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Binary Catalytic Therapy: A New Approach to Treatment of Malignant Tumors. Results of Pre-clinical and Clinical Studies

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Abstract—Novel "Oxicobalamin+Ascorbic acid," "Teraphthal+Ascorbic acid," and "Efiter+Ascorbic acid" binary catalytic systems (BCS) have been studied in the pre-clinical experiments and clinical trials. It was shown in experiments that Oxycobalamin, Teraphthal, and Efiter-based BCS demonstrated moderate antitumor efficiency against murine and rat transplanted tumors of various histogenesis, as well as high modifying activity for different official antistatic agents, radiotherapy, chemo-radiotherapy, and local laser-induced hyperthermia. The studied BCS had no pronounced toxicity in mice, rats, or dogs, so they were considered to be moderately hazardous pharmacologic agents. Revealed toxic and side effects of BCS were dose-dependent and totally reversible. Clinical trials of the BCS demonstrated good tolerability in cancer patients with exhausted possibilities of antitumor treatment and moderate antitumor efficacy. Partial regress and stabilization of the malignant processes were observed with various routes of catalysts administration.

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INTRODUCTION

Binary catalytic therapy (BCT) is a new direction in drug therapy of cancer, whose treatment effect is based on destruction of the tumor tissue by reactive oxygen species and other free radicals generated by catalytic oxidation of a biogenic substrate, specifically, of an ascorbic acid.

The research on binary catalytic systems with antitumor activity was initiated by the Member of the RAS M.E. Vol'pin and supported by the Corr. Members of the RAS G.N. Vorozhtsov and Yu.M. Luzhkov. Later the groups headed by Profs. E.A. Luk'yanets and I.Ya. Levitin in NIOPIK SRC and in the Institute of Organoelement Compounds, RAS, synthesized more than 50 cobalt and nickel complexes with macrocyclic chelating ligands, as well as cobalt com-plexes with tridentate ligands (Schiff bases), which together with a reducing agent (ascorbic acid) efficiently generated reactive oxygen species and other free radicals.

In the present work we have selected three cobalt complexes for the in-depth study of their chemical, physical, and biological properties:

– oxycobalamine (Coα-[α-(5,6-dimethylbenz-imidazolyl)]-Coβ-hydroxycobamide hydrochloride);

– teraphthal (octasodium salt of cobalt(II) octa-4,5-carboxyphthalocyanine) [1, 2];

– efiter (adduct of tetra[methylpenta(hexa)ethylene glycol] ester of cobalt(II) salt of phthalocyanin-

† Deceased.

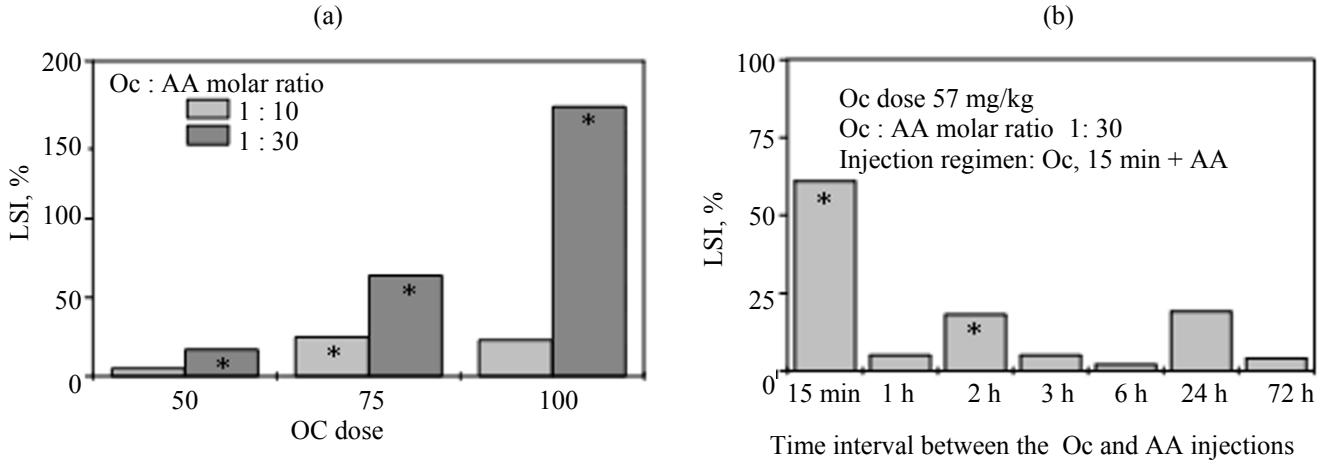

Fig. 1. Antitumor efficiency of the oxycobalamine (Oc) + ascorbic acid (AA) system: dependence of the lifespan increase (LSI) of EAC mice on the (a) Oc dose and Oc : AA molar ratio and (b) time interval between the Oc and AA injections. Female SHK mice. Tumor cells were implanted into peritoneum. The components of the binary system were injected intraperitoneally. The LSI of test mice reliably differed from that in control mice at $p < 0.05$. The test and control groups included 10 and 12 mice, respectively.

2,3,9,10,16,17,23,24-octacarboxylic acid with poly-*O*-(1-hydroxy-2-propyl)cycloheptadextrin [3]).

The research was performed along the following lines.

(1) Development of methodical approaches to testing the anticancer properties of binary catalytic systems in animals with implanted malignant tumors.

(2) Development of pharmaceutical formulations and medical drugs on their basis.

(3) Pre-clinical study of the antitumor efficiency and toxicity of the binary systems on the basis of oxycobalamine, teraphthal, and efiter, as well as their chemo- and radiomodifying effects.

(4) Clinical study of binary systems.

(5) Study of the possibilities to enhance the efficiency of catalytic systems.

(6) Study of the mechanism of action of binary catalytic systems.

Methodical approaches to testing the antitumor properties of "metal complex + ascorbic acid" systems in animals with implanted malignant tumors.

At first, we had to choose optimal protocols for catalyst and reducing agent administration to animals with transplanted tumors to ensure the highest efficiency of the binary system.

In mice with Ehrlich ascites carcinoma (EAC) and breast adenocarcinoma 755 (Ca-755) we studied the dependence of the antitumor effect of the binary systems, on the basis of oxycobalamine, efiter, and teraphthal, on the dose of the metal complex, molar (dose) ratio of the metal complex and ascorbic acid, as well as on interval and order of their injection. It was found out that the efficiency of the binary systems after intraperitoneal (EAC mice) or intravenous (Ca-755 mice) infusions enhanced with an increasing dose of the metal complex (at invariable molar ratio of the components) and molar ratio of the components (at invariable dose of the metal complex).

The antitumor efficiency of the binary systems injected intraperitoneally in EAC-bearing mice was more sensitive to the molar ratio of the components than to the dose of the metal complex. The highest efficiency of the "oxycobalamine + AA" and "efiter + AA" systems was observed after intraperitoneal injections at the components molar ratio of 1 : 30 (Fig. 1). The efficiency of the binary catalytic system on the basis of oxycobalamine in Ca-755-bearing mice was equally dependent on the dose of oxycobalamine and on the component molar ratio.

