with control. At least 3 independent experiments were performed for each drug or type of treatment.

Cellular Accumulation of Platinum Drugs. Exponentially growing cells were seeded in 5 cm diameter dishes in triplicate, and, 48 h later, they were exposed to platinum compounds for different times (30, 60, or 120 min) for time course experiments, or for 1 h in the case of dose response curves. The chosen concentrations did not display a direct toxic effect on cells, but produced an antiproliferative effect measurable 72 h after the end of 1 h exposure. After treatment with Pt drugs, cell monolayers were washed with ice-cold PBS, scraped, harvested and dissolved in 1 N NaOH.[13] Total cellular Pt content was determined by flameless atomic absorption spectroscopy (model 3300, Perkin-Elmer). Cellular Pt levels were expressed as ng of Pt/10^6 cells, with cell number determined by counting parallel cultures. For each type of treatment at least 3 independent experiments were performed.

DNA Platination. Cells, grown to near confluence, were exposed to platinum drugs for 1 h and recovered at the end of treatment and 5 or 24 h later. DNA was extracted according to standard procedures, and platinum content was measured by inductively coupled plasma mass spectroscopy (ICP-MS).[13] DNA-bound platinum levels were expressed as platinum pg/μg DNA, the amount of DNA being determined spectrophotometrically.

Apoptosis and Cell Cycle Analysis. Exponentially growing cells were seeded in 25 cm^2 flasks, and, 24 h later, they were exposed to different concentration of platinum compounds for 1 h. Floating and adherent cells were harvested after 24 h incubation in drug-free medium for cell cycle analysis and after 144 h incubation in drug-free medium for detection of apoptotic cells. For cell cycle analysis, cells were fixed and stained with a propidium iodide (PI)-containing solution (30 mg/mL PI, 66 U/mL RNase A in PBS). The cell cycle perturbations were measured by using a flow cytometer (Becton Dickinson, Mountain View, CA). Samples were analyzed for DNA content, and cell cycle distributions were calculated using Modfit software (Becton Dickinson). Apoptosis was evaluated by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay (Roche, Mannheim, Germany). After harvesting, cells were fixed in paraformaldehyde, permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate, and then incubated in the TUNEL reaction for 1 h. After washing, samples were analyzed by flow cytometry using Cell Quest software (Becton Dickinson).

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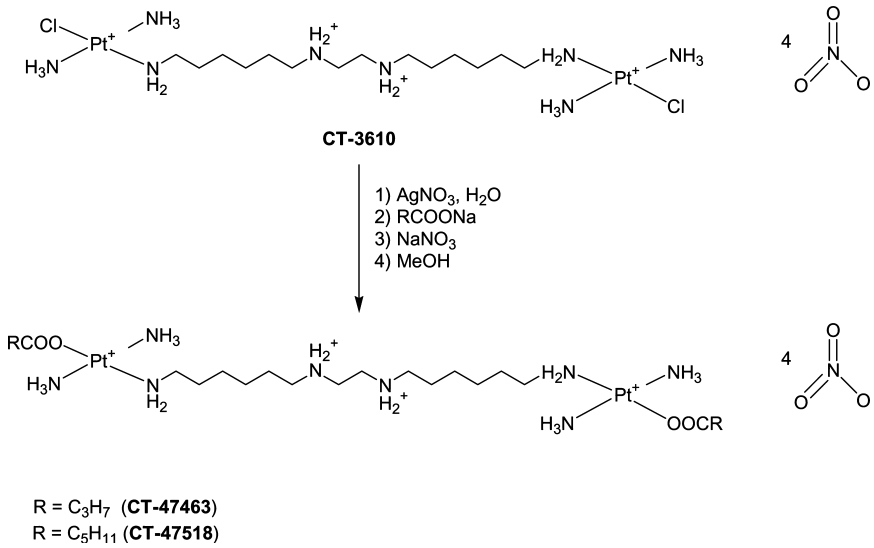

Figure 1. Structure of the studied bis-platinum complexes. CT-47518 and CT-47463 are derived from CT-3610.

