Experimental Evidence for Killing the Resistant Cells and Raising the Efficacy and Decreasing the Toxicity of Cytostatics and Irradiation by Mixtures of the Agents of the Passive Antitumor Defense System in the Case of Various Tumor and Normal Cell Lines in Vitro

Gyula Kulcsár

Abstract

Despite the substantial decline of the immune system in AIDS, only a few kinds of tumors increase in incidence. This shows that the immune system has no absolute role in the prevention of tumors. Therefore, the fact that tumors do not develop in the majority of the population during their lifetime indicates the existence of other defense system(s). According to our hypothesis, the defense is made by certain substances of the circulatory system. Earlier, on the basis of this hypothesis, we experimentally selected 16 substances of the circulatory system and demonstrated that the mixture of them (called active mixture) had a cytotoxic effect (inducing apoptosis) in vitro and in vivo on different tumor cell lines, but not on normal cells and animals. In this paper, we provide evidence that different cytostatic drugs or irradiation in combination with the active mixture killed significantly more cancer cells, compared with either treatments alone. The active mixture decreased, to a certain extent, the toxicity of cytostatics and irradiation on normal cells, but the most important result was that the active mixture destroyed the multidrug-resistant cells. Our results provide the possibility to improve the efficacy and reduce the side-effects of chemotherapy and radiation therapy and to prevent the relapse by killing the resistant cells.

Key words: chemotherapy, radiation therapy, passive antitumor defense system, increasing the efficacy of cytostatics and irradiation, protection of normal cells against toxicity of cytostatics and irradiation, killing multidrug resistant cells, Culevit

Introduction

In our previous papers, we assumed and proved the existence of a passive antitumor defense system (PADS). Briefly, it is well known that AIDS is associated with a substantial decline of the immune system. This is well demonstrated by the observation that in a patient whose AIDS was recognized after renal transplantation, the renal graft was not rejected, despite the significant, prolonged withdrawal of the usual immunosuppressive agents. This means that in AIDS, the decline of immune system reaches so high a degree, that it cannot reject even a foreign body. Consequently, if the immune system were the only mechanism to prevent the development of tumors, then in AIDS populations, all kinds of tumors should increase in incidence. However, only some kinds of them, mainly Kaposi's sarcoma (KS) and non-Hodgkin's lymphoma (NHL), has significantly increased incidence. Obviously, all the other kinds of tumors occur in AIDS patients, but the incidence of them is not higher than in the normal population. There are epidemiologic observations showing that even in the case of KS and NHL, the more frequent development in AIDS is not due to immune decline.
but other effects (e.g., viruses, Tat protein of HIV, and so forth). Considering similar observations made in the case of other immune deficiency disease or in immunosuppressed patients, it can be stated that the known immune system has no absolute role in the prevention of tumors.

Observing anomalies of the immune system, many research workers investigated the different active and passive mechanisms of immune escape possessed by tumors or the possibilities of immune response modification to find a tool for the activation of the immune system against cancer. Starting from similar observations, we got to the above conclusion about the inefficacy of the immune system and went to a third direction in our studies. Considering every logical possibility, we thought that the fact that tumors do not develop in the majority of the population during their lifetime, despite the ineffectiveness of the immune system, indicates the existence of other defense mechanism(s). Evidently, the components of a general defense mechanism (i.e., a "surveillance") must be in the circulatory system. It is well known that the uptake of many substances (e.g., amino acids, monosaccharides, nucleobases, and so forth) occurring in the circulatory system is strictly regulated by normal cells, whereas it is unregulated and elevated by tumor cells. This feature of tumor cells is well known and used for tumor detection (e.g., positron emission tomography). It is due to this mentioned feature that tumors accumulate the contrast medium and become detectable. Considering that many molecules in the living system have more than one role, we supposed that some of the accumulated substances, besides their usual role, might have a second function and might be the agents of a defense system capable of killing emergent cancer cells. To our hypothesis, the killing of tumor cells by the given substances forms the PADS.