The antitumor effect of the binary systems proved to be highly dependent on the time interval between catalyst and reducing agent injections. Thus, the best result in EAC-bearing mice was obtained when the metal complex was injected into peritoneum 1–5 min before the ascorbic acid infusion. When the time interval between the injections of oxycobalamine or efiter, and ascorbic acid, was prolonged from 30 min

to 1 h, the efficiency of the binary systems sharply decreased, while their toxicity increased. Inverse dependence of the therapeutic and toxic effects on the time interval between the injections of the system components was observed after intravenous injection of the oxycobalamine system in Ca-755-bearing mice. The highest efficiency was observed when the ascorbic acid was injected 1 h after the oxycobalamine (Fig. 2). The oxycobalamine and efiter systems showed antitumor activity only if the metal complex was injected before the reducing agent [4, 5]. The same regularities were revealed with the "teraphthal + AA" system [2].

Thus, optimal regimens have been developed for the treatment of animals bearing transplanted ascites and solid tumors by intraperitoneal and intravenous injections of metal complexes (oxycobalamine, efiter, and teraphthal) (Mc) and ascorbic acid (AA):

Mc, 0–15 min → AA (Mc : AA molar ratio 1 : 30); Mc, 60 min → AA (Mc : AA molar ratios 1 : 10 and 1 : 30).

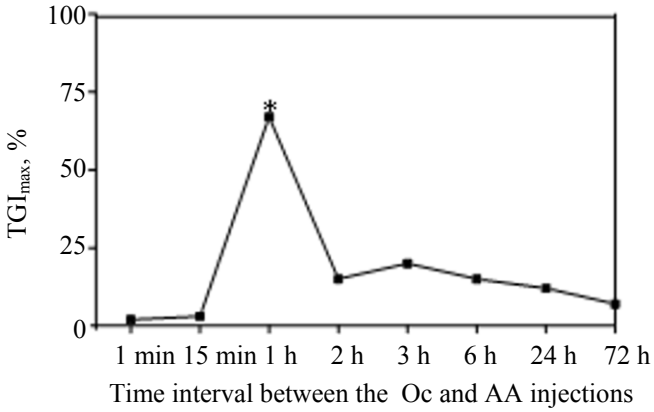

Fig. 2. Dependence of the effect of the oxycobalamine (Oc) + ascorbic acid (AA) system on tumor growth inhibition (TGI) in mice with Ca-755 carcinoma on the time interval between the Oc and AA injections. Female BDF1 hybrid mice. The Ca-755 tumor tissue was implanted subcutaneously. Oxycobalamine and ascorbic acid were injected intravenously in doses of 50 and 191 mg/kg, respectively (Oc : AA molar ratio 1 : 30). The tumor volume in test mice reliably differed from that in control mice at $p < 0.05$. The test and control groups included 10 and 12 mice, respectively.

Development of Pharmaceutical Formulations and Drugs for Binary Catalytic Therapy

The NIOPIK SRC, Hertsen MCRI, and Blokhin RCRC RAMS have developed technologies for manufacture of pharmaceutical formulations of oxycobalamine, teraphthal, and efiter, as well as drugs on their basis, including lyophilized powders for solutions for intravenous administration, with active ingredient contents of 0.5, 0.010, and 0.5 g, respectively.

Pre-clinical Study of the Binary Catalytic Systems

Anticancer Efficiency of the "Oxycobalamine + Ascorbic Acid" System in vivo

The pre-clinical study of the antitumor efficiency of the binary systems in monotherapy was performed in mice and rats with implanted tumors of different histogenesis and growth patterns: EAC, P-388, L-1210, Ca-755, LLC, B-16, and Pc-1.

The "oxycobalamine + AA" binary system showed antitumor activity against five of the seven studied tumors. Subcutaneously transplanted Ehrlich ascites carcinoma [life span increase (LSI) was 102%] and P-388 lymphatic leukemia [the tumor growth inhibition (TGI) was about 85–57% within 10 days post-treatment] were found to be the most sensitive to the therapy with this binary system. The P-388 lymphatic leukemia implanted to peritoneum showed medium sensitivity (LSI 25%); the same relates to Ca-755, LLC, and Pc-1 solid tumors (TGI 77–51% within 19–21 days of post-treatment). The L-1210 lymphatic leukemia implanted to peritoneum and solid B-16 melanoma proved to be insensitive to treatment with the binary system.

The resulting data provide evidence for broad-range antitumor activity and medium antitumor efficiency of the "oxycobalamine + AA" system. It should be noted that the efficiency of this binary catalytic system in mice with subcutaneously implanted EAC and P-388 lymphatic leukemia corresponds to that of cisplatin [4, 6].

General Toxicity of the "Oxycobalamine + Ascorbic Acid" System

The general toxicity of the binary system after single (acute toxicity) and multiple (chronic toxicity) administration of its components was studied in three animal species: mice, rates, and dogs [6].

The quantitative parameters of the acute toxicity of "oxycobalamine + AA" system demonstrate the broad range of its therapeutic, toxic, and lethal effects. It was revealed that the system is more toxic in

intraperitoneal than in intravenous administration. No gender dependence of the toxic effect was observed. Dogs were found to be more sensitive than mice to intravenous administration of the system in doses reduced to body weight, and less sensitive than mice and rats to doses reduced to body surface area. Thus, in terms of hazard classification according to the acute toxicity, the "oxycobalamine + AA" system can be rated as a moderately hazardous drug.

The selective acute and chronic toxicity targets of the "oxycobalamine + AA" system in rats are blood, kidneys, vessels, and central nervous system. The toxic and other side effects of this system in animals were found to be dose-dependent and reversible, and were observed at lethal, highly toxic, and low toxic doses. The functional reversibility of the toxic and other side effects, evaluated in chronic experiments with the oxycobalamine system, was 15–60 days.

The "oxycobalamine + AA" system showed the absence of pneumo-, cardio-, and gastrointestinal toxicity. In view of the dose dependence and reversibility of the toxic effects of the "oxycobalamine + AA" system and its accumulation index in chronic toxicity study, this system can be classified as a low-hazardous drug.

The binary system did not produce any damage in normal tissues with expressed proliferative activity. With few exceptions, all antitumor cytostatics used in clinics cause hematopoiesis inhibition and dyspepsia syndrome. The "oxycobalamine + AA" system caused no toxic leucopenia, granulocytopenia, or lymphopenia. No intestinal toxicity or anorexia were either observed.