***In Vivo* Activity.** The antitumor efficacy of bis-platinum complexes was evaluated in comparison with standard mononuclear platinum compounds (cisplatin, carboplatin and oxaliplatin), on several xenograft tumor models, including the cisplatin-resistant ovarian carcinoma A2780/CDDP, the colon carcinoma LoVo, HT-29 and HCT116 and the lung carcinoma LX-1. Human tumors were subcutaneously (sc) transplanted in female athymic (nu/nu) mice. The animals were randomized in experimental groups (6−8 mice each experimental group). Treatment started when the tumor reached an average weight of about 100−150 mm^3. Compounds were administered intravenously as a solution. Using a standard schedule employed for evaluation of platinum compounds (q7d×3), the optimal dose for each drug (defined as the maximum tolerated dose, i.e. dose which does not cause toxic deaths and is associated with acceptable body weight loss, ≤10%) was determined by dose−response curves in the range of 0.1−8 mg/kg. The drug effect is expressed as RTWI % = relative tumor weight inhibition % = 100 − [(mean tumor weight of treated)/(mean tumor weight of control or vehicle group)] × 100 evaluated 7 days after last treatment; TGD tumor growth delay: TG treated − TG vehicle/control (TG: mean time, in days, to reach a weight of 1 or 2 g). Statistical analyses were performed using one-way ANOVA with Bonferroni correction: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

Synthesis of Bis-platinum Complexes CT-47518 and CT-47463. The bis(carboxylatePt) complexes were prepared starting from CT-3610 by replacing the chlorine ligands with carboxylates (Figure 1). CT-3610 was activated with 2 equiv of $AgNO_3$ in H_2O to afford the diaquo form before reacting with sodium carboxylate (6 equiv). In the synthesis of the bis(butyratePt) complex, sodium butyrate was added as solid material, whereas for the bis(capronatePt) complex, a MeOH solution of sodium capronate was added to the activated CT-3610 solution at room temperature (all the syntheses were performed at room temperature due to the thermal instability of platinum complexes), in order to increase the critical micelle concentration (cmc) by the presence of the organic solvent (the ratio H_2O/MeOH was 1/1): in fact, a low cmc could prevent the salt from interacting with the aquo form. The isolation of bis(butyratePt) and bis(capronatePt) complexes was complicated by the high aqueous solubility of the complex skeleton. Eventually, we found that the use of an aqueous solution of $C_{12}H_{25}OSO_3Na$ (4 equiv) was instrumental in precipitating the complexes as tetra-dodecylsulfate salts (CT-47463 DS salt and CT-47518 DS salt) from the corresponding solutions in good yields. The DS salts showed a limited stability that was inadequate for further development. Therefore, we focused our attention on the optimization of the isolation of the water-soluble and more stable nitrate salts.

The tetra-nitrate salt of the bisbutyrate complex was initially prepared by reacting CT-3610 with excess C_3H_7COOAg (4 equiv). The use of the silver salt aimed at overcoming the isolation difficulties due to the solubility of the complex: C_3H_7COOAg is partially soluble in H_2O, and removal of the unreacted portion would leave an aqueous solution of the desired product. After a long optimization work, the best procedure to prepare CT-47463 and CT-47518 turned out to be the one illustrated in Figure 1, which was scaled up to 2 g of the starting CT-3610. This protocol was characterized by the use of sodium carboxylates (30 equiv of C_3H_7COONa, 6 equiv of $C_5H_{11}COONa$) and the precipitation of CT-47463 and CT-47518 with a large excess of $NaNO_3$ (488 equiv and 30 equiv, respectively), followed by the removal of the coprecipitated $NaNO_3$ with MeOH. Also, we observed that the activation of CT-3610 was driven to completion (HPLC) through the use of 2.5 equiv of $AgNO_3$. The yields of CT-47463 and CT-47518 were satisfactory (62% and 52%, respectively), and their purity profiles were high (>97%, HPLC).

***In Vitro* Study of Plasma Protein Binding.** The *in vitro* protein binding of the selected compounds was determined

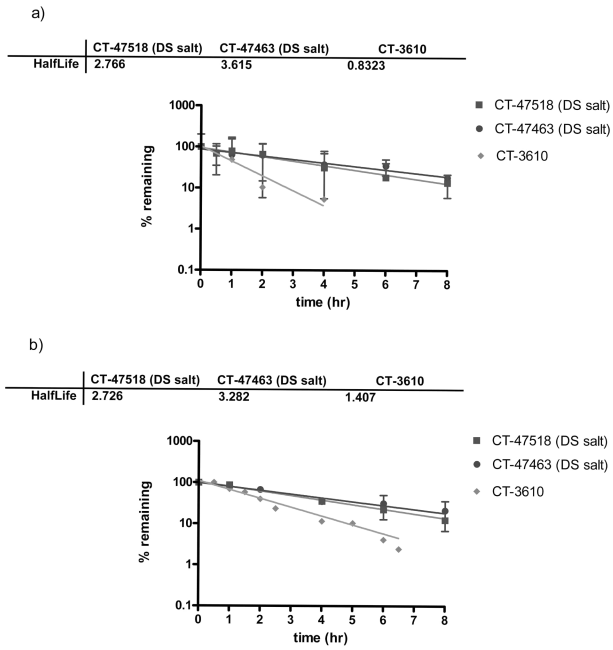

Figure 2. *In vitro* plasma protein binding of CT-47518 (DS salt) and CT-47463: (DS salt). Time course of free plus non covalent bound compounds in human (a) and mouse (b) plasma is shown. Binding/degradation kinetics was characterized through the estimation of the half-life.

