Synergistic antitumor effect of cisplatin and the platinum(IV) nitroxyl complex BC118 and the development of resistance to their combined action

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The efficacy of the combination treatment of P388 leukemia with cisplatin (cPt) and the platinum(IV) nitroxyl complex BC118 [*e*-ammine-*d*-(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)-*a*,*f*-bis(acetato)-*b*,*c*-dichloroplatinum(IV)] applied in low doses was found to be synergistically increased. Thus, the use of 1/10 of their LD_{50} cured 100% of animals. The monotherapy with these drugs used in the same doses and at the same schedules showed moderate efficacy, and no animals survived. A synergistic increase in the toxicity was not observed. The rate of the development of resistance decreases in the series cPt+BC118, cPt, and BC118. Strains resistant to cPt+BC118 and BC118 are highly sensitive to doxorubicin, etoposide, and cyclophosphane.

Key words: cisplatin, platinum(IV) complexes, mouse P388 leukemia, combination therapy, synergism, resistance.

Cisplatin (cPt) is a highly effective anticancer drug, which has been used for more than three decades for the treatment of various neoplasms both individually and as the main component in different combination chemotherapy regimes.[1—3] Like other Pt^{II} complexes, cisplatin binds to purine and pyrimidine bases of DNA to form intra- and interstrand cross-links.[4] DNA-platinum adducts disrupt the normal function of DNA and finally cause the cell death.

However, it is impossible to achieve the maximum efficacy of cisplatin because of its considerable overall toxicity and the rapid development of resistance. This gave impetus to the search for substitutes for cisplatin, resulting in the synthesis of numerous new platinum complexes. The most well known are carboplatin (the second-generation drug) and oxaliplatin (the third-generation drug), which are used in the clinic. The spectrum of toxicity of these drugs differs from that of cisplatin.[2,3] Both drugs do not exhibit nephrotoxicity typical of cisplatin, but they suppress hematopoiesis, the effect of carboplatin being stronger than that of oxaliplatin. Moreover, oxaliplatin showed experimental activity against tumors resistant to cisplatin and carboplatin. However, the situation has not radically changed. Hence, the extensive search for ways to increase the efficacy of platinum agents and reduce their drawbacks is going on.

The toxicity of cisplatin and its analogs is associated with high reactivity of divalent platinum. One of ways of improving the therapeutic properties of platinum drugs is based on transformations of Pt^{II} compounds to less reactive Pt^{IV} complexes and the introduction of biologically active ligands into their structures. We implemented this idea in the synthesis of a series of platinum(IV) nitroxyl complexes containing biologically active nitroxides covalently bound to the platinum pharmacophore. At low concentrations, nitroxides exhibit antioxidant properties,[5] whereas at higher concentrations ($\geq 10^{-3}$ mol L^{-1}) they have cytotoxic effect.[6] It should be noted that literature shows contradictory results regarding the influence of antioxidants on the cancer chemotherapy. Thus, depending on the tumor type and the nature of the drug, the effect may be either positive or negative.[7] The synthesized platinum(IV) nitroxyl complexes exhibited high antitumor activity comparable to that of cisplatin and lower toxicity.[8]

Most of the data indicate that amino complexes of Pt^{IV} are prodrugs. In cells, these complexes are reduced to more active Pt^{II} analogs, which perform cytotoxic functions.[9,10] We suggested that the combined use of cPt and platinum(IV) nitroxyl complexes can improve the treatment efficacy.

One of the main problems of modern anticancer therapy is that tumors develop resistance to the drugs used. Earlier,[11] we have found that the introduction of the nitroxyl group into the known drugs leads to a certain retardation of the development of resistance.

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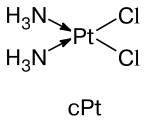
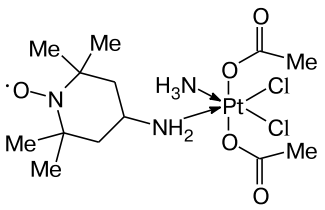
In the present study, we investigated the efficacy against P388 leukemia of the combination of cPt and the complex BC118 [*e*-ammine-*d*-(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)-*a*,*f*-bis(acetato)-*b*,*c*-dichloroplatinum(IV)], which was the most active in our earlier studies. We also examined the development of resistance of tumors to this therapy and to the monotherapy with the use of each component of the combination therapy. We studied the sensitivity of drug-resistant strains to known antitumor drugs.



