New platinum(IV) complex with adamantylamine ligand as a promising anti-cancer drug: comparison of *in vitro* cytotoxic potential towards A2780/cisR cisplatin-resistant cell line within homologous series of platinum(IV) complexes

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The aim of this study was to compare anti-tumor potency of platinum(IV) complexes with increasing hydrophobicity of their ligands. Cytotoxic potential of the new platinum(IV) complex, coded as LA-12 [(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)], was compared within the series of complexes of the general formula (*OC*-6-43)-bis(acetato)(alkylamine)amminedichloroplatinum(IV). Alkylamine ligands with increasing hydrophobicity were: isopropylamine, cyclohexylamine, 1-adamantylamine and 3,5-dimethyl-1-adamantylamine. Particular platinum(IV) complexes were coded as LA-4, LA-2 (known as JM-216), LA-12 and LA-15, respectively. Cytotoxicity was tested with the microplate tetrazolium (MTT) assay on the panel of cancer cell lines and the results were verified by microscopy. HPLC was used to measure hydrophobicity, stability of complexes in various buffers and velocity constants for their reactivity with glutathione. Platinum(IV) complexes with bulky hydrophobic ligands (LA-12 and LA-15) demonstrated about one order higher velocity constant for pseudo-first-order reaction with glutathione in comparison to cisplatin, LA-4 and LA-2, whose velocity constants were close to those measured for cisplatin and related platinum(II) complexes. Cytotoxicities of LA-12 and LA-15 towards cisplatin-resistant epithelial carcinoma A2780/cisR were superior to cisplatin, LA-4 and LA-2 in both 24- and 72-h continuous exposure MTT tests. Rapid induction of apoptosis in the treated cancer cell lines and no cisplatin cross-resistance were found for LA-12, which is a candidate for clinical testing. *Anti-Cancer Drugs* 15:537–543 © 2004 Lippincott Williams & Wilkins.

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Introduction

Many different analogs of cisplatin have been synthesized with the hope of reducing toxic side-effects to overcome multidrug resistance and to facilitate the preparation of a suitable formulation for application. About 1000 complexes have been studied in various laboratories so far, but only 10–20% of them turned out to be active against cancer cells in preclinical studies. A novel series of ammine/amine platinum(IV) dicarboxylate complexes represents the first class of complexes prepared as oral agents. Among them, JM216 [(*OC*-6-43)-bis(acetato)amminedichloro(cyclohexylamine)platinum(IV)] has been found to show significant antitumor activity via the oral route and now is currently in phase II/III trials [1–3].

Preclinical *in vitro* studies of the new hydrophobic platinum(IV) complex (*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV), coded as LA-12, showed that the complex is very efficient against many cancer cell lines resistant towards cisplatin [4] and its favorable *in vivo* toxicological profile is auspicious for human application.

To address some question on the effect of the hydrophobic ligand on the cytotoxicity of platinum(IV) complexes, their stability in various media and reactivity towards biological thiols, we have prepared platinum(IV) complexes forming a homologous series with respect to hydrophobicity and bulkiness of ligands.

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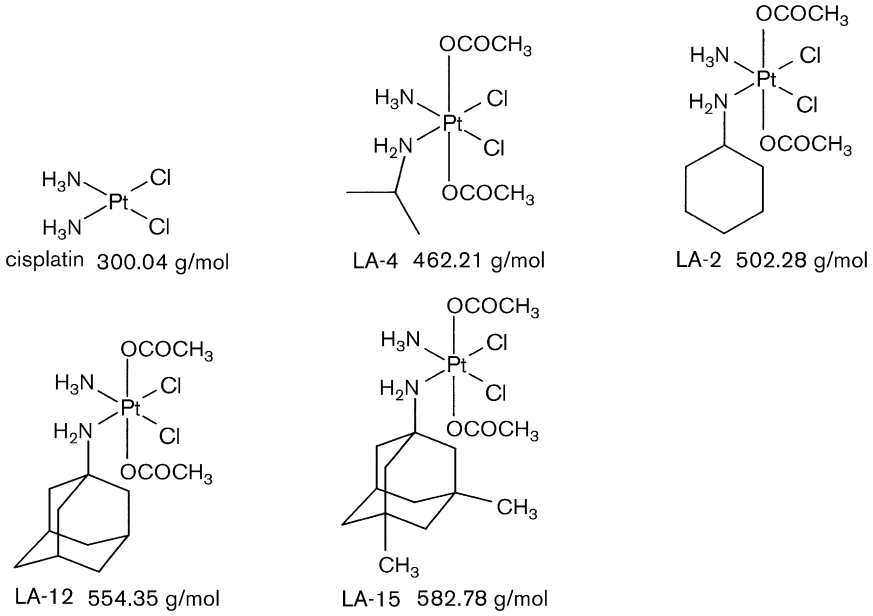
Fig. 1



