Research Article **ISSN 2835-6276**

American Journal of Medical and Clinical Research & Reviews

Mechanism of action of oxazaphosphorine cytostatics and anti metastatic experimental therapy with SUM-IAP a new Ifosfamide derivative adapted to the mechanism of action

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Received: 29 Jan 2023; *Accepted:* 15 Feb 2023; *Published:* 25 Feb 2023

Citation: Georg Voelcker. Mechanism of action of oxazaphosphorine cytostatics and anti metastatic experimental therapy with SUM-IAP a new Ifosfamide derivative adapted to the mechanism of action. AJMCRR 2023; 2(2): 1-12.

ABASTRACT

SUM-IAP is an Ifosfamide derivative adapted to the mechanism of action of Ifosfamide (IF). IF and other oxazaphosphorines (OX) are hydroxylated in the liver by cytochrome P450 enzymes. The formed 4-hydroxyoxazaphosphorine (OXOH) forms an equilibrium mixture with its tautomer OX-aldophosphamide (OXALD). OXALD is the pharmacologically active metabolite from which the two therapeutically active metabolites OX-phosphoreamidemustard (OXPAM) and 3-hydroxypropanal (HPA) are formed by enzymatic cleavage with esterases. OXPAM damages the DNA by alkylation and thus initiates apoptosis like other alkylating substances. What is special about OX, however, is that alkylation-initiated apoptosis is enhanced by the pro apoptotic HPA. Now OX were found by a happy coincidence and were not tailor-made for the mechanism of action described, so that the therapeutic possibilities hidden in the mechanism of action are not fully exploited.

SUM-IAP is a new developed substance tailored to the mechanism of action. Chemically speaking, SUM-IAP is the I-aldophosphamide-perhydrothiazine with a modified alkylating function. In contrast to the alkylating function of IF, the modified alkylating function of SUM-IAP generates DNA intra strand crosslinks that are difficult or impossible to repair by cellular repair, thereby increasing the apoptosis yield compared to IF, which generates easily repairable inter strand crosslinks. With I-aldophosphamide-perhydrothiazne (IAP) containing the alkylating function of IF and with SUM-IAP, therapy experiments with P388 tumor bearing CD2F1mice were carried out. It is shown that SUM-IAP the alkylating function of which is adapted to the mechanism of action of OX, is in vivo orders of magnitude more effective than IAP.

Therapy experiments with SUM-IAP and CD2F1 mice bearing subcutaneously transplanted P388 tumors showed that although the transplanted primary tumor is eradicated, the mice die from SUM-IAP-resistant metastases between days 40 and 70 after tumor transplantation. The following article describes experiments demonstrating the mechanism of action of OX and experiments to prevent metastasis formation.

Key words

Mechanism of action of oxazaphosphorine cytostatics, Aldophosphamide-perhydrothiazines, SUM-IAP, anti metastatic therapy, N-methylformamide, Immunstimulation by SUM-IAP.

Introduction

OX were developed with the aim of making nitrogen mustard, which was used as a cytostatic at the time, less toxic and more tumor-specific. Nitrogen mustard should be transported to the tumor bound in a phosphoric acid amide bond as a non-toxic transport form where it is then cleaved by enzymes that catalyze splitting of phosphorus-nitrogen bonds (phosphamidases) into a non-toxic transport component and nitrogen mustard. At that time it was (wrongly) assumed that the activity of phosphoamidases in tumor cells was higher than in normal cells [1]. Of all the substances synthesized according to this specification, the OX “Cyclophosphamide” (CP) proved to be the most effective in animal experiments and was introduced into the clinic. Very quickly it turned out that the assumption on which the development of CP was based was wrong, but that a very effective anti-tumor substance had been "found" as a kind of happy coincidence. Studies on the metabolism of CP showed very quickly that CP - after hydroxylation in the liver - is converted into the DNA-alkylating phosphoreamide mustard (PAM).