cPt



BC118

Experimental

Analysis of the platinum content was performed by atomic absorption spectroscopy on an AAS-3 spectrometer, the accuracy of the determination was ±3 rel.%. The IR spectra were recorded in the range of 400—4000 cm^{-1} on a Specord 75-IR spectrometer in Nujol mulls. The electronic spectra were measured in the range of 200—800 nm on a Specord UV—Vis spectrophotometer. The ESR spectra were obtained at ~20 °C on a SE/X 2544 instrument at the microwave power of 2 mW and modulation amplitude of 0.032 mT.

Synthesis of the complex BC118. The starting complex BC114 [*e*-ammine-*d*-(4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)-*a*,*f*-dihydroxo-*b*,*c*-dichloroplatinum(IV)] was synthesized according to the procedure described earlier.[12] Freshly distilled acetic anhydride (7 mL) was added with stirring and cooling with ice to the thoroughly ground complex BC114 (1.20 g, 2.46 mmol). After dissolution of BC114, the ice bath was removed. The course of the reaction was monitored by TLC on Silufol plates with the use of a MeCN—MeOH mixture (2 : 1) as the eluent, R_f was 0.35 and 0.77 for BC114 and BC118, respectively. No starting complex BC118 was detected in the reaction mixture after ~0.5 h. The overall reaction time was 1 h. The reaction product was precipitated by the slow addition of a 1 : 1 diethyl ether—hexane mixture (20 mL). The suspension was kept for 20 min until the crystallization was completed. The product was filtered off, washed with a 1 : 1 diethyl ether—hexane mixture (3×3 mL), and dried in air. The complex BC118 was obtained in a yield of 1.14 g (81%) as orange crystals, m.p. 222—224 °C (decomp.) (from MeOH—Et_2O). Found (%): C, 27.10; H, 4.82; Cl, 12.50; N, 7.24; Pt, 34.76. $C_{13}H_{28}Cl_2N_3O_5Pt$ (molecular weight 572.37). Calculated (%): C, 27.28; H, 4.93; Cl, 12.39; N, 7.34; Pt, 34.08. UV (H_2O), λ_{max}/nm (ε/L mol^{-1} cm^{-1}): 442 sh (19), 372 sh (120), 216 (33000). IR (Nujol mulls), ν/cm^{-1}: 1573, 1670, 3108, 3178, 3242 (N—H, C=O). ESR (H_2O): three lines, *g* factor 2.0056, a_N = 1.693 mT.

P388 Leukemia was maintained in DBA/2 mice by the tumor transplantation of 10^6 cells per mouse weekly. Experiments on the chemotherapy and the induction of resistance were carried out in hybrid mice (DBA/2xC57/Bl)F1.

The following drugs were used: cisplatin, etoposide, methotrexate, 5-fluorouracil (Ebeve, Austria), doxorubicin (Teva, the Netherlands), rubomycin, cyclophosphamide (Russia), gemzar (Lilly, France), and mitomycin C (Kiowa, Japan). All drugs were injected intraperitoneally in a volume of 0.2 mL per 20 g mouse. Cisplatin, 5-fluorouracil, and cyclophosphane were dissolved in a sterile physiological solution. Methotrexate, adriamycin (doxorubicin), rubomycin, mitomycin C, and BC118 were dissolved in sterile water. The sensitivity to the therapy was evaluated from the number of cured animals (animals that remained alive for 60 days after the tumor transplantation), as well as from the index ILS characterizing the increase in the median life span of treated mice compared to control animals. In the calculations of ILS, the life span of the cured animals was taken to be equal to 60 days.

The toxicity of the drugs was estimated according to conventional procedures.[13,14]

Resistance was induced by the successive transplantation of tumor cells from animals treated with cPt (strain P388/cPt), the complex BC118 (P388/BC118), or their combination (P388/cPt+BC118). The drugs were used in equitoxic doses and were injected on days 1, 3, 5, and 7 after the tumor transplantation. The induction was started with the injection of low doses of the drugs, which were gradually increased after each two successive transplant generations synchronously in all strains. In each generation, the sensitivity of each strain to the inductor drug and to the other two medications applied in therapeutic doses was investigated. The resistance was assumed to be formed when the sensitivity of the tumor to the drug-inductor decreased by at least 80%.