Structural formulae of the tested platinum complexes.

Materials and methods

Chemicals

Cisplatin [CDDP, (SP-4-2)-diamminedichloroplatinum (II)].

JM-216 [(*OC*-6-43)-bis(acetato)amminedichloro(cyclohexylamine)platinum(IV)], coded in this article as LA-2.

LA-4 [(*OC*-6-43)-bis(acetato)amminedichloro(isopropylamine)platinum(IV)].

LA-12 [(*OC*-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)].

LA-15 [(*OC*-6-43)-bis(acetato)(3,5-dimethyl-1-adamantylamine)amminedichloroplatinum(IV)].

The platinum(IV) complexes were synthesized and kindly donated by PLIVA-Lachema (Brno, Czech Republic). The structural formulae are shown in Figure 1.

MTT–based cytotoxicity test

We used the microplate tetrazolium (MTT) assay [5,6] to measure the cytotoxicity of the tested drugs in cells in exponential growth phase. The cells were seeded on 96-well flat-bottom microplates at densities of $2.5–3.0 \times 10^4$/ml, 100 µl/well, and let to grow for 16–24 h in culture medium (RPMI supplemented with 10% fetal calf serum). The drugs dissolved in phosphate-buffered saline (PBS) (total volume 20 µl) were added to wells and the cytotoxic effect was evaluated after 24 and 72 h of exposure to the concentration range 0.3–160 µM.

MTT (Sigma, Czech Republic) was dissolved in PBS at a concentration of 5 mg/ml and sterilized by filtration. MTT solution was added into all wells of 96-well flat-bottom microplates with cells at a dose of 20 µl/well. The plates were incubated at 37°C and 5% CO_2 for 3 h. To enhance the dissolution of dark-purple crystals of formazan, 110 µl of 10% SDS in PBS (final pH 5.5) was added to all wells. The microtiter plates were stored in a light-tight box at room temperature and evaluated the next day using the micro-plate reader iEMS (Labsystem, Finland) at 540 nm. All experiments were performed in triplicates.

Cancer cell lines

Cancer cell lines having $IC_{50} \geq 40$ µM (for cisplatin, 24 h continual exposure) were selected from the panel of cancer cell lines tested in our laboratory and were used for the study of cross-resistance. Highly resistant A2780/cis90 ($IC_{50} \sim 90$ µM for cisplatin, 24 h exposure) was established from A2780/cis ($IC_{50} \sim 40$ µM for cisplatin) during 15 months by cultivation in medium with increasing concentrations of cisplatin (1–12 µM). HBL100, MCF-7 (breast carcinoma and adenocarcinoma), HT-29, HT-29N and HCT-116 (colon carcinoma and adenocarcinoma) were obtained from the Masaryk Memorial Institute of Oncology (Brno). K-562 (chronic myelogenous leukemia), KG-1 (chronic myelogenous leukemia), ML-2 (acute myelogenous leukemia) and B16 (mouse melanoma) were obtained

from the Institute of Hematology and Blood Transfusion, Prague. A427, CORL23/CTR (cisplatin resistant) (lung large cell carcinoma), A2780, A2780/cis (ovarian carcinoma) were purchased from ECACC.

The cell lines were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Gibco, Czech Republic), 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 300 μg/ml L-glutamine. A2780 cell line and its cisplatin resistant sublines were cultivated in RPMI 1640 medium supplemented with insulin (40 IU/100 ml of medium).