in human and murine plasma. The remaining percent of the unchanged drug is comprehensive of the free and the noncovalent bound fraction. The disappearance of the compounds over time probably reflects the combination of two mechanisms, i.e., the irreversible binding to plasma proteins and the biotransformation of the compound into metabolites. Binding/degradation kinetics was characterized through the estimation of the half-life. The half-life of CT-47463 DS salt and CT-47518 DS salt in murine and human plasma were estimated to be similar, but 2- and 3-fold longer compared to CT-3610 in murine and human plasma, respectively, reflecting a slower protein binding/degradation rate compared to CT-3610 (Figure 2).

Cell Sensitivity to Bis-platinum Complexes of Cells with Acquired Resistance to Platinum Drugs. The multinuclear complexes were employed in cellular pharmacology studies using cell systems of different tumor types which included drug-resistant sublines generated by chronic exposure to cisplatin, oxaliplatin or BBR3464 (Table 1). The degree of resistance to the selecting agent ranged between 2.5 and 100, and for cisplatin was lower than that observed for oxaliplatin (around 100, see IGROV-1/OHP) and BBR3464 (around 30, A2780/BBR3464). For all cell systems, resistance was associated with multiple alterations including reduced sensitivity to drug-induced apoptosis.[14,16] In addition, defects in the MMR pathway have been documented.[12,13] IGROV-1/Pt1 and IGROV-1/OHP cells also exhibited up-regulation of survival pathways.[15]

Sensitivity of cisplatin-sensitive and -resistant cell lines to new bis-platinum compounds was assessed by growth-inhibition assay after 1 h exposure, and the ratio between IC_{50} of resistant and parental cells was determined to define the level of cross-resistance between new bis-platinum complexes and other platinum drugs. The compounds exhibited a marked cytotoxic potency as compared with cisplatin and oxaliplatin. In particular, we observed a certain degree of cross-resistance between CT-47518/CT-47463 and other mononuclear compounds (cisplatin, oxaliplatin) in ovarian carcinoma Pt-resistant sublines (IGROV-1/Pt1 and IGROV-1/OHP), whereas U2-OS/Pt cells were not cross-resistant to CT-47518 and displayed a weak collateral sensitivity to CT-47463 (Tables 1 and 2). A high degree of cross-resistance between such complexes and BBR3464 was found in ovarian carcinoma cells displaying acquired resistance to BBR3464 (A2780/BBR), suggesting a similar mechanism of action at a cellular level (Tables 1 and 2). Interestingly, A431/Pt cells, which are characterized by collateral sensitivity to BBR3464 and are not resistant to oxaliplatin,[6,17] also displayed increased sensitivity to bis-platinum complexes when compared to A431 parental cells (Tables 1 and 2).

Cell Sensitivity to Bis-platinum Complexes of Cells with DNA Mismatch Repair Defects. Since the loss of mismatch repair (MMR) has been linked to cisplatin resistance in cellular models of different tumor types and this mechanism of resistance has been shown to be bypassed by oxaliplatin,[18,19] we examined the effect of the MMR status on sensitivity to bis-platinum complexes. This study was performed in HCT15 and HCT15/chr2 cells (respectively deficient and proficient in MSH6), in HEC59 and HEC59/chr2 (respectively deficient and proficient in MSH2), and in HCT116 cells deficient (HCT116/chr2) or proficient (HCT116/chr3) in MLH1. In HCT116/chr3 cells a functional copy of MLH1 had been introduced by chromosome 3 transfer, whereas in HCT116/chr2 cells chromosome 2 had been inserted as a control. As shown in Table 3, CT-47518 was capable of overcoming resistance mediated by DNA MMR defects in different model systems. Also CT-47463, tested in HCT116 cell system (proficient or not for MLH1), showed the capability to overcome resistance mediated by DNA MMR defects (Table 3).