We demonstrated by experiments that the PADS really works in living systems. It was collected by us from the scientific literature and published in another paper, including a lot of experimental data, as well as epidemiologic and clinical observations that support the existence and operation of the PADS. To determine the agents of PADS, we investigated 89 substances of the circulatory system. In the experiments with cancer cells, 16 substances were selected on the basis of their ability to increase the effect of each other and kill the cells. Considering that in the living system the arising cancer cells are extinguished by the simultaneous action of molecules of PADS, we used, in the experiments, the mixture of 16 substances called the active mixture. It was demonstrated that the active mixture had a cytotoxic effect in vitro and in vivo on different tumor cell lines, but not on normal cells and animals—as would be expected for agents of a defense system. It was detected by different methods that the active mixture induced the apoptosis of cancer cells selectively.

Knowing the 16 agents of the PADS, the possibility arose of a practical usage as medication. The compositions have been protected by patent in Hungary and in many other countries. On the basis of these patents two products (Culevit tablets and Culevit cream) have been developed and marketed by Immunal Ltd. (Budapest, Hungary). The development of medicines in different pharmaceutical forms (e.g., powder for drinking water and infusion) is at the preclinical phase. Obviously, the mentioned products contain the above substances in different concentrations.

The rationale for combining different treatments is to broaden the therapeutic index. It is assumed that different treatments have overlapping anticancer effects but a decreased overlapping spectrum of side-effects. There are a lot of reports about the combination therapy of cytostatics with each other or with radiotherapy or with other agents, including ascorbate. Because the active agents of PADS are the nontoxic ingredients of different products developed for medication and the components of active mixture as well, in this study, we investigated the effect of the active mixture on the efficacy of different cytostatics or irradiation in vitro.

In this paper, we provide evidence that there was a significant addition of cytotoxic effects of the active mixture and cytotoxic drugs as well as irradiation, resulting in enhanced cancer-cell killing. In contrast with tumor cells, in the case of normal cells, the toxicity of some cytostatics and irradiation was decreased by the combination with the active mixture. Some cell lines were resistant, to a certain extent, to cytostatics, while the active mixture was effective against all of them and had a significant cell-killing effect on multidrug-resistant cells.

Materials and Methods

Materials

Adenine, l-arginine, l-phenylalanine, l-histidine, l-tryptophan, l-tyrosine, L-methionine, 2-deoxy-D-ribose, L(-)malic acid, D-biotin, pyridoxine, riboflavin, L-ascorbic acid sodium salt, D(+)-mannose, N-acetyl-D-glucosamine, myo-inositol, D(-)-glucosamine, DL-6,8-thiocetic acid, glutathione, and sodium bicarbonate (all of them were cell-culture-tested biochemicals) were purchased from Sigma-Aldrich Co. (Budapest, Hungary). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, trypsin blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), folic acid, hypoxanthine, D-pantothenic acid hemicalcium salt, orotic acid monosodium salt, hippuric acid sodium salt, allantoin, L(+)fucose, betaine, leucinial acid, propionic acid sodium salt, and catalase (no. C-40) were also purchased from Sigma-Aldrich. An amino-acid kit (AS-30), nicotinamide, and thiamin hydrochloride were obtained from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany), succinic acid disodium salt, D(-)-ribose from FLUKA AG (Buchs, SG, Switzerland), and fetal calf serum (FCS) from Sebak GmbH (Aidenbath, Germany). Cytarabine, mitoxantrone, doxorubicin, etoposide, mitomycin, cisplatin, vinblastine, and fluoroauracil were all obtained from the University Pharmacy (Medical School, University of Pécs, Pécs, Hungary). All other chemicals were of the purest grade available from Reanal Finechemical Co. (Budapest, Hungary).