The cytotoxicity of LA-12 against cancer cell lines was examined by the MTT test, and the results were confirmed by Hoffman modulation contrast and fluorescent microscopy (epifluorescent inverted microscope T200; Nikon, Japan) exposing morphological changes of the cells treated with platinum complexes. Propidium iodide and YO-PRO-1 (Molecular Probes, Eugene, OR) were used to distinguish dead or apoptotic cells from vital living ones [7].

Reactivity with glutathione

Reactivity of platinum complexes with glutathione was determined as a velocity constant for the pseudo-first-order reaction at pH 7.5 (20 mM sodium phosphate, 150 mM NaCl), 37°C, 1 mM glutathione. Concentration of platinum complex in reaction mixture was 10 μM. Samples of reaction mixture were analyzed by HPLC and reaction was monitored in 30-min intervals for 3 h. Cisplatin was analyzed on the column Zorbax NH_2, 25 cm × 4.6 mm. Mobile phase: 90% methanol, 10% water; flow rate 1 ml/min; 25°C. Chromatographic conditions for analysis of LA-2, LA-4, LA-12 and LA-15 are described in the following section. Reaction conditions were chosen to approximate the *in vivo* conditions with respect to reactant concentrations, pH and ions.

Hydrophobicity of platinum complexes

Hydrophobicity of platinum complexes was estimated by HPLC and expressed as the capacity factor k' for each complex. Chromatographic separations were run on the column ABZ plus Supelcosil LC-18-S, 150 × 3 mm, 5 μm (Supelco, Prague, Czech Republic). Mobile phase: 40% methanol in water. Flow rate: 0.5 ml/min. Temperature: 40°C. Detection: UV 210 nm. Instrument: Waters chromatographic system consisting of the Waters 600 gradient pump, the Waters 717 plus autoinjector and the Waters 996 diode array detector. The system was controlled by the programme Millennium 2010. Mobile phase: methanol with water in the ratio 41:59 (v/w), flow rate 0.7 ml/min, room temperature, detection at 206 nm.

Stability of platinum complexes in various media

Stability of platinum(IV) complexes LA-4, LA-2, LA-12 and LA-15 (10 μM) was tested after 24 h incubation in various media at pH 7.2, 37°C (media: 50 mM sodium phosphate buffer, pH 7.2, 50 mM Tris–HCl, pH 7.2, PBS, pH 7.2). The contents of platinum(IV) complexes were analyzed by HPLC and expressed as a percentage of the original concentration.

Statistics

Survival curves, IC_{50} and velocity constants were calculated by the programme GraphPad Prism, version 3.03. (GraphPad Software, San Diego, CA).

Results

Hydrophobicity of platinum complexes

Hydrophobicities of platinum complexes were measured to get a better insight into the effect of the hydrophobic ligand on the ability of various drugs to penetrate through the cell membrane. Hydrophobicity of platinum complexes was estimated by HPLC and expressed as the capacity factor k' for each complex. This method is sensitive and relatively simple to allow comparison of various ligands with respect to their contribution to the overall hydrophobicity of various platinum complexes within the homologous set. The expected hydrophobicities of platinum complexes based on their structural formulae are in a good agreement with the capacity factor k' calculated from chromatographic data (Fig. 1 and Table 1).

Stability of platinum complexes in various media

The platinum complexes loose their chloride ligands in media containing low concentrations of chloride to form positively charged monoaqua and diaqua species which migrate much faster on chromatographic reverse phase column. The comparison within the set of platinum(IV) complexes showed that there were no substantial differences among them with regard to stabilities in media with both low and high chloride contents (50 mM phosphate buffer versus PBS). Stabilities in PBS were somewhat higher, as expected. Replacement of chloride by water in the platinum(IV) complexes was more rapid in Tris–HCl buffer in comparison to phosphate buffer. Results are summarized in Table 2.

Reactivity of platinum complexes with glutathione

Platinum(IV) complexes LA-12 and LA-15 with bulky hydrophobic adamantylamine or 3,5-dimethyl-1-adamantylamine ligands exerted about one order higher velocity constant for the pseudo-first-order reaction with

Table 1 Hydrophobicity of platinum complexes expressed as capacity factor k'

Platinum complex	Capacity factor k'
Cisplatin	0.00
LA-4	0.41
LA-2	2.30
LA-12	9.54
LA-15	40.95

Mobile phase 41% methanol in water $K' = R_t/R_0$, where R_t is the retention time and R_0 is the dead volume (R_t for non-retained standard).