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Table 1. Cell Sensitivity of Drug-Sensitive and -Resistant Cells to Conventional Platinum Compounds and to BBR3464[a]

	IC_{50} (μM)		
cell line	cisplatin	oxaliplatin	BBR3464
IGROV-1	7.05 ± 2.1	14.0 ± 4.2	0.06 ± 0.01
IGROV-1/Pt1	105 ± 7.0 (10.5)[b]	171.5 ± 0.2 (12.2)	0.54 ± 0.008(9)
IGROV-1/OHP	50.96 ± 9.0 (7.2)	1467 ± 250(105)	5.9 ± 0.9 (98)
A2780	3.3 ± 1.1	nd[c]	0.037 ± 0.0003
A2780/BBR3464	8.6 ± 2.1 (2.6)	nd	1.19 ± 0.2 (32)
U2-OS	13.6 ± 2.6	76.8 ± 13	0.89 ± 0.5
U2-OS/Pt	71.0 ± 3.3 (5.2)	80.6 ± 2.5	0.96 ± 0.8 (1.07)
A431	35.7 ± 8.3	154.9 ± 3.	2.5 ± 1.4
A431/Pt	90.0 ± 18.3 (2.5)	138.5 ± 20	0.024 ± 0.008 (0.09)

[a] Cell sensitivity to drugs was assessed by growth-inhibition assays. Twenty-four hours after seeding, cells were exposed to drug for 1 h, and cell growth was assessed by cell counting 72 h after treatment. [b] Resistance index, ratio between the IC_{50} value (drug concentration inhibiting cell growth by 50%) of resistant and parental cells. [c] Not determined.

Table 2. Cell Sensitivity of Drug-Sensitive and -Resistant Cells to the Bis-platinum Complexes CT-47518 and CT-47463[a]

	IC_{50} (μM)	
cell line	CT-47518	CT-47463
IGROV-1	0.025 ± 0.016	0.037 ± 0.02
IGROV-1/Pt1	0.108 ± 0.03 (4.3)[b]	0.77 ± 0.4 (19.25)
IGROV-1/OHP	0.31 ± 0.09 (12.4)	0.76 ± 0.3 (19)
A2780	0.00165 ± 0.0007	0.00285 ± 0.001
A2780/BBR3464	2.65 ± 1.4 (1606)	2.065 ± 0.09 (724)
U2-OS	0.048 ± 0.001	0.064 ± 0.01
U2-OS/Pt	0.0695 ± 0.009 (1.45)	0.041 ± 0.01 (0.64)
A431	0.056 ± 0.03	0.044 ± 0.02
A431/Pt	0.044 ± 0.01 (0.78)	0.003 ± 0.001 (0.07)

[a] Cell sensitivity to drug was assessed by growth-inhibition assays. Twenty-four hours after seeding, cells were exposed to drug for 1 h, and cell growth was assessed by cell counting 72 h after treatment. [b] Resistance index, ratio between the IC_{50} value (drug concentration inhibiting cell growth by 50%) of resistant and parental cells.

Table 3. Cell Sensitivity of DNA Mismatch Repair-Deficient and -Proficient Cells to the Bis-platinum Complexes CT-47518 and CT-47463[a]

	IC_{50} (μM)	
cell line	CT-47518	CT-47463
HCT15 ($Msh6^{-/-}$)	0.0436 ± 0.03	nd[b]
HCT15chr2 ($Msh6^{+/+}$)	0.0547 ± 0.03	nd
HEC59 ($Msh2^{-/-}$)	0.031 ± 0.01	nd
HEC59chr2 ($Msh2^{+/+}$)	0.093 ± 0.09	nd
HCT116 ($Mlh1^{-/-}$)	0.00715 ± 0.004	0.035 ± 0.008
HCT116chr2 ($Mlh1^{-/-}$)	0.00785 ± 0.002	0.027 ± 0.02
HCT116chr3($Mlh1^{+/+}$)	0.0057 ± 0.003	0.032 ± 0.009

[a] Cell sensitivity to drug was assessed by growth-inhibition assays. Twenty-four hours after seeding, cells were exposed to drug for 1 h, and cell growth was assessed by cell counting 72 h after treatment. [b] Not determined.