Tumor cells and culture

The K562 human erythroleukemia, the HeLa human cervix epithelioid carcinoma, the HEP-2 human larynx epidermoid carcinoma, the MCF7 human breast adenocarcinoma, the LLC-MK2 rhesus monkey (Macaca mulatta) kidney, and the MDCK canine (Canis familiaris) kidney cell lines were generously provided by Júlia Szekeres, M.D., D.Sc., (Department of Microbiology, Medical School, University of Pécs). The Sp2/0-Ag14 mouse myeloma, the A20 mouse B-cell lymphoma, and the 89 substances of the circulatory system have more than one role, we supposed that some of the accumulated substances, besides their usual role, might have a second function and might be the agents of a defense system. To our hypothesis, the killing of tumor cells by the given substances forms the PADS.
FIG. 1. Effects of the indicated amounts of six various cytostatic drugs on MCF7 human breast adenocarcinoma cells alone (solid triangles) or in combination with the 75% (hollow circles) or 50% (hollow squares) active mixtures. The 75% active mixture contained 3.75 mM of L(-)-malic acid disodium salt, 3.75 mM of D(+)-mannose, 3.75 mM of hippuric acid sodium salt, 1.88 mM of L-phenylalanine, 1.88 mM of L-arginine hydrochloride, 1.88 mM of L-histidine, 1.88 mM of 2-deoxy-D-ribose, 1.5 mM of L-tyrosine, 0.75 mM of orotic acid sodium salt, 0.56 mM of L-methionine, 0.38 mM of L-tryptophan, 0.38 mM of D-biotin, 0.38 mM of pyridoxine hydrochloride, 0.23 mM of L-ascorbic acid sodium salt, 0.15 mM of adenine hydrochloride, and 0.0023 mM of riboflavin. The 50% active mixture contained 2.5 mM of L(-)-malic acid disodium salt, 2.5 mM of D(+)-mannose, 2.5 mM of hippuric acid sodium salt, 1.25 mM of L-phenylalanine, 1.25 mM of L-arginine hydrochloride, 1.25 mM of L-histidine, 1.25 mM of 2-deoxy-D-ribose, 1.0 mM of L-tyrosine, 0.5 mM of orotic acid sodium salt, 0.38 mM of L-methionine, 0.25 mM of L-tryptophan, 0.25 mM of D-biotin, 0.25 mM of pyridoxine hydrochloride, 0.15 mM of L-ascorbic acid sodium salt, 0.1 mM of adenine hydrochloride, and 0.0015 mM of riboflavin. The concentrations are given as final concentrations in a well. The cell cultures and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for five independent experiments.

Concentration of cytostatic drugs

The Jurkat human acute T-cell leukemia cell lines were kindly provided by Péter Németh, M.D., D.Sc., (Department of Immunology and Biotechnology, Medical School, University of Pécs). The AT3B-1 multidrug-resistant rat prostate cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA). The Sp2/0-Ag14, A20, Jurkat, K562, and MCF7 cells were cultured in RPMI-1640 medium containing L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FCS. The AT3B-1 cells were cultured in RPMI-1640 medium containing the above detailed amounts of L-glutamine, penicillin, streptomycin, and FCS, but it was supplemented with sodium pyruvate (1.0 mM) and doxorubicin (0.001 mM). The HeLa, MDCK, and LLC-MK2 cells were grown in DMEM containing 10% FCS. HEp-2 cells were grown in the same medium containing 5% FCS. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. All cell lines were free of Mycoplasma.

Cytotoxicity assay

The toxicity was assessed by adding the tested compounds (i.e., active mixtures and/or cytostatic drugs) dissolved directly in the applied medium immediately before each experiment in the indicated concentrations specified under, or in,
Cytarabine (pg/L)  
Etoposide (pg/L)  
K562 cells  
Mitoxantrone (pg/L)  

- Cytostatic  
- Cytostatic + 50% active mixture  
- Cytostatic + 75% active mixture  

% of untreated cells

Concentration of cytostatic drugs

FIG. 2. Effects of the indicated amounts of three different cytostatic drugs on K562 human erythroleukemia cells alone (solid triangles) or in combination with the 75% (hollow circles) or 50% (hollow squares) active mixtures. The composition of the 75% and 50% active mixtures are given in the legend of Figure 1. The cell cultures and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for three independent experiments.

According to the literature, from the cytostatic drugs used in this study, only doxorubicin and cisplatin may have a stability problem, having half-lives of 29 and 18.5 hours, respectively, in medium at 37°C. Even though the drugs were utilized for 48 hours in culture medium, no correction for the relative instability of them was made. In the case of Sp2/0-Ag14, A20, Jurkat, and K562 cell lines, the logarithmically growing cells were harvested from the medium and resuspended to a final concentration of $4 \times 10^4$ cells of Sp2/0-Ag14, Jurkat, and $2 \times 10^4$ cells of A20, K562, in $250 \mu$L of appropriate medium per well containing the tested materials in the indicated concentrations. In the case of MCF7, HeLa, AT3B-1, MDCK, and LLC-MK2 cell lines, the cultured cells were harvested from 75% confluent tissue-culture flasks with 0.2% trypsin, 0.025% versene solution, and resuspended in the appropriate medium at a density of $10^5$ cells/mL. Aliquots (100-μL) were dispensed into 96-well microplates, made up to 250 μL with the appropriate medium, and incubated for 24 hours. Then, the medium was gently discarded and replaced with 250 μL of fresh medium containing the tested compounds in the indicated concentrations. All kinds of cells were allowed to proliferate for 48 hours.