Antitumor Efficiency of the "Teraphthal + Ascorbic Acid" System in vitro and in vivo

The optimal conditions for the highest cytotoxic effect of the "teraphthal + AA" system, established in *in vitro* experiments are as follows [7]: simultaneous or consecutive (first teraphthal and then ascorbic acid, time interval $\leq$ 1 h) injections of the components in concentrations of 50 μmol and 500 μmol (molar ratio 1 : 10), respectively.

The cytotoxic effect of this system was observed *in vitro* against 9 human cancer cell lines of various histogenesis: CaOv ovarian cancer, K562 myeloleukemia, A549 lung cancer, MCF7 breast cancer, and other cell lines.

Higher sensitivity (in comparison to the parent cell lines) to this system was observed in tumor cell lines with the multidrug resistance (MDR) phenotype associated with the hyperexpression of the MDR1 gene and its daughter PgP170 transporter protein [8]. The research was performed using cell cultures sensitive to MDR-inducing drugs: colchicin, doxorubicin, and vincristine. The cytotoxicity of the binary system with the 1 : 10 component ratio was assessed by means of the cell viability assay. The PgP-mediated MDR was induced after the parent cells were selected by viability in the presence of colchicin (McA-RH 7777/0.4 cell subline), and doxorubicin (K562/4 and MCF7/Dox cell sublines), and transfected with MDR1 cDNA gene [K562i/S9 cell subline provided by I. Roninson (Chicago, USA)] [9]. The COR/L23R cell subline with the MDR-mediated MDR phenotype was obtained after the cells were selected by viability in the presence of doxorubicin. This subline was provided by P. Twentimann (Cambridge, England).

It was found that teraphthal alone in concentrations less than 1×10^{-4} M does not show cytotoxicity either to the parent cell lines or to those with induced resistance. At the same time, the "teraphthal + AA" combination (1 : 10 component molar ratio) proved to be double as toxic to MDR cells as to the parent cell lines [8]. At the same time, neither teraphthal (10^{-5} M), nor its combination with ascorbic acid, affected the cytotoxicity of vincristine or doxorubicin. However, in the same cell system verapamil (the PgP-mediated MDR modulator) in 5×10^{-6} M concentration demonstrated pronounced chemosensitizing activity. Thus, the "teraphthal + AA" system does not inhibit the PgP protein, because its single administration does not enhance the cytotoxicity of doxorubicin. However, K562/4 cells with induced resistance to doxorubicin demonstrated enhanced sensitivity to doxorubicin after treatment with this system through 8 passages of cells. The IC50 values for sensitive and resistant K562 cells became equal in the 8th cycle. The fact that the resistant cells recover their sensitivity to doxorubicin is probably explained by faster death of resistant cells compared to sensitive ones. The obtained data suggest that during repeated treatment cycles with the binary system tumors may recover their sensitivity to MRD-inducing drugs.

The antitumor activity of the "teraphthal + AA" system was studied in mice and rats with implanted tumors using intravenous, intra-arterial, intraperitoneal, and intra-pleural routes of administration [10].

The highest therapeutic effect was obtained after a single intravenous injection by the following scheme: teraphthal (20–40 mg/kg) → 1 h → ascorbic acid. Fifteen types of tumors of different histogenesis and growth patterns were implanted in mice and rats: hemablastoses (P-388, L-1210, MOPS 106), Ehrlich ascites carcinoma, G-22A hepatoma, Zeidel ascites hepatoma, Ca-755, LLC, B-16, and Pc-1 solid tumors, etc. Tumors were implanted subcutaneously on the body's side, as well as intraperitoneally, or intravenously. The binary system was injected in 20 + 44, 30 + 66, and 40 + 88 mg/kg doses using the optimal therapeutic scheme.

As a result, much extended life spans and significantly inhibited growth of solid tumors were observed in the animals bearing EAC and hepatomas. Nine of 15 studied tumors, including five solid tumors in mice, one leukemia, two ascites hepatomas, and one solid rat cancer, proved to be sensitive to binary therapy. After intraperitoneal injection, successful therapy in a part of animals with ascites hepatomas was observed only when the binary system was injected in doses close to the maximum tolerated dose (MTD).

Antitumor Efficiency of the "Teraphthal + Ascorbic Acid" System in Treatment of Plural Tumors

Tumoral (metastatic) pleuritis is a common complication of lung, breast, and ovarian cancers, as well as lymphomas and leukemia, and it complicates treatment of cancer patients. The ability of reactive oxygen species to damage serous membranes formed the basis for the assessment of the biological activity of the catalytic system injected intrapleurally for treatment of experimental pleuritis tumor. In was shown in healthy mice that teraphthal and ascorbic acid injected separately caused no sclerosis of the pleural cavity. At the same time, joint injection of the components of the binary system in the teraphthal doses ≥50 mg/kg (component molar ratio 1 : 10) led to pleurodesis. The selectivity of the pleural sclerosing effect, as measured by the therapeutic index (TI) (ratio of the maximum tolerable and effective pleural sclerosing doses in the catalytic pair with a fixed dose of teraphthal and the component ratio 1 : 100), was 1.82. At the component ratio 1 : 10, the therapeutic index was 1.26, i.e., the therapeutic window increased by 1.5. This result implies that at a fairly low therapeutic dose of teraphthal, the increasing dose of ascorbic acid not only does not reduce tolerance to therapy with the catalytic system, but also extends the therapeutic potential of the latter [11].

Toxicity studies on animals (mice and dogs) showed a highly efficient pleural sclerosing effect in intrapleural injection of the teraphthal binary system in a concentration of no less than 0.5% in doses comprising 0.5 MTD for intravenous injection in mice. The good tolerance to therapy is associated with the attenuation of the resorptive effect of teraphthal because of the development of a local cytotoxic reaction in the pleural cavity. Pharmacokinetic study showed that teraphthal injected intrapleurally is absorbed slower from the pleural cavity and not completely, unlike the intravenously injected teraphthal [12, 13].

The resulting data allow the "teraphthal + AA" system to be recommended for clinical trials in cancer patients with pleural tumor [14,15].

Efficiency of the "Teraphthal + Ascorbic Acid" System on Regional Intra-Arterial Administration

The experiments were performed on outbred male rats with a PC-1 tumor suspension (40 mg) injected subcutaneously in the left leg so that tumors developed in the femoral basin [11]. Within 11–14 days after implantation, the tumor volume reached 2525± 641 mm^3. After intra-arterial injection of the "teraphthal + AA" catalytic system, the effect was observed in all used therapeutic treatment schemes. The tumor growth delay was 8–20 days, and the longest delay was at the teraphthal dose of 2.5 mg per rat (5 mg/kg, component molar ratio 1 : 10). Complete remission lasting a month was observed in a part of rats.

Selective Occlusion Technique for Treatment of Liver Cancers

Among organs with arterial blood supply, the best pharmacokinetic parameters of teraphthal were observed with liver (affinity factor 116, average retention time of teraphthal 163 h). To ensure the closest contact of the components of the "teraphthal + AA" system and a long interaction time with the tumor, a selective occlusive technique was developed [16]. The technique consists of the enclosure of the tumor-containing lobe of the lung. This allows one to create a high concentration of teraphthal in the blood stream and prevent its washout into the systemic circulation and delivery to other tissues. As a result, the second component of the binary system (ascorbic

acid) is introduced on the background of a high level of teraphthal in the liver.