Cellular Accumulation of Bis-platinum Complexes. Defects in intracellular drug accumulation has been shown to play an important role in resistance to platinum drugs.[20] A comparison of cellular drug accumulation after 1 h exposure to the same concentrations of CT-47518 (DS-salt) and CT-47463 (DS salt) was performed in IGROV-1 cells

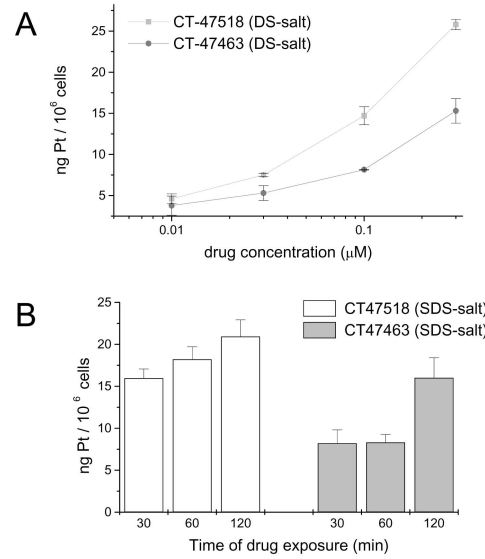

Figure 3. Cellular accumulation of platinum in IGROV-1 cells exposed to bis-platinum complexes. Cells were exposed to different concentrations of bis-platinum complexes for 1 h (A) or, in time-course experiments, for 30, 60, or 120 min (B), and then harvested for analysis of platinum by ICP-MS. The reported values are the mean (±SD) of three independent experiments.

(Figure 3A). We found that the accumulation of CT-47518 was higher than that of CT-47463. Moreover, lower levels of accumulated Pt were detected when cells were exposed to equitoxic concentrations of conventional Pt drugs.[16,21] The level of cellular accumulation of bis-platinum complexes was also determined in time-course experiments 30, 60, and 120 min after exposure to equimolar concentrations (0.1 μM) of

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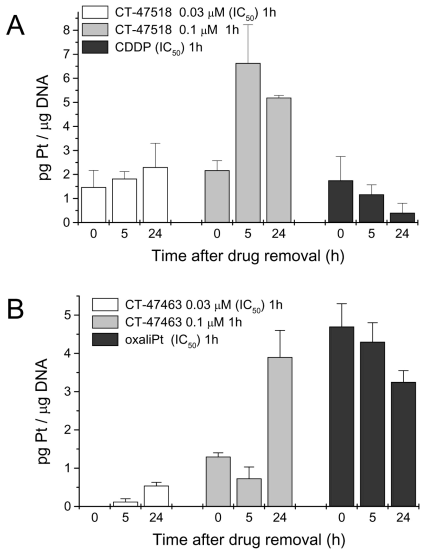

Figure 4. DNA platination in IGROV-1 cells exposed to the bis-platinum complexes. Cells were exposed to CT-47518 or cisplatin (A) and to CT-47463 or oxaliplatin (B) as indicated, and then harvested for DNA extraction and analysis of DNA-bound platinum. The reported values are the mean (±SD) of three independent experiments.

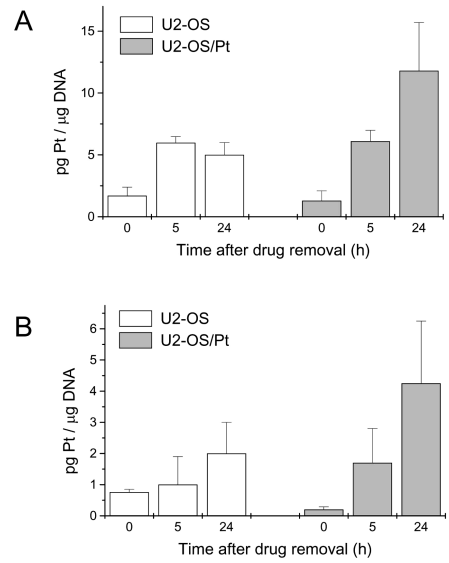

Figure 5. DNA platination in U2-OS and U2-OS/Pt cells exposed to the bis-platinum complexes. Cells were exposed to 1 μM CT-47518 (A) or CT-47463 (B) and then harvested for DNA extraction and analysis of DNA-bound platinum. The reported values are the mean (±SD) of three independent experiments.

the two drugs (Figure 3B). Similarly to what observed in dose response curves, CT-47518 displayed a higher accumulation than CT-47463; the increased accumulation was already evident at early time points (Figure 3B).