The number of viable Sp2/0-Ag14, A20, Jurkat, and K562 cells was then counted microscopically with the trypan blue dye exclusion method. The viability of MCF7, HeLa, AT3B-1, MDCK, and LLC-MK2 cells was assessed with the MTT colorimetric assay. Because of the disturbing effect of the tested compounds, the modified assay was used. In brief, after 48 hours of incubation, the medium was removed from the wells and the cells were washed with sterile phosphate-buffered saline (PBS). To the cells in each well was added 50 μL of a 5-mg/mL sterile filtered solution of MTT in the applied medium. After incubating the plate for 4 hours in 5% CO₂ at 37°C, the untransformed MTT was removed from the wells and the cells were washed with PBS. In all cases, the addition and removal of solutions were made carefully so as...
FIG. 3. Effects of the indicated amounts of three different cytostatic drugs on Jurkat human acute T-cell leukemia cells alone (solid triangles) or in combination with the 75% (hollow circles) or 50% (hollow squares) active mixtures. The composition of the 75% and 50% active mixtures are given in the legend of Figure 1. The cell cultures and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for three independent experiments.

not to disturb the attached cells. Then, 50 μL of isopropanol was added to all wells of the plate and thoroughly mixed in order to solubilize the formazan crystals. The quantity of formazan product formed was assessed by its absorbance at 550 nm on a Dynatech MR7000 (Dynatech Laboratories, Chantilly, VA). Peripheral wells of each plate were utilized for blank (N = 3) background determinations. Background values were subtracted from each reading. Results were expressed in the case of all cell lines as the percentage of the untreated control systems. All values are expressed as the mean ± standard error.

Irradiation of cells

The in vitro radiosensitization assay was done, as described above, for the cytotoxicity assay, with the addition of irradiation of the active mixture–treated cells. In the experiments, a final concentration of 2 × 10⁴ cells of Sp2/0-Ag14, Jurkat, K562, A20, and 5 × 10³ cells of HeLa, HEp-2, MDCK, and LLC-MK² were used in 250 μL of appropriate medium per well. After 4 hours of exposure to different concentrations of the active mixture, two plates of every cell line were transported to the Radiation Therapy Department, where one of the plates was irradiated at a dose of 4 Gy with a Theratron 780C 80 (MDS Nordion, Ottawa, Ontario, Canada) cm isocenter ⁶⁰Co clinical irradiator (Radiology Department, Medical School, University of Pécs). Following radiation treatment, the cultures were returned to the 37°C incubator. The viability of the cells was assessed 3 days after irradiation by the trypan blue dye exclusion method or the modified MTT colorimetric assay, as described above.

Statistical analysis

The two-tailed Student’s t-test was used to determine the statistical significance of any changes we observed.
Results

Comparison of the effect of different cytostatics alone or in combination with active mixtures on the growth of various tumor cell lines

To investigate the effect of the active mixture on the efficacy of various cytostatic drugs, different cancer cells were either treated with cytostatics alone (solid triangles) or in combination with the 50% or 75% (in the case of A20 cells, only 20% or 40%) of the 100% active mixture used in our earlier experiments.\(^1\) The exact compositions of the 50% and 75% mixtures are shown in the legend of Figure 1. The concentration ranges of cytostatics used in this study were chosen based upon preliminary dose-escalation experiments (data not shown).

The effects of six different cytostatics in the indicated concentrations alone or in combination with an increasing amount (from 50% to 75%) of active mixtures on the growth of MCF7 human breast adenocarcinoma cells are shown in Figure 1. It can be seen that the active mixtures increased the effect of all cytostatics. The combination of every cytostatic with the 75% active mixture (hollow circles) resulted in higher cell-killing effects than the combination with the 50% active mixture (hollow squares). The curves are almost parallel.

In the case of K562 human erythroleukemia (Fig. 2) and Jurkat human acute T-cell leukemia (Fig. 3) cell lines, the effect of three cytostatics were investigated. In all cases, more cancer cells died when the different cytostatic drugs were applied in combination with active mixtures. However, the cytostatic drugs only moderately enhanced the effect of active mixture on the death of K562 cells (Fig. 2), especially at low concentrations of the given drugs, as can be seen from the slope of the curves.