A measure of the therapeutic quality of a substance is the therapeutic quotient, which indicates the ratio of the toxic dose to the therapeutically effective dose. For a long time it was puzzling why the therapeutic quotient - measured in Yoshida ascites sarcoma in rats - for CP and their hydroxylation product 4-hydroxycyclophosphamide (CPOH) is greater than 100, but less than 5 for the actual DNA-alkylating metabolites PAM [2]. The reason for the different antitumor effects of CPOH and PAM was a mystery for decades and the reason why all attempts to further develop OX failed. There are two reasons why the difference in the antitumor effect of CPOH and the alkylating agent formed from it remains a mystery. Firstly, because the results of in vitro tests with CPOH have been uncritically transferred to in vivo conditions. In vitro, from the tautomer of CPOH, that is ALD, alkylating PAM is formed by ß-elimination of acrolein, but in vivo by enzymatic cleavage. This reaction does not produce acrolein but 3-hydroxypropanal (HPA) [3]. The second reason is that it was not known for a long time that DNA damage initiates apoptosis because the phenomenon "apoptosis" was not yet discovered. Only after the discovery of HPA as an OX metabolite and the discoveries that HPA is a pro apoptotic aldehyde [4] and that cell death after OX application in animals and patients is caused by apoptosis [5] it was possible to determine the mechanism of action of OX and, based on this, to develop new "cyclophosphamides" adapted to the mechanism of action. Experiments with the model substance SUM-IAP, which is adapted to the mechanism of action, are described below.

Metabolism and mechanism of action of oxazaphosphorine cytostatics

The oxazaphosphorine ring is a common chemical structural feature of CP, IF and trofosfamide. They are therefore referred to as "oxazaphosphorines" (OX). The metabolism of OXis described below to the extent that it is necessary for understanding the mechanism of action (see Fig. 1).

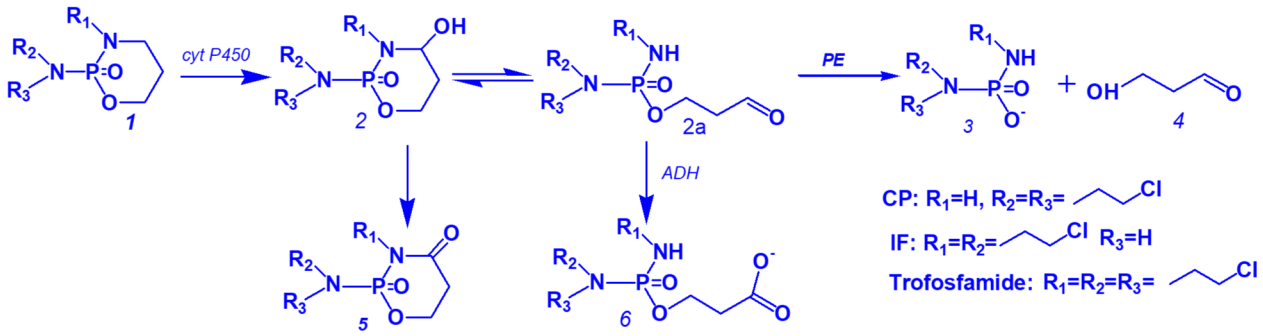


Fig.1 Main metabolic pathways of OX

OX (1) are hydroxylated by cytochrome P450 enzymes in the liver to OXOH (2). In a side reaction, toxic chloroacetaldehyde is formed as a result of side chain hydroxylation (not shown). OXOH forms an equilibrium mixture with its tautomer OX-ALD (2a). OX-ALD is the pharmacologically active metabolite that is enzymatically cleaved by esterases into DNA-alkylating PAM (3) and pro apoptotic HPA (4). The bulk of the tautomer mixture is detoxified prior to enzymatic cleavage to form ineffective Keto-OX (5) and carboxy-OX (6).

After intravenous injection of 100 mg/kg CP in mice, 92% is hydroxylated to CPOH. 80% of this is detoxified to Keto-OX and carboxy-OX so that less than 20% of the applied CP is ultimately available for the formation of OXPAM and HPA [6].