DNA Platination by Bis-platinum Complexes. DNA platination studies, performed in ovarian carcinoma IGROV-1 cells, indicated a similar level of DNA-bound Pt after 1 h exposure to equitoxic concentrations (IC_{50}) of CT-47518 and cisplatin (see Tables 1 and 2), as shown in Figure 4A. Interestingly, CT-47518 produced Pt adducts that were more persistent than those produced by cisplatin, because a high level of DNA-bound Pt was still detected 24 h after drug removal (Figure 4A). We also tested in IGROV-1 cells the effect of 1 h exposure to equitoxic concentrations (IC_{50}) of CT-47463 and oxaliplatin (see Tables 1 and 3), and a higher level of Pt adducts was found with oxaliplatin in comparison to such bis-platinum compounds (Figure 4B). However, the level of oxaliplatin decreased upon drug removal. Thus, both bis-platinum complexes produced Pt adducts more persistent than those produced by conventional platinum compounds.

Since the bis-platinum complexes were capable of overcoming resistance to cisplatin in U2-OS/Pt cells, this model was selected for further analyses of DNA platination. A comparison of DNA platination between CT-47518 and CT-47463 was performed in U2-OS and U2-OS/Pt cells after 1 h exposure to equimolar concentrations (1 μM) of the two bis-platinum complexes (Figure 5). We observed that the level of DNA-bound Pt was higher with CT-47518 than CT-47463 in both cell lines. In addition, the cisplatin-resistant subline appeared to show a high level of DNA-bound Pt also 24 h after removal of both drugs, suggesting an increased tolerance to DNA damage in comparison to the sensitive parental cell lines.

Percentage of Pt Accumulation and of DNA Platination in IGROV-1 Cells Exposed to Platinum Compounds. We have compared the different platinum drugs employed in our study (cisplatin, oxaliplatin, CT-47518 and CT-47463) in terms of percentage of accumulated Pt and of DNA-bound Pt in IGROV-1 cells. To this aim, cellular accumulation and DNA binding of Pt were evaluated at drug concentrations feasible for detection of a Pt signal for each compounds under the used experimental conditions. Since the differences in cytotoxic potency among the considered Pt drugs did not allows us to measure Pt accumulation and DNA-binding at equimolar concentrations, the comparison was done at equitoxic concentrations (IC_{50}, see Table 1 and 2). As detailed in Table 4, the percentage of intracellular Pt (ng of Pt/10^6 cells) and extracellular Pt (ng of Pt/L) indicated that accumulation of new bis-platinum complexes was much higher than that of cisplatin and oxaliplatin. The percentage of DNA-bound Pt (pg of Pt/μg of DNA) and extracellular Pt (pg of Pt/L) suggested that, also in terms of platination, the new bis-platinum complexes produced a higher level of DNA binding with respect to cisplatin and oxaliplatin. The DNA platination, also evaluated as percent of DNA-bound Pt (pg of Pt/μg of DNA) and extracellular Pt (pg of Pt/10^6 cells), resulted comparable between CT-47518 and cisplatin, whereas the percentage observed with oxaliplatin was slightly increased.

Apoptosis and Perturbation of Cell Cycle. The more potent bis-platinum complex, i.e., CT-47518, was chosen to investigate cell cycle perturbations following treatment and the mode of cell death. Cell cycle perturbation analysis indicated the occurrence of CT-47518-induced G2 accumula-

Table 4. Percentage of Pt Accumulation and of DNA Platination in IGROV-1 Cells Exposed to Platinum Compounds[a]

	% of Pt accumulation[b]	% of DNA platination[c]	% DNA-bound Pt vs intracellular Pt[d]
cisplatin	0.0003	7.6×10^{-8}	0.025
oxaliplatin	0.00014	7.5×10^{-8}	0.052
CT-47518	0.064	13.6×10^{-6}	0.02
CT-47463	0.042	2.7×10^{-6}	0.006

[a] Cells were exposed for 1 h to Pt compounds (IC_{50}) and then harvested for analysis of Pt accumulation and DNA-bound Pt by ICP-MS. The results from at least three independent experiments have been normalized to calculate the percentage. [b] Percent values of the intracellular amount of Pt (ng/10^6 cells) relative to the extracellular concentration used in the drug accumulation studies. [c] Percent values of DNA-bound Pt (pg of Pt/μg of DNA) relative to extracellular concentration used for the DNA platination studies. [d] Percent values of amount of DNA-bound Pt (Pg of Pt/μg of DNA) relative to the intracellular amount of Pt (pg/10^6 cells).