Preclinical study of the selective occlusion technique was performed on rats with implanted liver tumors: hepatoma 27 and mucus-secreting cholangiocellular cancer RS-1 [17]. At teraphthal doses varying from 0.3 + 0.66 mg/kg (1/100 MTD) to 1.5 + 3.3 mg/kg (1/10 MTD), a more than 90% tumor growth inhibition and marked tumor pathomorphism were observed. Thus, this injection technique ensures a high antitumor effect of the binary catalytic system at teraphthal doses less than 1/10 MTD for systemic injection.

Safety assessment (toxicity and adverse effects on organs and tissues) of the "teraphthal + AA" system on the selective occlusion injection in liver was performed using beagle dogs [18]. Teraphthal solutions (7.5–25 mL) of concentrations of 0.1–0.003% (1/10 and 1/100 toxic dose for dogs, adequate to the nominal dose for humans, used at phase II of clinical trial of systemic intravenous administration) were injected. The following adverse effects were observed: changes in the peripheral blood (leukocytosis, accelerated ESR), changes in the liver (reduction of albumin, increased activity of ALT, AST, and LDH transaminases and alkaline phosphatase), pulmonary engorgement (microstasis) associated with temporary microcirculatory disorders, microthrombus formation, and adhesion.

The functional and morphologic changes in organs and tissues, observed after selective occlusive injection of the "teraphthal + AA" system in dog liver, are fully reversible and not specific of teraphthal. These changes were considered to follow surgery and anesthesia. In terms of local irritation, 0.05–0.025% teraphthal solutions in volumes of up to 20 mL were found to be the best choice. Selective occlusion made it possible to produce a high liver concentration of teraphthal at low-dose injection, which correlates with the high degree of tumor pathomorphosis. These data provide evidence for the advantage of selective occlusion over systemic administration in the treatment of liver cancers.

Thus, the "teraphthal + AA" system injected by the selective occlusion technique shows significant efficiency and produces no adverse effects that would prevent its admission to phase I clinical trials for treatment of liver tumors in humans.

General Toxicity of the "Teraphthal + Ascorbic Acid" System

It was found out that single intravenous injection of 0.1% and 0.01% teraphthal solutions in animals produces no local irritation effect. Mikhailova et al. [19] performed quantitative assessment of the toxicity (safety) of teraphthal and the "teraphthal + AA" binary system in terms of calculated doses (LD10 = MTD, LD16, LD50, LD84) for mice and rats, and toxic doses (LD, TDH, TDL, and HNTD[1]) for rats and dogs [19]. Acute and chronic toxicity parameters of teraphthal and the binary system on single and multiple (10) intravenous injections were established. The general toxicity parameters of the "teraphthal + AA" catalytic system are as follows:

mice: LD10 = 42 (36.2–48.7) mg/kg;
LD50 = 61 (52.6–70.0) mg/kg;

rats: LD10 = 20 (16.8–23.8) mg/kg;
LD50 = 26 (21.8–30.9) mg/kg;

(teraphthal doses are given, teraphthal : AA molar ratio 1 : 10, time interval between teraphthal and ascorbic acid injections 1 h).

The toxicity study revealed narrow toxic and lethal ranges and a lack of gender-related difference in the toxicity of the binary system. The "teraphthal + AA" binary system after a single injection is 1.5–2 times more toxic than teraphthal. The most sensitive to the toxic action of teraphthal and the binary system on its basis on intravenous injection in doses both per body weight and per body surface area were found to be rats (LD50 26 mg/kg), and then mice (LD50 61 mg/kg), and dogs (LD50 160 mg/kg). The species sensitivity factor to the binary system dosed per body surface area is 20.8, which points to marked differences in the sensitivity of different animal species to this system and suggests human specificity. The accumulation indices of the "teraphthal + AA" system are 5.6% in rats, and 7% in dogs. These values show that the teraphthal + AA system possesses no cumulative properties.

The targets for the toxic effects of both teraphthal and the binary system are lungs, liver, kidney, red blood cells, platelets, cardiovascular system, skin, and CNS. Species-specific dose-dependent reversible toxic complications were revealed in animals. At high toxic

[1] High nontoxic dose.

and lethal doses, toxic pulmonites in rodents, bronchites or bronchopneumonias in dogs, toxic myocardial distrophia, changes in the ECG, and reduction of blood pressure by 15–30 mm were observed. Hepatotoxic and nephrotoxic effects were also revealed. A dose-dependent effect of teraphthal on the blood coagulation system was observed in animals [20]. In the maximum tolerated dose and high toxic doses, the preparations caused reversible toxic coagulopathy with hemorrhagic syndrome. Transient coloring of skin and mucous membranes were also observed. Teraphthal and the "teraphthal + AA" binary system induced allergic reactions.

The dose-limiting toxicities of the catalytic system are associated with the cardiovascular system (reduced blood pressure, collapse), blood coagulation system (deep changes in the hemostasis system) [19], and toxic pulmonary complications (toxic pulmonites).

The "teraphthal + AA" system applied in high nontoxic doses and in doses of 1/10 MTD caused no toxic effects in animals. No impact of normal tissues with expressed proliferative activity was observed. The bluish-green coloring of skin and mucous membranes were reversible and dependent on the teraphthal dose.

Based on the toxicity parameters of the binary system we determined its starting safe dose for humans at phase I clinical trials: teraphthal 12.95 mg/m^2 and ascorbic acid 28.49 mg/m^2. Further on a modified Fibonacci dose escalation scheme was applied.

Antitumor Efficiency and Toxicity of the Binary System on the Basis of Efiter

Pre-clinical and clinical trials of the "teraphthal + AA" system revealed side effects, likely due to the molecular structure of the metal complex. The presence of eight carboxy groups in the teraphthal molecule favors binding of bivalent metal ions (Ca^{2+}, Mg^{2+}) in blood and, as a result, leads to aggregation of the metal complex, which, in its turn, decreases its catalytic activity and entails undesirable toxic effects in animals and humans.

Aimed at improving the pharmacological properties of teraphthal we modified it to obtain tetra[methyl-penta-(hexa)ethylene glycol] ester of teraphthal [3]. Having no free carboxy groups, this ester exhibits no reactivity over a wide pH range and does not act as a complex-forming agent. For improved solubility the ether was adducted with poly-*O*-(1-hydroxy-2-propyl)-cycloheptadextrin. This substance was used to develop an efiter drug (lyophilisate for solution for intravenous injection).