Table 2 Stability of platinum(IV) complexes LA-4, LA-2, LA-12 and LA-15 after 24 h incubation in various media at pH 7.2, 37°C

Medium	Platinum(IV) complex (% of original content)			
	LA-4	LA-2	LA-12	LA-15
Phosphate buffer	86.1	88.3	83.0	81.4
TRIS	71.1	72.7	72.0	71.3
PBS	88.3	92.3	88.4	93.3

Media: 50 mM sodium phosphate buffer, pH 7.2, 50 mM Tris–HCl, pH 7.2, PBS, pH 7.2.

Table 3 Velocity constants for the pseudo-first-order reaction of platinum drugs with glutathione

Platinum drug	Velocity constant (s^{-1})
Cisplatin	3.23×10^{-5}
LA-4	2.20×10^{-5}
LA-2	2.56×10^{-5}
LA-12	3.28×10^{-4}
LA-15	3.53×10^{-4}

Velocity constant for the pseudo-first-order reaction at pH 7.5 (20 mM sodium phosphate, 150 mM NaCl), 37°C, 1 mM glutathione. Concentration of platinum complexes in reaction mixture was 10 μM.

Table 4 Effect of pH on the velocity constants for pseudo-first-order reaction of LA-12 complex with glutathione

	pH	
	6.5	7.5
k ($\times 10^{-4}$ s^{-1})	1.37	3.28

Velocity constant for the pseudo-first-order reaction at pH 6.5 and 7.5 (20 mM sodium phosphate, 150 mM NaCl), 37°C, 1 mM glutathione. Concentration of platinum complexes in reaction mixture was 10 μM.

glutathione in comparison to cisplatin, LA-4 and LA-2. Results are shown in Table 3. The values of velocity constants for cisplatin, LA-4 and LA-2 were very close to those ones measured for platinum(II) complexes related, with respect to the same alkylamine ligands, to LA-2, LA-4, LA-12 and LA-15 (results not shown). Reactivity of LA-12 was studied at pH 6.5 and 7.5 to get experimental evidence on the higher reactivity of $-S^-$ in comparison to –SH function in glutathione. The obtained data (Table 4) supported the view that $-S^-$ is more efficient than –SH with respect to nucleophilic attack of the central platinum atom.

Antitumor evaluation

We tested cytotoxicities of a new platinum complex LA-12 on the panel of cisplatin-resistant cancer cell lines to find the boundaries of cisplatin cross-resistance. Cytostatic effects of LA-12 after 24 h exposure were compared with those of cisplatin and LA-2, which represent drugs of clinical relevance or drug in clinical trials, respectively. Results are summarized in Table 5. The cytotoxic effect of LA-12 on cisplatin-resistant cell lines is rapid and strong in comparison to cisplatin and LA-2. Lung large cell carcinoma cell line CORL23/CTR, which is cisplatin resistant and slow growing, showed the highest degree of resistance towards LA-12, even if lower than towards both compared drugs. A427 (cisplatin-resistant lung large cell carcinoma) was the only cell line sensitive to LA-2 in a 24-h exposure test. LA-12 did not demonstrate cisplatin cross-resistance within the series of A2780 cell sublines (Table 5). The cisplatin-resistant A2780 sublines represent the types of cisplatin-resistant cells in which several mechanisms of resistance are well balanced; hence, we have used these cell lines to compare the cytostatic effect of various platinum(IV) complexes. Both short continuous exposure (24 h) and long continuous exposure (72 h) were used to compare cytostatic effects of various platinum(IV) complexes. Cisplatin non-resistant parent epithelial ovarian carcinoma cell line A2780 demonstrated high sensitivity towards LA-12 and LA-15. Surprisingly, LA-2 and LA-4 were not as effective as cisplatin in both 24- and 72-h exposure experiments. The difference in cytotoxicity between LA-2 and LA-4 became negligible after 72 h exposure. Dose–response curves are shown in Figure 2.