Table 5. Cell Cycle Perturbations in IGROV-1 and IGROV-1/Pt1 Ovarian Carcinoma Cells after Exposure to CT-47518[a]

		cell cycle phase (percent of cells)		
cell line	compd (concn, μM)	G1	S	G2/M
IGROV-1	control	53.54	23.31	10.37
	CT-47518 (0.1)	41.49	14.25	24.16
	CT-47518 (0.03)	45.84	14.76	19.40
IGROV-1/Pt1	control	36.52	24.49	25.49
	CT-47518 (1)	24.53	22.14	30.04
	CT-47518 (0.1)	27.69	24.12	29.13

[a] Cells were exposed for 1 h to equitoxic concentrations of CT-47518, and after 24 h incubation in drug-free medium, they were harvested and processed for cell cycle analysis. A representative experiment is reported.

Table 6. Analysis of Apoptosis in IGROV-1 and IGROV-1/Pt1 Ovarian Carcinoma Cells Exposed to CT-47518[a]

		apoptotic cells (%)			
		IGROV-1		IGROV-1/Pt1	
drug	concn (μM)	72 h	144 h	72 h	144 h
	0	nd[b]	1.4	nd	7
CT-47518	0.03	nd	16	nd	17
CT-47518	0.1	nd	42	nd	29
CT-47518	1.0	6	53	4	46
cisplatin	30	45	61	11	16

[a] Cells were exposed for 1 h to different concentrations of CT-47518 or to cisplatin and processed for TUNEL assay after 72 or 144 h incubation in drug-free medium. [b] Not determined.

tion both in Pt drug-sensitive (IGROV-1) and -resistant cells (IGROV-1/Pt1) after 1 h exposure to equitoxic drug concentrations and 24 h incubation in drug-free medium (Table 5). The treatment of cells with CT-47518 resulted in the occurrence of marked apoptosis, particularly evident 6 days after drug exposure, both in both cisplatin-sensitive and -resistant cells (Table 6). However, cisplatin was capable of inducing higher levels of apoptosis than CT-47518 in parental cells 72 as well as 144 h after exposure, but not in the cisplatin-resistant variant (Table 6). Thus, the bis-platinum complex can overcome resistance associated with reduced susceptibility to apoptosis.

Table 7. Antitumor Activity of CT-47463 and CT-47418 on Human Tumor Xenografts[a]

cell line	compd	dose (mg/kg)	RTWI %[b]	TGD[c]
	Human Ovarian Carcinoma			
A2780/CDDP	CT-47463	4.3	80	20*[d]
	CT-47518	2.1	80	18*
	CT-3610	0.1	62	11
	cisplatin	6	59	9
	carboplatin	80	nd	nd
	oxaliplatin	13.5	nd	nd
	Human Lung Carcinoma			
LX-1	CT-47463	4.3	76*	21***
	CT-47518	2.1	73*	21***
	CT-3610	0.1	77	28
	cisplatin	6	46	13
	carboplatin	80	43	0
	oxaliplatin	13.5	nd	nd
	Human Colon Carcinoma			
LoVo	CT-47463	4.3	80*	22***
	CT-47518	2.1	72*	24***
	CT-3610	0.1	58	15
	cisplatin	6	55	9
	carboplatin	80	38	5
	oxaliplatin	13.5	27	1
HT-29	CT-47463	4.3	75**	36***
	CT-47518	2.1	65**	28***
	CT-3610	0.1	51	20
	cisplatin	6	49	13
	carboplatin	80	34	6
	oxaliplatin	13.5	0	0
HCT-116	CT-47463	4.3	82**	52
	CT-47518	2.1	67	28
	CT-3610	0.1	68	25
	cisplatin	6	50	13
	carboplatin	80	nd	nd
	oxaliplatin	13.5	46	6

[a] Tumors were implanted s.c. and treatments were started when tumors were measurable. Drugs were delivered intravenously every seventh day for 3 times. [b] RTWI%, relative tumor weight inhibition: 100-(mean tumor weight of treated/mean tumor weight of untreated group)x100 evaluated 7 days after last treatment. [c] TGD, tumor growth delay (days): TG treated-TG untreated (control); TG: mean times to reach a tumor weight of 1 or 2 g; n.d., not determined. [d] *$p < 0.05$; **$p < 0.01$ *versus* carboplatin and oxaliplatin; ***$p < 0.001$ *versus* standard platinum compounds (cisplatin, carboplatin, oxaliplatin).