The optimal therapeutic regimen for treatment of ascites tumors with the "efiter + AA" system includes consecutive single injections of efiter at a dose of 1250 mg/kg and ascorbic acid at a dose of 443 mg/kg (efiter : acid molar ratio 1 : 30). The best results of treatment using this scheme were observed in EAC mice: The lifespan increase (LSI) was 92%. The efficiency of treatment on animals with ascitic lympholeukemia L1210 was much lower (LSI 25%), whereas P-388 ascitic lympholeukemia and S-37 sarcoma proved to be resistant to the "efiter + AA" system (the LSI of mice with these tumors were $\leq$ 2%).

With mice with solid tumors (LLC, Ca-755, P-388, CC5, ACATOL), the best results were obtained after protracted treatment (5 days) with daily injection of efiter at a dose of 1250 mg/kg, followed within 60 min by injection of ascorbic acid at a dose of 443 mg/kg (efiter : acid molar ratio 1 : 30).

Treatment using this scheme was found to be the most efficient in mice with implanted Ca-755 breast carcinoma, solid P-388 lympholeukemia, and Lewis lung carcinoma. Thus, in Ca-755-bearing mice the tumor growth inhibition reached 89–67% (the effect persisted for 20 days after treatment), in P-388-bearing mice the effect was 79–52% (11 days), and in LLC mice it was 85–50% (14 days).

The CC5 cervical cancer showed medium sensitivity to therapy with the "efiter + AA" binary system (the TGI at a level of 50% was observed for 14 days after treatment), and ACATOL proved to be insensitive to this drug therapy.

The general toxicity of the "efiter + AA" system on single (acute toxicity) and multiple (chronic toxicity) administration of its components was studied on three animal species: mice, rates, and dogs.

The acute toxicity parameters (LD10, LD16, LD50, LD84) obtained in mice and rats point to broad-range toxic and lethal effects of this binary system. No gender-dependent differences in toxicity were found. Rats were found to be the most sensitive among small rodents to the toxic effects of the binary catalytic system dosed per body weight and compared in sensitivity with mice to the catalytic system dosed per body surface. The toxicometry results allow the "efiter + AA" system to be referred to low-toxicity drugs in terms of acute toxicity hazards.

Chronic toxicity study of the "efiter + AA" system on rats and dogs gave the following results. Experiments on rats showed that the binary system at high and low toxic doses induces reversible dose-dependent toxic effect on liver, destroying its barrier function, and has no impact on the peripheral blood, kidney, digestive tract, and CNS. Experiments on dogs showed that the catalytic system at high and low toxic doses affects the peripheral blood (reduces the red blood cell count, hemoglobin content, and hematocrit number) and protein-synthesis liver function.

After multiple injections in dogs no toxic impacts of the "efiter + AA" catalytic system on the functional state of cardiovascular system, lungs, kidney, pancreas, and other body organs and systems were revealed. At the same time, pathomorphology showed that multiple intravenous injections of the catalytic system in dogs at high and low toxic doses caused long-term but reversible destructive and dystrophic changes in liver and kidney, well-defined inflammatory changes in lungs, destructive changes in the thyroid epithelium, damage of ovarian follicles, and dystrophic changes in adrenal gland zona glomerulosa cells. These findings allowed the "efiter + AA" binary system to be classified as a moderately hazardous drug in terms of chronic toxicity hazards.

Clinical Study of the Binary Catalytic Systems on the Basis of Oxycobalamine and Teraphthal

Application of the "Oxycobalamine + Ascorbic Acid" System

Based on the results of pre-clinical study of oxycobalamine, the Ministry of Health of the Russian Federation allowed its clinical trials both as a single substance (for safety assessment, Phase I) and in combination with ascorbic acid (safety and efficiency assessment, Phases I and II).

Phase I clinical trials. Fair tolerability of both oxycobalamine and the "oxycobalamine + AA" binary catalytic system was demonstrated, dose-limiting toxicity was not reached. Doses of the substances for Phase II clinical trials were determined: oxycobalamine 296 mg/m^2, and ascorbic acid 370 mg/m^2.

Phase II clinical trials of the "oxycobalamine–ascorbic acid" binary catalytic system were performed at the Blokhin RCRC RAMS, Petrov Research Institute of Oncology (Petrov RIO), and Herzen MCRI. The therapy was applied to patients with developed cancers (stage IV), which were found to be impossible to treat by other methods (surgery, radiotherapy, and chemotherapy). Evidence for the safety of the "oxycobalamine + AA" system was obtained, no adverse toxic reactions were observed.

Study of the therapeutic efficiency of the "oxycobalamine + AA" system gave the following results: partial regression of tumors was observed by instrumental methods in 3 patients (4.8%), stabilization of the process in 30 patients (48.2%), and progress of the disease in 29 patients (47%). Most of the patients (67%) showed improvement of the quality of life: reduction of weakness, increase of activity and physical stamina, improvement of appetite. Taking into account the severe initial state of the patients, the efficiency of the binary system can be evaluated as moderate.

Both the clinical and experimental results suggest that the efficiency of the therapy could be improved by using other regimens of treatment with this binary system, which would allow active formation of antitumor oxidants due to the reaction of the system components. A promising approach is a local application, e.g., an injection of oxycobalamine and ascorbic acid to bladder, in the case of bladder cancer, to create a high concentration of free-radical reaction products directly in the tumor zone.

Application of the "Teraphthal + Ascorbic Acid" System

Phase I clinical trials were performed at the RICO Blokhin RCRC RAMS and Petrov RIO [21, 22].

The aims of the trials were to assess the toxic and side effects (to determine dose-limiting toxicity), the maximum tolerated and therapeutic doses, and antitumor efficiency of the agents. Forty seven patients with disseminated cancer and exhausted possibilities for specific treatment were included in the research. To fulfill the aims of the trials, escalation of single doses of teraphthal alone (Protocol I) and in combination with ascorbic acid (Protocol II) was performed.

The teraphthal monotherapy was applied to 12 patients. The starting dose of teraphthal, taken as 100%, was 23.13 mg/m^2; the dose was increased to 523% (121.4 mg/m^2).

The therapy with the binary system was applied to 35 patients. The starting doses of teraphthal and ascorbic acid, taken as 100%, were 12.95 and 28.49 mg/m^2; the doses were increased to 925% (teraphthal 119.8 mg/m^2, ascorbic acid 263.5 mg/m^2).

Neither dose-limiting toxicity nor maximum tolerated dose were found both for teraphthal and the binary system. The following results were obtained: in 36% patients treated with teraphthal and the binary system no subjective or objective signs of toxicity were revealed; in 60% of patients side effects were weak, specifically skin itch, small tortoiseshell, paresthesia, reduced blood pressure, temporary gray coloration of skin and mucous membranes, and phlebitis at the teraphthal injection site, were observed (Grade I and II by the WHO Toxicity Grading Scale). In four patients suffering hypertension increased blood pressure was observed after teraphthal infusion (3.3%); two patients complained of heart pain during teraphthal infusion; changes in the ECG were registered in four patients with chronic ischemic heart disease.