It was an interesting result that when we used the cytarabine alone (solid triangles), then first, the number of surviving K562 cells markedly decreased (to 23.4% ± 1.9% of the untreated cells) with an increasing cytarabine concentration (from 2 to 200 \(\mu\)g/L), but all the remaining 1.6 \(\times\) 10⁵ (23.4%) cells survived, despite the further 100-fold increase of the cytarabine concentration (from 200 to 20,000 \(\mu\)g/L). In other words, cytarabine had not any effect on the rest of the 1.6 \(\times\) 10⁵ K562 cells, although 5.2 \(\times\) 10⁵ cells from the same cell line were killed by it at its lower concentrations. A similar, but not so marked, an ineffectiveness of mitoxantrone on 19.8% ± 3.9% of K562 and doxorubicin on 9.1% ± 1.7% of Jurkat cells was observable. Since we observed the most remarkable inefficiency on the remaining cells in the case of cytarabine, it seemed useful to confirm the above observations by a study of its effect on another cell line. The result in the case of Sp2/0-Ag14 mouse myeloma cells (Fig. 4) was very similar. Using the Sp2/0-Ag14 cell line, 6.9% ± 0.8% of cells (3.3 \(\times\) 10⁵) were not destroyed, but only the number of them was slightly reduced by the further 100-fold increase of the cytarabine concentration.

In the case of the A20 mouse B-cell lymphoma cell line, only 20% and 40% active mixture was used because a 60% mixture killed all the cells even without cytostatics. The compositions of the mixtures are shown in the legend of Figure 5. In these experiments, the active mixtures increased the effect of doxorubicin end etoposide (Fig. 5). The enhancing effect was also practically additive and increased by the amount of the active mixture. It can be seen that at the highest concentrations of the cytostatic drugs, the combined treatments with the 40% active mixture (hollow circle) destroyed all the cells in both cases.

Figure 6 shows that the active mixture had a higher enhancing effect on the influence of mitomycin against HeLa human cervix epithelioid carcinoma than in the preceding experiments in the case of any cytostatics. Mitomycin, at 1 mg/L concentration, could kill only 19.4% of HeLa cells, compared with untreated cells, but together with a 50% active mixture, it destroyed 59.8%, and with a 75% mixture, 94.6% of the cells.

Effect of active mixtures on the survival of AT3B-1 multidrug-resistant rat prostate cancer cells compared to control mixtures

Using a multidrug-resistant AT3B-1 rat prostate cancer cell line, the active mixture decreased significantly the growth of cells (hollow circles) as a function of mixture amount (Fig. 7). However, the control mixture (solid circles) was not cytotoxic at any concentration, but it rather slightly increased the proliferation of the cells. The composition of control mixture was the same as in our previous papers.\(^1\) It was composed of physiologically and chemically similar mole-
**AGENTS OF PASSIVE ANTITUMOR SYSTEM**

---

**Concentration of cytostatic drugs**

**FIG. 5.** Effects of the indicated amounts of doxorubicin or etoposide on A20 mouse B cell lymphoma cells alone (solid triangles) or in combination with the 40% (hollow circles) or 20% (hollow squares) active mixtures. The 40% active mixture contained 2 mM of L(-)malic acid disodium salt, 2 mM of D(+)-mannose, 2 mM of hippuric acid sodium salt, 1 mM of L-phenylalanine, 1 mM of L-arginine hydrochloride, 1 mM of L-histidine, 1 mM of 2-deoxy-~ribose, 0.8 mM of L-tyrosine, 0.4 mM of orotic acid sodium salt, 0.3 mM of L-methionine, 0.2 mM of L-tryptophan, 0.2 mM of D-biotin, 0.2 mM of pyridoxine hydrochloride, 0.12 mM of L-ascorbic acid sodium salt, 0.08 mM of adenine hydrochloride, and 0.0012 mM of riboflavin. The concentrations are given as final concentrations in a well. The cell cultures and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for three independent experiments.