Table 5 Cytotoxicity of cisplatin, LA-2 and LA-12 platinum complexes against cisplatin-resistant tumor cell lines

Cancer cell lines	IC_{50} (μM)		
	Cisplatin	LA-12	LA-2
K562	>80	3	63
KG-1	48	2	63
ML-2	>80	1	56
B16	>80	6	>80
HT-29N	>80	12	>80
HT29	50	8	70
HCT116	>80	9	>80
A427	63	6	13
HBL100	63	6	>80
MCF-7	71	8	70
CORL23/CTR	>80	25	56
A2780	9	6	37
A2780cis40	40	3	39
A2780cis90	>80	5	34

IC_{50} was calculated for 24 h exposure time.

The cisplatin high-resistant cell line A2780/cis90 did not demonstrate substantial differences in sensitivity towards platinum(IV) complexes in comparison with the cisplatin-sensitive A2780 parental cell line. A great difference was only seen in the sensitivity towards cisplatin. It was found for both the cisplatin-sensitive A2780 parental cell line and the high-resistant cell line A2780/cis90 cell subline, that within the series of the tested platinum(IV) complexes the cytotoxic effect was in direct proportion to their hydrophobicity. In contrast to LA-12, LA-15 and cisplatin, both LA-4 and LA-2 did not prevent a substantial fraction of A2780/cis90 cells from survival after 72 h exposure (Fig. 3). Results obtained by the MTT assay were confirmed by microscopy (results not shown).

Morphological changes of cancer cell lines after treatment by platinum complexes were observed by fluorescent microscopy. Well-developed morphological symptoms of apoptosis, such a forming of blebs and increased

Fig. 2

Dose–response cytotoxicity curves for the cisplatin-sensitive ovarian epithelial carcinoma A2780 cell line treated with various platinum(IV) complexes and cisplatin. (A) Continual exposure to various complexes for 24 h. LA-12 (IC_{50} 5.5 μM), LA-15 (IC_{50} 2.7 μM), LA-2 (IC_{50} 36.6 μM), LA-4 (IC_{50} 67.9 μM) and cisplatin (IC_{50} 8.7 μM). (B) Continual exposure to various complexes for 72 h. LA-12 (IC_{50} 2.6 μM), LA-15 (IC_{50} 1.0 μM), LA-2 (IC_{50} 15.6 μM), LA-4 (IC_{50} 17.2 μM) and cisplatin (IC_{50} 6.9 μM).

Fig. 3

Dose–response cytotoxicity curves for the cisplatin-resistant ovarian epithelial carcinoma A2780/cis90 cell line treated with various platinum(IV) complexes and cisplatin. (A) Continual exposure to various complexes for 24 h. LA-12 (IC_{50} 4.7 μM), LA-15 (IC_{50} 4.2 μM), LA-2 (IC_{50} 33.9 μM), LA-4 (IC_{50} 65.0 μM) and cisplatin (IC_{50} 83.8 μM). (B) Continual exposure to various complexes for 72 h. LA-12 (IC_{50} 1.6 μM), LA-15 (IC_{50} 1.2 μM), LA-2 (IC_{50} 19.4 μM), LA-4 (IC_{50} 18.3 μM) and cisplatin (IC_{50} 18.4 μM).

permeability of the cell membrane to YO-PRO-1, were seen in dying cells. A photograph of apoptotic HCT-116 cells is presented in Figure 4.

Discussion

With various platinum(II) complexes, an increase in ligand hydrophobicity is positively correlated with an increase of cytotoxic effects due to enhanced penetration of particular platinum complexes via non-specific diffusion to the cancer cells [8]. We have obtained a similar correlation within the set of platinum(IV) complexes for both cisplatin-sensitive and -resistant A2780 cancer cell lines. However, mere correlation between hydrophobicity and cytotoxic effects of platinum drugs is a simplification which does not take into account possible specific interactions of ligands with cellular structures, especially with the proteins involved in recognition and repair of platinum–DNA adducts. The hydrophobic character and the structure of a particular ligand inevitably influences the structure of the molecules of water solvating the hydrophobic platinum complex. Differences in the structure of the water coat could be responsible for different reactivities of platinum(IV) complexes with glutathione. Approximately one order higher reactivity of LA-12 and LA-15 with glutathione, in comparison to cisplatin, LA-2, LA-4 and other related platinum(II) complexes (results not shown), represents data pointing to the relevancy of the water coat to the reactivity of platinum complexes. The comparison of X-ray crystallographic data showed only minor differences between LA-12 and LA-2 (JM216) with respect to the bond length and angles [4]. However, the geometry of solvated complexes in the water environment may be different from those seen by X-ray crystallography.