Teraphthal monotherapy caused moderate changes in the hemostasis system: decreased fibrinogen concentrations and moderately slower platelet aggregation rates, which normalized within 2.5–3 weeks, were observed in 60–70% of patients. In three patients (6.5%) idiosyncrasy of teraphthal, manifested in a sharp fall of blood pressure up to collapse, was observed. To prevent this complication, corticosteroid premedication was recommended.

The antitumor effect of the "teraphthal + AA" catalytic system was revealed in 25.7% of patients: minimal tumor regressions in patients with metastatic kidney cancer and malignant adrenal pheochromocytoma, as well as long stabilization in the case of malignant carcinoid and metastatic adrenal cancer.

Phase II clinical trials. The following therapeutic doses were recommended: teraphthal 68 mg/m^2, and ascorbic acid 150 mg/m^2. To prevent hypersensitivity reactions (hypotension, collapse), premedication by intramuscular injection of dexamethasone (20 mg), and 1% dimedrol (1 mL) 30 min before teraphthal infusion is obligatory.

Trials of the binary system (intravenous injection) were performed at the RICO Blokhin RCRC RAMS, Petrov Research Institute of Oncology, Herzen MCRI, and City Clinical Hospital no. 40 (Moscow) [23]. The goals of the trials were to determine the spectrum of antitumor activity and the nature of side effects. The test group included 95 patients with morphologically verified disseminated malignant neoplasms.

The following results were obtained: no complete tumor regressions were observed, minimal regressions were registered in four patients, and 25 patients showed stabilization of the process, accompanied by improvement of the general state. Thus, controlled tumor growth was obtained in 26.3% of patients. The most controllable were found to be malignant carcinoid, kidney and adrenal cortex cancer, skin melanoma, and malignant pheochromocytoma. Metastases in lymph nodes, adrenal glands, and lungs, too, showed sensitivity to the binary therapy. Slowdown of the accumulation of fluid in the pleural cavity was also observed. The median stabilization time was 8 weeks. Six of 36 patients (16.6%) with disseminated breast cancer showed stabilization for 6–16 weeks. The spectrum of anti-tumor activity, good tolerability to the binary system, and lack of side effects commonly associated with cytostatic therapy gave us reasons to continue Phase II clinical trials, focusing on a limited group of tumors.

Study of the Efficiency of the "Teraphthal + Ascorbic Acid" System on Intrapleural Injection in Patients with Pleural Tumors

Phase I clinical trials were performed at the RICO Blokhin RCRC RAMS [24]. The test group included 10 patients with plural tumor resistant to specific chemotherapy and indications for pleural sclerotherapy (pleurodesis).

After injections of the binary system in doses lower than 246.75 mg/m^2 teraphthal and 542.5 mg/m^2 ascorbic acid no side effects were detected. Dose-limiting toxicity, specifically, chest pain of different intensity, characteristic of pleurodesis, developed immediately post-injection. The pain syndrome was observed in seven of 10 patients (70%) exclusively in doses equal or higher than 246.75 mg/m^2 teraphthal and 542.85 mg/m^2 ascorbic acid. The pain intensity increased with increasing dose of the preparations. Therefore, futher dose escalation was not applied. Pain management was performed using standard narcotic analgesics.

The other side effects were fever (temperature up to 38.5 C) in 20% of patients and hypotension in 10% of patients. The intensity of side effects was dose-dependent. Weak short-time blue coloration of urine was observed in 100% of patients at any dose. Of 10 patients treated by intrapleural catalytic system, eight (80%) showed partial regression of plural tumor as an X-ray verified encystation of pleural effusion and relieved clinical symptoms of respiration failure (dyspnea). Further observation established that after

the encystation of pleural effusion no fluid accumulation took place until patient's death. In 20% of patients pleuritis progression with continuing fluid accumulation and dyspnea aggravation took place.

Phase II clinical trials. The following therapeutic doses were recommended: teraphthal 247 mg/m^2, and ascorbic acid 543 mg/m^2. The test group comprised 13 patients with indications for pleurodesis [25]. Before trials all the patients received chemotherapy, i.e., they had pleural tumor resistance to systemic treatment. The efficiency of treatment by intrapleural catalytic system was assessed in 11 (84.6%) of 13 patients. Among those 11 patients, nine (81.8%) showed partial regression of pleural tumor and weaker clinical symptoms of respiratory failure. In two patients (18.2%) pleuritis progression with continuing fluid accumulation and dyspnea aggravation took place. The research is in progress.

Study of the Efficiency of the "Teraphthal + Ascorbic Acid" System on Selective Occlusive Injection into Liver Vessels of Patients with Malignant Liver Tumors

Phase I clinical trials were performed at the Blokhin RCRC RAMS and Sechenov Moscow Medical Academy. The test group included 10 patients [24], among them eight with metastatic colorectal cancer, one with liver metastases from gastric cancer, and one with liver metastases from prostate cancer. The patients all received multiple chemotherapy and hormone therapy courses which gave no positive results.

Therapeutic effects of the components of the binary system at concentrations comprising 300–450% of the starting dose were assessed. Most patients fairly well tolerated treatment. The observed side effects included short-time drop of blood pressure (down to 70/40 mmHg) during and after teraphthal infusion and VCI occlusion (4 patients), anaphylactic shock in one patient (though this patient received dexamethasone premedication he had a reduced blood pressure for a long time after surgery); and elevated ALT enzymes and alkaline phosphatase in blood (2 patients). The temporary fall of arterial blood pressure to 70/40 mmHg, observed in all patients, was not considered as a complication, because it could be explained both by the effect of the drug in itself and by the hemodynamic disorder associated with placing of a tourniquet around the VCI. The dose-limiting side effect of teraphthal was cholangitis.

By the end of the trials, four patients with colorectal cancer were alive, and their observation was continued. The life span was 17 months, and no progress of the diseases took place (teraphthal dose 0.9 mg/m^2). Three patients died within 8, 4, and 3 months after initiation of the binary therapy.

Phase II clinical trials were not performed in view of the f that most patients included in the test group needed a complicated surgery, and the efficiency of surgical treatment and treatment by the selective occlusion method (effect on lifespan without disease progression) proved to be impossible to assess separately.

Pharmacokinetics of Teraphthal

In 10 patients received teraphthal in single doses varying from 75 to 230 mg, the following pharmacokinetic parameters were established: half-life in blood serum 1.3–3.6 h, average retention time in blood 11.38 h. Teraphthal is excreted from the body with urine: within 3 days from 26 to 38% of the administered dose is excreted.

The pharmacokinetic parameters did not change when teraphthal was applied together with ascorbic acid.

Pre-clinical Study of the Chemo- and Radiomodifying Properties of the Binary Systems on the Basis of Oxycobalamine, Teraphthal, and Efiter

To extend the range of possible applications of the binary catalytic systems, pre-clinical studies on the efficiency of their combination with traditional conservative antitumor therapies, such as chemotherapy, radiotherapy, and hyperthermia, were performed.