---

**Comparison of the toxicity of different cytostatic drugs alone or in combination with active mixtures on normal cell lines compared to untreated cells**

It can be seen that every cytostatic drug tested in the previous experiments against cancer cells had also a toxic effect on LLC-MK2 rhesus monkey kidney (Fig. 8) and MDCK canine kidney (Fig. 9) normal cells, especially at their higher concentrations (solid triangles). It is an important result that the active mixtures decreased the toxicity of cisplatin, vinblastine, and mitoxantrone (only at the 75% mixture concentration) in the case of LLC-MK2 normal cells and the toxicity of etoposide, vinblastine, doxorubicin, Fluorouracil, and Cisplatin in the case of MDCK normal cells, as can be seen in Figures 8 and 9.

**Comparison of the effect of active mixtures alone or in combination with irradiation on the growth of various tumor and normal cell lines**

To investigate the effect of active mixture on the efficacy of radiation therapy (Fig. 10), different cancer and normal cells were either treated with an active mixture alone (white columns) or in combination with 4 Gy of irradiation (black columns). The dilution of the active mixture was expressed as a percentage. To get representable results, the starting mixture was only 50% of the 100% active mixture used in our earlier experiments. The exact composition of the 50% mixtures is shown in the legend of Figure 1. The dashed lines represent the level of the effect of irradiation alone. The results show that active mixture enhances radiation cytotoxicity as a function of concentration in all kinds of cancer cells, compared to untreated cells (first white columns). The lymphoma cell line (A20) was the most sensitive to both
ness and decrease the side-effects of them. It appears rational therapeutic combinations, in which the efficacy of traditional treatments, is complemented by using nontoxic agents. Based on this rationale, we investigated the influence of the active mixture containing nontoxic components in combination with commonly used chemotherapeutic agents and radiation to test their efficacy against various cancer and normal cell lines. The cytostatics used in our experiments were selected from different groups of chemotherapeutic agents, using the data of the special literature:

**Discussion**

According to our previous results, a PADS exists in the living system besides the known immune mechanisms and the effective agents of this defense are certain small substances of the circulatory system. These substances were selected experimentally out of 89 substances. It was demonstrated that the mixture of them (called the active mixture) had a selective toxic effect on cancer cells. It induced apoptosis of tumor cells.

Using the active agents of the defense system, two products (Culevit tablets and Culevit cream) were developed and marketed by Immunal Ltd. (Budapest, Hungary), and the development of medicines in different pharmaceutical forms are in the preclinical phase. There are a lot of reports about different combination therapies of cytostatic drugs and radiation therapy in vitro and in vivo to increase the effective-
and cytarabine (pyrimidine analogs) from antimetabolites, vinblastine (Vinca alkaloid), etoposide (epipodophyllotoxin), doxorubicin and mitomycin (antibiotics) from natural products, cisplatin (platinum coordination complex), and mitoxantrone (anthracycinedione) from miscellaneous agents. Comparing the effect of cytostatics alone or in combination with the active mixture, we chose those kind of tumor cell lines that are usually treated by the given cytostatics in clinical practice. In preliminary experiments, we determined the effective concentration range of the cytostatics against the mentioned cell lines and used them in this range in different concentrations alone or in combination with active mixtures. We applied only 50% and 75% (in the case of A20 cells only, 20% and 40%) active mixtures, because the 100% active mixture used in our earlier experiments destroyed all the cells in combination with the different cytostatics and the results were not figurable.

Because in clinical practice the breast tumors can be treated with many kinds of chemotherapeutic agents, we investigated the influence of active mixtures on the effect of six different cytostatics against the MCF7 human breast adenocarcinoma cell line (Fig. 1). The combined treatments enhanced tumor-cell killing, compared with active mixtures or cytostatics alone. Because of the almost parallel curves, it appears that the combinations of treatments result in practically additive effects. The fact that the combination with the 75% active mixture resulted always in higher cell death, compared to the effect with the 50% mixture, shows that the effect of cytostatics is increased by the active mixtures in a dose-dependent manner and the cytotoxic interaction is not an accidental event, but a real result of the combined treatment. The total cell death caused by the combination of drugs with the 100% active mixture (data not shown) suggests that the simultaneous application of cytostatic drugs and the active mixture can be really a powerful treatment. In our earlier experiments, the 100% active mixture contained its components in such amounts in which they could not decrease the number of cancer cells and could not induce apoptosis, compared to untreated cells, when they were used alone. Obviously, this is even more true for the 75% and 50% mixtures, in which the amounts of active materials were 25% and 50% less than in the 100% mixture. This excludes the possibility that the individual toxicity of any substances of active mixtures has a role in the additive cell-killing effect.
Concentration of cytostatic drugs

FIG. 9. Effects of the indicated amounts of eight different cytostatic drugs on MDCK canine (Canis familiaris) kidney normal cells alone (solid triangles) or in combination with the 75% (hollow circles) or 50% (hollow squares) active mixtures. The composition of the 75% and 50% active mixtures are given in the legend of Figure 1. The cell cultures and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for five independent experiments.