In all experiments, six—ten mice per group were used.

Results and Discussion

The determination of the toxicity of both compounds showed that the complex BC118 is almost four times less toxic than cisplatin (Table 1).

The results of the combined use of cPt and BC118 are presented in Table 2.

As can be seen from Table 2, the combination treatment was always more effective than the monotherapy. In the case of the combination treatment, a considerable number of cured animals was observed in all groups. The maximum antitumor effect (100% of survivors) was achieved by combining low doses of the drugs and their fourfold injections. In this case, the dose of cisplatin and the complex BC118 was only 1/10 of their LD_{50}. It should be noted that the monotherapy with each drug of the com-

Table 1. Toxicity of cisplatin and the complex BC118 for mice

Agent	LD_{100}	LD_{50}	MTD*
	mg kg^{-1}		
Cisplatin	16	12	8
Complex BC118	50	46	30

* MTD is the maximum tolerated dose.

Table 2. Sensitivity of P388 leukemia to the combination therapy with cisplatin and the complex BC118 and the monotherapy with each agent (agents were given once in the morning)

Agent	Dose /mg kg^{-1}	Dosage schedule (days after transplantation)	Percentage of survived animals	ILS
			%	
cPt	0.6	1, 3, 5, 7	0	130
	1.2	1, 3, 5, 7	0	264
	2	1, 3, 5, 7	66	362
BC118	2.3	1, 3, 5, 7	0	100
	4.6	1, 3, 5, 7	0	187
	7.7	1, 3, 5, 7	50	347
cPt+BC118*	0.6 + 2.3	1, 3, 5, 7*	66	366
	1.2 + 2.3	1, 3, 5, 7*	66	409
	2.0 + 2.3	1, 3, 5, 7*	100	446
	1.2 + 4.6	1, 3, 5, 7*	100	506
cPt	4	1, 4, 7	67	392
BC118	15.3	1, 4, 7	33	327
cPt	1	1, 5, 9	0	236
	2	1, 5, 9	0	289
	4	1, 5, 9	50	367
BC118	5	1, 5, 9	0	133
	10	1, 5, 9	0	242
	15.3	1, 5, 9	50	340
	17	1, 5, 9	0	266
cPt+BC118*	1 + 10	1, 5, 9*	33	433
	1 + 15	1, 5, 9*	50	428
	1 + 17	1, 5, 9*	50	467
	2 + 10	1, 5, 9*	83	539
	2 + 15	1, 5, 9*	67	542
	2 + 17	1, 5, 9*	17	144

* The agents were injected simultaneously.

bination applied in the same doses and schedules gave moderate efficacy, and there were no survivors. This synergistic effect of the combination therapy can be attributed to the effect of both drugs on the same target. It is known[15] that the formation of adducts with DNA is the key step in the mechanism of action of cPt and its analogs. Its quite possible, BC118 has an effect of its own. Model experiments showed that amino complexes of Pt^{IV} can oxidize guanine to 8-oxoguanine in double-stranded oligonucleotides,[16] which can be indicative of the additional effect on DNA. The efficacy and/or selectivity of the antitumor effect can be influenced by possible differences in the pharmacokinetics of Pt^{II} and Pt^{IV} complexes. The effect of the nitroxide own cannot be ruled out as well.

The high antitumor activity of the cPt+BC118 combination immediately gave rise to the question about the development of drug resistance. To induce the resistance to the combined action of cPt and the complex BC118, we used the schedule, which cured 100% of animals (the fourfold injections of cPt (1.2 mg kg^{-1}) and BC118 (4.6 mg kg^{-1})). The results are shown in Fig. 1.