It was found that the binary systems on the basis of oxycobalamine and efiter act as efficient modifiers of the therapeutic effects of official antitumor drugs (cisplatin, taxol), radiotherapy, as well as local laser-induced hyperthermia [26–29].

The chemomodifying properties of the "oxycobalamine + AA" and "efiter + AA" systems are most pronounced when they are applied in combination with cisplatin. In this case, the dose of such a highly toxic cytostatic can be reduced by half (from 8 to 4 mg/kg) without sacrificing the efficiency of treatment [26, 28]. The incorporation of these binary systems in the protocol of chemoradiotherapy combined with cisplatin administration for treatment of mice with Lewis lung carcinoma resulted in a biologically significant tumor growth inhibition (50–68% and 70–

79%, respectively) for 21 day post-treatment and enhanced the therapeutic effect of radio-/chemotherapy as individual antitumor agents. The efficiency of combined antitumor therapy in EAC mice was slightly lower [27–30].

The therapeutic efficiency of a combination of the binary systems on the basis of oxycobalamine and efiter and local distant laser-induced hyperthermia was assessed by the following schemes:

oxycobalamine (150 mg/kg), 1 h + AA (190 mg/kg),
5 min > hyperthermia (43°C, 10 min);

efiter (1250 mg/kg), 1 h + AA (443 mg/kg),
5 min > hyperthermia (43°C, 10 min).

Treatment of animals with the solid form of sarcoma 37 (tumor volume ~3.5 cm^3) resulted in a 51–88% tumor growth inhibition, and the effect persisted 18 days post-treatment; at the same time, the efficiency of separate treatment with the binary system or hyperthermia, estimated in terms of the TGI criterion, was no higher than 34%.

Yakunina et al. [31] performed pre-clinical study of the antitumor efficiency of combinations of the binary system on the basis of teraphthal and cytostatics of different classes. The best results were obtained with combined treatment of rats with cancer PC-1 by intra-arterial injection of the teraphthal + AA and cisplatin or doxorubicin. In the case of doxorubicin, the efficiency of combined therapy is strongly dependent on the order the combinants are injected. The best results were obtained when doxorubicin was injected before the binary system: in 66% of rats full remission was observed, and in the others, long-term partial remission.

The model of rat kidney cancer was used to study the antitumor efficiency of the "teraphthal + AA" system in combination with interferon α-2. The treatment protocol included single injection of the binary system 2 days after transplantation of the tumor, followed by daily injections of interferon at the second through sixth day. Reliable doubling of the duration of the antitumor action of the combination of the catalytic pair and interferon compared to each combinant separately.

The antitumor efficiency of combined therapy with teraphthal or the "teraphathal + AA" system and radiotherapy (local irradiation) was studied on mice with the ELD Ehrlich carcinoma, B-16 melanoma, and Lewis lung carcinoma transplanted into leg [32]. Single irradiation (dose 31 Gy) of all the three tumors after pretreatment with the binary system resulted in a deeper tumor regression and a longer lifespan compared to what could be reached by single irradiation alone. The same results as with the binary system were obtained with teraphathal alone. Thus, single irradiation with teraphthal pretreatment prolonged the tumor doubling time in mice with Ehrlich cancer from 15 to 24 days and their lifespan from 45 to 50 days, and by this time one the eight mice was still alive.

In view of the fact that the clinical radiation treatment of cancer involves dose-fractionated local irradiation, the radiomodifying efficiency of the catalytic system and teraphthal alone in mice experiments was studied using double irradiation with a single dose of 20 Gy. The tumor doubling time of B16 melanoma after single irradiation was 16 days (lifespan 50 days), whereas the respective parameters for combined therapy with teraphthal (30 mg/kg) were 24 days and more than 50 days, and by this time three of eight animals were alive (Fig. 3). Combining teraphthal with radiotherapy was recommended for clinical study.

Experiments on spontaneous dog tumors revealed enhancement of the antitumor effect of the "teraphthal + AA" system by its combination with microwave or laser hyperthermia [33]. The objective results in dog experiments were as follows: complete tumor regression 38% and tumor growth inhibition 68% (against 23 and 40% in control, respectively). The best results with complete regression in 80–100% of animals were obtained on the case of skin histiocytoma and squamous cell cancer (n = 7). No limiting effects of the new method were revealed.

Enhancement of the Efficiency of the Catalytic System by Inhibiting Activity of the Antioxidant Enzymes

Clinical trials revealed the ability of the "teraphthal + AA" system to cause minimal regressions and stabilization of the process in 26.3% of patients with exhausted possibilities of specific therapy of such cytostatic-resistant malignant tumors as kidney cancer, adrenal cancer, malignant carcinoid, and others.

In searching for ways to enhance the antitumor efficiency of the catalytic system, *in vitro* and *in vivo* research on the effect of pharmacological modulation of certain antioxidant enzymes (heme oxygenase 1, catalase, peroxidase) on the efficiency of catalytic

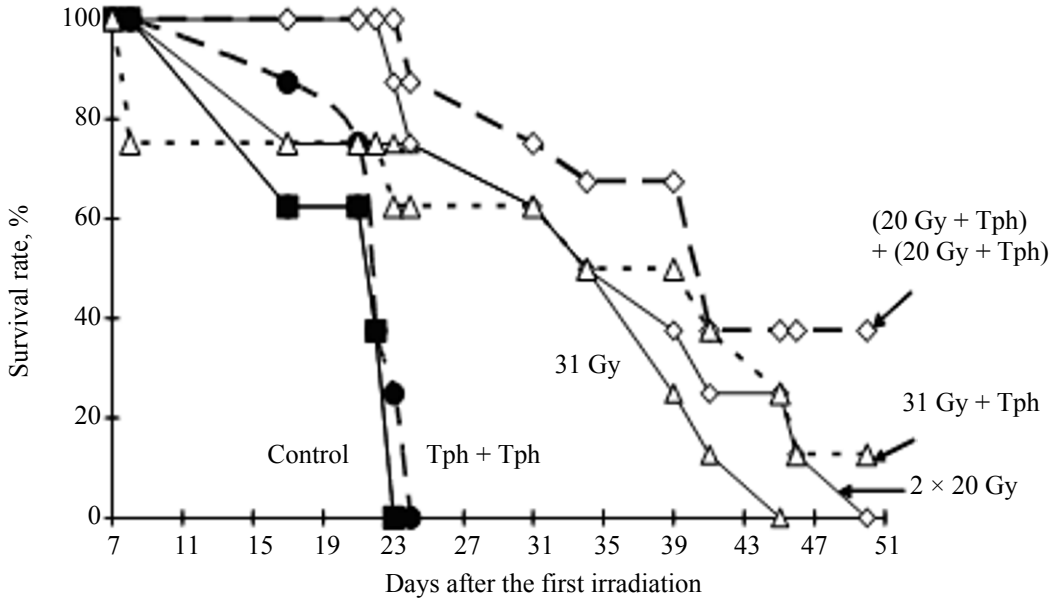

Fig. 3. Survival of animals with B-16 melanoma after local single (31 Gy) and double (20 Gy) irradiation in combination with teraphthal (Tph) injection in a single dose of 25 mg/kg post-radiation.

therapy whose mechanism of action is associated with intracellular generation of hydrogen peroxide. As known, these proteins are necessary to protect cells from the oxidative stress caused by free radicals, as well as H_2O_2. In was shown that catalases are the only among the enzymes studied *in vitro* and *in vivo*, which are able to enhance the efficiency of the "teraphthal + AA" catalytic system two times [34].