Octahedral platinum(IV) complexes undergo ligand substitution reactions that are slow compared to those of their platinum(II) analogs and have been considered as the compounds which are unable to react directly with DNA [9,10]. It was shown that reactivity of hydroxoplatinum(IV) complexes with glutathione is low in comparison with chloroplatinum(IV) complexes [11]. Because the hydrophobic ligands did not change the velocity of the chloride substitution reaction in platinum(IV) complexes, as seen from the results on their stability after 24 h incubation in various media at pH 7.2, 37°C (Table 2), the differences in reactivity with glutathione were not related to the different stability of chloroplatinum(IV) complexes.

The anti-tumor activity of platinum(IV) compounds has been suggested to require *in vivo* reduction to the kinetically more labile, and therefore reactive, platinum(II) derivatives [17,18]. The presence of reduced glutathione is required for the reaction of tetraplatin with DNA *in vitro*. It was postulated that platinum(IV) complexes are activated intracellularly by glutathione or other intracellular reducing agents [11,12]. On the other hand, there is evidence that platinum(IV) species can enter cells and react with DNA [10]. With respect to involvement of glutathione in activation of platinum(IV) complexes, the role of glutathione in the resistance of cancer cells towards cisplatin-based drugs is equivocal, and results based on a simple correlation between intracellular level of glutathione and cytotoxicity are misleading. A more significant role of glutathione is linked with an organic anion pump, multidrug resistance protein (MRP), which could be involved in cisplatin resistance. High expression of MRP2 was found in the A2780/cis70 cell line together with increased levels of glutathione, whose conjugates are the substrates for this ATP-driven pump [9]. The ovarian epithelial carcinoma cell line A2780 and the cisplatin-resistant A2780/cis90 subline represent the types of cisplatin-resistant cells in which several mechanism of resistance are well coordinated [9]. Cisplatin-resistant A2780/cis70 cells demonstrated enhanced efflux as compared to parent A2780 cells [13]. In spite of that, cytotoxic effects of LA-12 and LA-15 towards cisplatin-resistant A2780/cis90 cells were