That is why we studied only the influence of the active mixture and did not investigate the individual effect of its components in combination with cytostatics and radiation.

Besides the repeated demonstration of the additive effect, the experiments with K562 (Fig. 2) and Jurkat cells (Fig. 3) brought up the problem of resistant cancer cells. Some amounts of cells could not be destroyed by the cytostatic drugs alone (solid triangles), despite the further increase of the concentration of the drugs, although the mentioned drugs in lower concentrations could kill a large part of the same kind of cells. This inefficacy, on some part of the cells, was especially well detectable in the case of cytarabine. Repeating the experiment with another (Sp2/0-Ag14) cell line (Fig. 4), a very similar phenomenon could be observed. If the reason of ineffectiveness was really the resistance of cells, it is difficult to imagine that drug-resistant cells arose within 48 hours of treatment as a result of selection induced by the treatment. It is more probable that the drug resistance of remaining cells existed before treatment. Contrary to the above observations with cytostatics, the number of remaining cells decreased when the mentioned cytostatics were applied in combination with the active mixtures. Because the combined effects of cytostatics and active mixtures were practically additive and the cell-killing effect increased as the amount of active mixture changed from 50% to 75% it could be assumed that the death of remaining cells was really due to the active mixture. It is a plausible explanation for the above results (Figs. 2–4) that the active mixture could not reverse the resistance of cells to the cytostatic drugs but could destroy the resistant cells. Theoretically, the killing of resistant cells is a logically expectable feature of the active mixture because the reason of resistance is that the cancer cells can recognize that the cytostatics are foreign molecules, and as a result, they exhibit a wide repertoire of drug-resistance stratagems. A quite general pattern of drug resistance is that the cells overexpress P-glycoprotein, which acts as a drug-efflux pump in the plasma membrane and decreases the intracellular concentration of the cytostatics. Contrary to cytostatics, the constituents of the active mixture are not foreign molecules for the living system because they are the active agents of the PADS. Therefore, the cancer cells cannot recognize these substances and the behavior of cells does not change toward them. Because it is well documented that the uptake of the substances of the active mixture by tumor cells is increased, unregulated, and proportional to their availability, it is obvious that the cancer cells have a fundamentally different relation to the compo-
AGENTS OF PASSIVE ANTITUMOR SYSTEM

The active mixture had a cytotoxic effect on MCF7/ADR adriamycin-resistant cells, which exhibited a multidrug-resistant phenotype and was cross-resistant to a wide range of antineoplastic agents, including Vinca alkaloids, anthracyclines, and epipodophyllotoxins.25 Because in cancer chemotherapy multidrug resistance of tumor cells is a great problem, the above results suggest that the combination of traditional therapies with the products containing the agents of PADS could provide a therapeutic benefit for patients who have a high risk for disease recurrence.

It was demonstrated earlier3 that A20 mouse B cell lymphoma cells have a higher sensitivity to active mixture than the other cell lines. We observed a similar result in the present experiments, in which the effects of the active mixture alone and in combination with irradiation (Fig. 10) were investigated. That is the reason that in the experiments with this cell line, only the 20% and 40% active mixtures were used in combination with cytostatics, but in spite of that, the combination of treatments was highly toxic to the A20 cells (Fig. 5). The observations that the sensitivity of the A20 cell