Catalase was inhibited using a specific inhibitor of this enzyme 3-amino-1,2,4-triazole in an nontoxic dose. The average volumes of the transplanted mice tumors treated with teraphthal in a single dose of 40 mg/kg and, followed after 1 h by ascorbic acid in a single dose 88 mg/kg, were 223 ± 141, 2097 ± 813, and 4992 ± 2003 mm^3 by 5th, 8th, and 11th day post-treatment. The tumor growth inhibition (53, 23, and 6%, respectively) did not significantly differ statistically from control. Toxicity-induced deaths were not observed. The average tumor volumes in the group of mice premedicated 2 h before start of the binary therapy with triazole (1000 mg/kg) were 147 ± 78, 1323 ± 661, and 3347 ± 1207 mm^3 by 5th, 8th, and 11th day post-treatment, respectively. Thus, the proposed method allows suppression of tumor cell proliferation *in vitro* and enhancement of tumor growth inhibition 1.3–6.0 times, depending on the recording time of the effect. The same effect was obtained both *in vitro* and *in vivo* with combined application of the "oxycobalamine + AA" system and specific catalase inhibitor.

The experimental results allow us to conclude that the catalase inhibitor 3-amino-1,2,4-triazole in a dose of 1000 mg/kg enhances the antitumor effect of the binary catalytic system without toxicity enhancement.

Study of the Mechanism of Action of the Binary Catalytic Systems

Until recently the main hypothesis explained the development of biological effect by oxygen-centered free radicals, including those formed on the contact of cells with the binary catalytic system, that induce oxidative stress of cells, damage of DNA and proteins, mutagenesis, degenerative processes, and cell death [30, 35]. Thus, oxycobalamine and ascorbic acid act on tumor cells, causing formation and accumulation of double-strand DNA same system fragments, and this effect compares with the DNA damage induced by γ radiation (150 Gy). At the same time, the development of oxidative stress, accompanied by mitochondrial damage and intracellular H_2O_2 formation is likely to be a consequence rather than a reason for the apoptotic cell death. Over the past years evidence has appeared showing that free radicals can act as secondary participants of signaling pathways and enhance signal transmission from various membrane receptors, including those responsible for physiological functions

such as vascular tonus regulation, platelet aggregation, cytokine production, etc. [36].

The results of the research on the mechanism of action of the "teraphthal + AA" catalytic system give a new view of the antitumor and side effects of the catalytic system, observed in the pre-clinical and clinical study of the binary therapy of malignant tumors. Below we present the principal results of these studies [37–40].

The "teraphthal + AA" system inhibits DNA reparation.

The binary catalytic systems on the basis of teraphthal and oxycobalamine increase the intracellular level of oxygen free radicals in the CaOv cell line: the "oxycobalamine + AA" system at the component concentration ratios 10^{-5} M : 10^{-4} M and 10^{-7} M : 10^{-6} M increases the level by 1.9 and 1.5 times, respectively, and the "teraphthal + AA" system, by 1.3 times. At the same time, the "efiter + AA" system does not affect the level of free radicals. The "teraphthal + AA" system decreases the glutathione level, which implies that it affects the cellular redox homeostasis.

Teraphthal has specific binding sites on tumor cell membranes, identified as α_1- and β-adrenoreceptors. Cells containing functionally active membrane adreno-receptors, i.e. cells conjugated with effector proteins (guanylate cyclase and adenylate cyclase) of the cAMP- and cGMP-dependent signaling pathways are targets for the catalytic system on the basis of teraphthal.

The activity modulation of the cAMP- and cGMP-dependent signaling pathways by oxygen free radicals under the action of the "teraphthal + AA" system on target cells contributes much to the mechanism of the cytotoxic and antitumor effects of the catalytic therapy, as well as to such its side effects as hypotension and reduction of fibrinogen concentration and platelet aggregation.

Intensification of intracellular production of oxygen free radicals was also observed in K562 and U937 cells under the action of the "oxycobalamine + AA" system, and the effect of the latter system is stronger compared to the "teraphthal + AA" system. Both these systems induce a long-time (up to 2 h) oxidative stress in the U937 cell line, which may further activate cell apoptosis.

The mechanism of tumor cell death under the action of the 'teraphthal +AA' system is caspase-dependent apoptosis [40].

Evidence for the cell damage induced by intra-cellular formation of oxygen free radicals comes as the enhancement of radiation-induced cell damage upon combined tumor therapy by using γ-radiation and the binary systems on the basis of oxycobalamine and teraphthal. The fact that this effect gets weaker, when the binary systems are applied after irradiation, suggests that they inhibit the cell reparation process initiated immediately after the radiotherapy is complete.

The effect of combined treatment with teraphthal and interleukin 2 (IL-2) on the cytolytic activity of lymphocytes with respect to K562 cells was studied. It was shown that teraphthal in the tested concentrations induces enhancement, to the same extend as IL-2, specifically by 25%, of spontaneous cytotoxicity of mononuclear cells with respect to K562 cells. However, combined application of teraphthal and Il-2 does not enhance their effects on killer T-lymphocyte activity [44].

Thus, the mechanism of the antitumor action of the binary catalytic systems consists in the damage of intracellular targets by oxygen free radicals and activation of certain components of cellular immunity.

CONCLUSIONS

Medicinal substances (oxycobalamine, teraphthal, and efiter) whose combinations with ascorbic acid (binary catalytic systems) are designed for binary cancer therapy have been developed. The binary systems on the basis of these substances showed a moderate antitumor activity with respect to a broad spectrum of experimental tumor-growth models.

The toxic and side effects that develop in the presence of maximum tolerated doses of the binary systems are reversible, whereas in the presence of high nontoxic doses no toxic effects are observed in animals. The binary systems have no effect on normal tissues with expressed proliferative activity. The general toxicity of the binary catalytic systems is dose-dependent.

Clinical trials of the binary systems on the basis of teraphthal and oxycobalamine in patients with malignant neoplasms (with exhausted possibilities for specific therapy) revealed objective effects such as partial regressions and stabilization of the process at different routes of administration.

To introduce the binary catalytic therapy into clinical practice, further research into pro-oxidant binary systems is required.

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