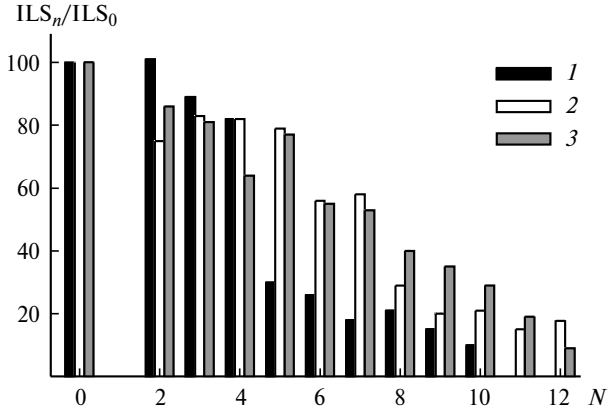


Fig. 1. Development of resistance to the combination therapy with cPt+BC118 (*1*), the monotherapy with cisplatin (*2*), and the monotherapy with the complex BC118 (*3*); *N* is the number of successive transplantation generations, ILS_0 is ILS in the 0th generation, *i.e.*, before the induction of resistance, $ILS_{1,2,3...n}$ is ILS in each particular successive transplant generation.

As can be seen from Fig. 1, the resistance to BC118 was most slowly developed (in the 11th transplant generation). The resistance to cPt was developed in the ninth generation. The resistance was most rapidly developed to the cPt+BC118 combination (in the seventh generation). The more rapid development of resistance to the combination therapy can be associated with the fact that divalent platinum complexes are active agents in both cases,[9,10] and high cytotoxicity causes the enhanced DNA repair[15] (the main mechanism of the development of resistance). In this case, the formation of resistance should occur more rapidly compared to the monotherapy following the principle that the stronger the action the stronger the counteraction. In the case under consideration, the rate of the development of resistance to cPt differs from that to the cPt+BC118 combination by only two generations. It should be noted that the efficacy of this combination holds promise that the complete cure will be achieved before the development of resistance.

If the cancer treatment leads to the development of drug resistance, the question arises as to what drugs should be used for the further treatment. To answer this question, we studied the sensitivity of the drug-resistant tumors to a series of conventional antitumor drugs: mitomycin C (1.5 mg kg^{-1}; the injection on days 1 and 6 after the tumor transplantation), etoposide (20 mg kg^{-1}; on days 1 , 5, and 9), cyclophosphane (100 mg kg^{-1}; on days 1 and 5), cisplatin (4 mg kg^{-1}; on days 1, 5, and 9), 5-fluorouracil (15 mg kg^{-1}; on days 1—6), methotrexate (1 mg kg^{-1}; on days 1—6), doxorubicin (7.5 mg kg^{-1}; on 1 day), and gemzar (25 mg kg^{-1}; on days 1 and 5). All drugs were injected intraperitoneally. The results are presented in Table 3.

As can be seen from Table 3, the tumor P388/cPt+BC118 proved to be most sensitive to chemotherapy.

Table 3. Efficacy of antitumor drugs in drug-resistant strains and the initial P388 tumor*

Drug	P388/cPt+BC118		P388/cPt		P388/BC118		P388	
	Y	ILS	*Y*	ILS	*Y*	ILS	*Y*	ILS
Etoposide	83	440	83	467	83	403	100	567
Cyclophosphane	50	338	0	271	83	462	17	433
Cisplatin	0	81	0	23	0	121	67	480
Mitomycin C	17	245	0	68	0	71	0	158
Doxorubicin	100	471	33	298	50	372	0	242
5-Fluorouracil	0	126	0	31	0	90	0	98
Methotrexate	0	159	0	100	0	116	0	126
Gemzar	0	79	0	75	0	71	0	87

* The percentage of survivors (Y (%)) and ILS (%) are given.

Thus, doxorubicin cured all animals of this strain; etoposide and cyclophosphane also showed high efficacy. The sensitivity of the strain P388/BC118 to the drugs is comparable with that for P388/cPt+BC118, although the particular values are somewhat different. It should be emphasized (it is very important) that both strains under consideration retain partial sensitivity to cisplatin. The sensitivity of the strain P388/cPt to the drugs is somewhat lower than that for the other two drug-resistant tumors, except for etoposide. In the latter case, the sensitivity was approximately equal for all three drug-resistant tumors.

Therefore, the combination treatment of the P388 leukemia with cisplatin and BC118 applied in low doses leads to a synergistic increase in the efficacy (100% of animals were cured) compared to the monotherapy with these drugs. It should be noted that the synergistic increase in the toxicity was not observed, which is very important because this therapy can lead to a decrease in toxic effects typical of cisplatin and its analogs. The rate of the development of resistance decreases in the series cPt+BC118, cPt, and BC118. The strains resistant to cPt+BC118 and BC118 are highly sensitive to doxorubicin, cyclophosphane, and etoposide.

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