FIG. 10. Effect of the indicated amounts of active mixtures alone (white columns) or in combination with 4 Gy of irradiation (black columns) on the growth of six various tumor and two normal cell lines, compared to untreated cells (first white columns). The dashed lines represent the level of the effect of irradiation alone. The composition of the 50% active mixture is shown in the legend of Figure 1. The cell cultures, the irradiation of cells, and cytotoxicity assay were made as described in Materials and Methods. The results are expressed as a percentage of untreated cells. The values are the mean ± standard error (bars) for five independent experiments.
line to doxorubicin and etoposide was similar to the K562 (Fig. 2) and Jurkat cells (Fig. 3) and to irradiation is similar to Sp2/0-Ag14 and Jurkat cells (Fig. 10), but to the active mixture is higher than the mentioned cells, as well as the higher sensitivity of another lymphoma (EL4) cell line to the active mixture detected earlier support that the powerful efficacy on lymphoma cells is really a remarkable feature of the active mixture. The reproducible results on the two cell lines (A20 and EL4) in different experimental conditions offer a potentially valuable therapy for lymphomas, which frequently develop in the case of AIDS and in other immune deficiency diseases or in immunosuppressed patients with the products (Culevit) (used either alone or in combinations with cytostatic drugs) developed on the basis of PADS. Because the majority of cytostatic drugs and the irradiation have a different mechanism of action, it has a low probability that the active mixture can interfere with all of them. Without intervention in the action of each other, the cooperative growth inhibition can only be additive. Obviously, in the case of additive effects, the cytostatics, irradiation, and active mixtures kill the cancer cells independently from each other; in spite of that, they are used simultaneously. The additive effects detected in the above experiments make it clear how the active mixture has a general effect and can enhance the growth-inhibitory effect of irradiation and every cytostatic drug investigated in all cancer cells examined (Figs. 1–6 and 10).

In the cases of all cancer cells, the curves showing the combined effects (hollow symbols) are in a dose-dependent manner under the curve presenting the effect of the given cytostatic alone (solid triangles). In contrast with cancer cells, the graphs showing the results of experiments in which LLC-MK2 (Fig. 8) and MDCK (Fig. 9) normal cells were treated by cytostatics in combination with active mixtures run in the case of etoposide, vinblastine, doxorubicin, fluorouracil, and cisplatin (hollow circles and squares) above the curve of single cytostatics (solid triangles). These results suggest that the active mixture decreases, to a certain extent, the toxicity of the above mentioned cytostatics. Similarly, the protective effect on normal cells could be observed in the case of irradiation (Fig. 10). Although the mechanism responsible for the protective effect is not entirely clear, several potential explanations are possible. It can be seen in Figure 10 that the active mixture slightly increased the proliferation of the LLC-MK2 and MDCK normal cells. Thus, one consideration is that the higher proliferation rate of normal cells can overcompensate the toxicity of cytostatics and irradiation. This assumption is supported by the finding that the rate of increase of proliferation (Fig. 10) is commensurable with the rate of protective effect. Another possibility in the case of cytostatics is that the active mixture inhibits the penetrations of the drugs into the cells, because the uptake of components of active mixture by normal cells is strictly regulated, and therefore, they may engage the transport systems.

Despite rapid progress in diagnostic and operative techniques, cancer is one of the most difficult human diseases to treat, which is partly due to the resistance of tumor cells to chemotherapeutic drugs and/or radiotherapy. In light of that, it appears a promising chance to increase, with nontoxic agents, the efficacy and decrease, to a certain extent, the side-effects of traditional therapies with the simultaneous killing of resistant cells that decreases the risk for disease recurrence and can result in a complete recovery of patients whose illness was diagnosed in an early stage and the therapy was started in time.

**Conclusions**

Our results support that the products that were developed or whose development is in progress on the basis of PADS provide the above mentioned chance. This is corroborated by animal experiments where significant inhibitory effects in the case of all tumors (P-388, S-180, B-16, MXT, Colon-26, Hc/De, Ne/De, and HL-60) investigated and significant potentiating effect on cytostatics (5-FU, cisplatin) of Culevit infusion and/or powder have been observed (Gaál D. National Institute of Oncology, Budapest, Hungary) and by clinical observations using a combination of bleomycin, vincristine, and methotrexate chemotherapy and Culevit tablets for the treatment of head and neck squamous cell cancer (Olasz L. Department of Oral and Maxillofacial Surgery, Faculty of Medicine, University of Pécs). Future experimental efforts will include the investigations of mechanism and human clinical trials.

**Acknowledgments**

The author thanks Prof. B. Sümegi, Ph.D., and L. Lex, Ph.D., for their continuous support, and also H. Halász for excellent technical assistance and Sz. Belyei for his invaluable help.

**Disclosure Statement**

No competing financial interests exist.

**References**