In Vivo **Antitumor Activity Studies.** The effects of the novel bis-platinum complexes against human tumor xenografts of different tumor types including cisplatin-resistant ovarian carcinoma (A2780/CDDP), as well as lung (LX-1) and colon (LoVo, HT-29 and HCT116) carcinomas, were investigated in comparison with CT-3610 and mononuclear platinum compounds used as reference drugs (Table 7). Such xenografts were selected among tumor types which are clinically treated with regimens including platinum compounds. Treatment with the optimal drug dose resulted in

inhibition of tumor growth as shown by relative TWI measurements in all the examined models. A marked activity of the two bis-platinum complexes (TWI of 80%) was observed in the A2780/CDDP and LX-1 models, in which cisplatin activity was marginal. In the colon carcinoma xenografts, a significant improvement of TWI was reached with CT-47463 in the three tested models as compared with CT-3610 and standard mononuclear compounds. Also CT-47518 appeared more active than CT-3610 and conventional compounds, except than in the HCT116 model systems. Thus, both compounds appeared endowed with substantially improved antitumor activity as compared with mononuclear compounds. A comparison of effective doses (i.e., doses producing TWI > 60%) and toxic doses (LD_{10}) supported an improvement of the therapeutic index of the multinuclear compounds as compared to conventional platinum drugs.

Discussion

In spite of the promising preclinical profile of first generation multinuclear platinum complexes, the clinical development of this class of drugs has been disappointing due to dose-limiting toxicities and low therapeutic index, in particular of BBR3464.[7−11] Among novel bis-platinum complexes, two derivatives of BBR3610 were selected for preclinical studies on the basis of their reduced binding to plasma proteins (Figure 2). In fact, binding to human plasma proteins has been considered the most likely cause for the poor therapeutic index of previously generated multinuclear complexes. The present study provides evidence that two novel bis-platinum complexes display an improved pharmacological profile and therapeutic behavior. Indeed, CT-47518 and CT-47463 exhibited a strong cytotoxic potency and an increased antitumor activity. In particular, a potent cytotoxicity of CT-47518 and CT-47463 in comparison to conventional mononuclear agents was somehow related to the extent of cellular accumulation, DNA binding and to DNA damage persistence. Indeed, a comparison of the percentages of Pt compounds that entered tumor cells with respect to the amount of drug used for cell treatment indicated that, despite their 4-fold greater molecular weight, accumulation of the new complexes was much higher than that of cisplatin and oxaliplatin. The superior cytotoxic activity of the CT-47518 and CT-47463 could also be explained by the observation that the relative amount of DNA-bound Pt—in particular after exposure to CT-47518—was much higher (2 orders of magnitude) than that found for mononuclear compounds. This behavior has been previously reported for Pt compounds capable of overcoming cisplatin resistance.[22] The enhanced DNA damage persistance of the bis-platinum complexes as compared to cisplatin and/or oxaliplatin is reminiscent of what was observed for BBR3464.[23,24] The chemical features of CT-47518 and CT-47463 may be useful to explain the high platination level. Indeed, an increased affinity of the drugs for DNA is expected from (a) the presence of positive charges that should favor electrostatic and hydrogen binding to DNA as well as (b) the presence of two reactive Pt centers. Another major feature of the two compounds, relevant for therapeutic implications, was the ability to overcome resistance associated with DNA MMR defects as shown by using isogenic MMR-deficient and proficient cells (Table 3). Moreover, the two compounds could overcome cisplatin-resistance in human osteosarcoma and in squamous cell carcinoma cells. A partial overcoming of resistance to cisplatin and oxaliplatin by CT-47518 was observed in the IGROV-1/Pt1 and IGROV-1/OHP sublines, which are characterized by a very high level of resistance to the respective selecting agent, whereas the degree of resistance to CT-47463 resulted quite high (Tables 1 and 2). Such a behavior could be explained by the better profile of cellular accumulation of CT-47518 observed in IGROV-1 cells, in comparison to CT-47463 (Figure 3).

A partial reversal of resistance to cisplatin by CT-47518 was observed also in IGROV-1/Pt1 cells in which G2 accumulation and apoptosis occurrence was documented after exposure to drug concentrations equitoxic with respect to those employed in parental cells. However, both compounds failed to overcome resistance to BBR3464, suggesting that the three compounds share a common mechanism of action. The improved pharmacological profile of the selected bis-platinum complexes likely reflects a more favorable *in vivo* behavior, which may be dependent on their chemical−physical features. This interpretation is consistent with the improved behavior of CT-47518 and CT-47463 over the closely related CT-3610 bis-platinum complex. Both CT-47518 and CT-47463 displayed a marked antitumor activity in terms of tumor weight inhibition and tumor growth delay. Interestingly, such an effect was observed in an ovarian carcinoma with acquired resistance to cisplatin (A2780/CDDP). Thus, the selected compounds are promising candidates for clinical development.

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