Fig. 2

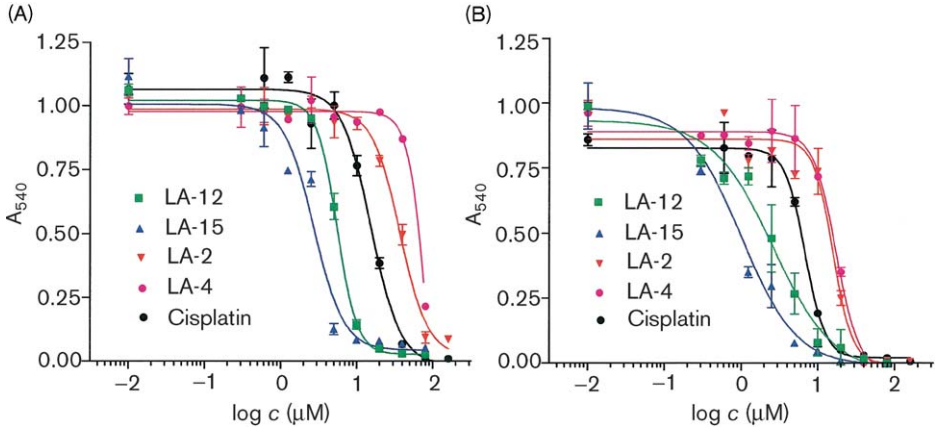


Fig. 3

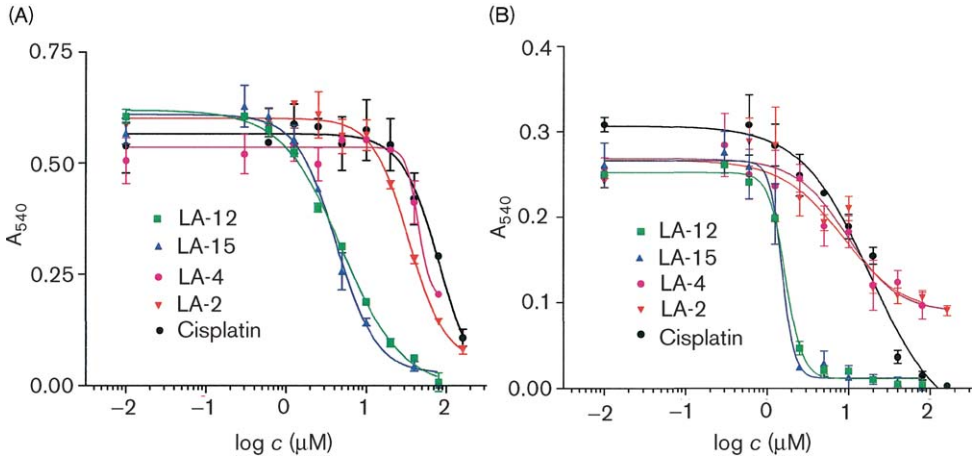
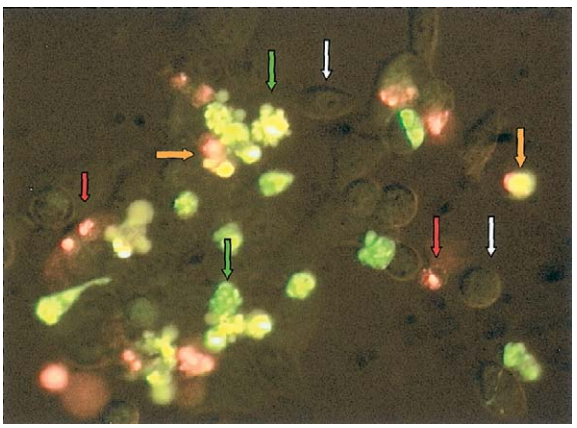


Fig. 4



Morphology of HCT116 exposed to 10 μM LA-12 for 4 h. Various stages of apoptotic cells were stained by YO-PRO-1 (green arrows). Well-developed blebs and apoptotic bodies are apparent. Dead cells are stained red by propidium iodide (secondary necrosis) (red arrows). Vital cells (white arrows) are not stained and remain adherent. The cells in the transition phase between apoptosis and secondary necrosis are stained by both dyes (orange arrows).

strong and rapid. It can be explained by rapid penetration through the cell membrane, activation by glutathione and interaction with DNA. Rapid induction of apoptosis, when the first morphological changes were seen after 3–4 h of incubation (Fig. 4), implicates that LA-12 and LA-15 can activate pro-apoptotic pathways immediately after entering the cell and forming DNA adducts. Such a rapid onset of apoptosis in treated cells can avoid the replication bypass.

In conclusion, the phenomenon of drug resistance is very complex and little information is available on how disparate mechanisms are coordinated with each other. Resistance to cisplatin is multifactorial, and in general it may consist of mechanisms either limiting the formation of DNA adducts and/or increasing the tolerance of DNA adducts. It has been shown that the tolerance to cisplatin-induced damage of DNA was the fundamental mechanism which caused increased cisplatin resistance in some ovarian carcinoma cell lines. Resistance towards cisplatin displayed by the cisplatin-resistant A2780 cell subline could not be solely explained by enhanced repair

of the lesions, but was more likely related to some mechanisms of damage tolerance [14–16]. Both signaling and executive mechanisms of apoptosis can be suppressed in cisplatin-resistant cancer cells to induce the tolerance towards DNA damage. LA-12 proved to be very cytotoxic in the 24-h MTT test against various cancer cells representing different types of cancer and employing different mechanisms of cisplatin-resistance towards cisplatin (Table 5). The strong cytotoxic effect is relevant to the presence of bulky hydrophobic ligands in the platinum(IV) complex. Further studies on the mechanism of cytotoxic effects of LA-12 are focused on its interaction with DNA, the DNA repair system and apoptotic pathways. Highly cytotoxic effects against leukemic, melanoma and colorectal cancer cell lines is promising for the prospective application of the therapy of the corresponding tumors.

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