HPA is an antibiotic which is produced by Lactobacillus reuteri and also known as Reuterin. Experiments by Iyer [4], showed that HPA is a pro apoptotic aldehyde which stimulates apoptosis by inhibiting anti-apoptotic proteins Bcl-2 and Bcl-xL and the TNF dependent NF-κB activation.

All DNA-alkylating substances initiate apoptosis But contrary to other anticancer drugs like doxorubicin and cisplatin [7] in which the activation of caspase 8 is the initial apoptotic event, CPOH activates the intrinsic caspase 9-dependent p53-controlled apoptosis pathway [5]. This and the discovery of HPA as a CP metabolite [3], together with the results published by Iyer et al. [4], showing that HPA is a pro apoptotic aldehyde, lead to the scheme for the mechanism of action of OX, as shown in fig. 2.

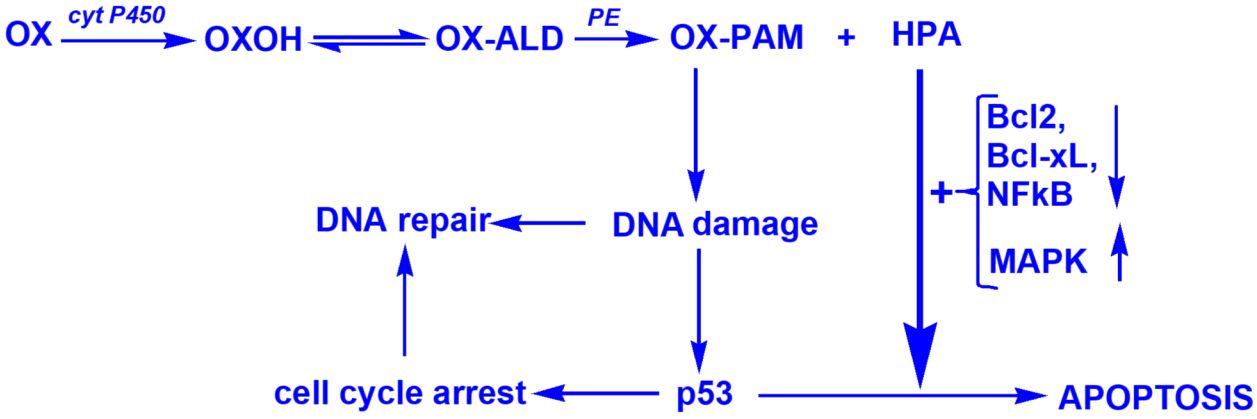


Fig.2: OX are hydroxylated by P450 enzymes in the liver to OXOH. OXOH are in equilibrium with its tautomeres OX-ALD. OX-ALD is decomposed by esterases (PE) to the alkylating OX-PAM and pro apoptotic HPA. OX-PAM

damages DNA by alkylation. The alkylated DNA is either repaired immediately or, if this is not possible, the tumor suppressor protein p53 is activated, which induces cell cycle stop to give the cell time to repair the damage. If DNA repair is not possible, p53 induces apoptosis, which is - and this is special for OX – strengthened by HPA.

Mechanism of action of MESNA

Hemorrhagic cystitis, successfully treated with 2-mercaptoethanesulphonate (MESNA)) is dose-limiting in OX therapy [8]. According to the prevailing opinion, the cause of the urotoxicity is acrolein formed in the metabolism of OX, which forms non-toxic thioesters with MESNA. However, acrolein does not appear in the scheme for the main metabolic pathways of OX presented in Fig.1. This raises the question of who caused the hemorrhagic cystitis and the mechanism of action of MESNA. In the metabolism of OX, apart from chloroacetaldehyde, which is thought to produce nephrotoxic and neurotoxic side effects [9], only OXOH is formed as a toxic metabolite. OXOH is an electrophile with a pronounced local toxic effect that reacts with nucleophilic groups of macromolecules such as SH groups of proteins and is neutralized by MESNA (see Fig. 3).

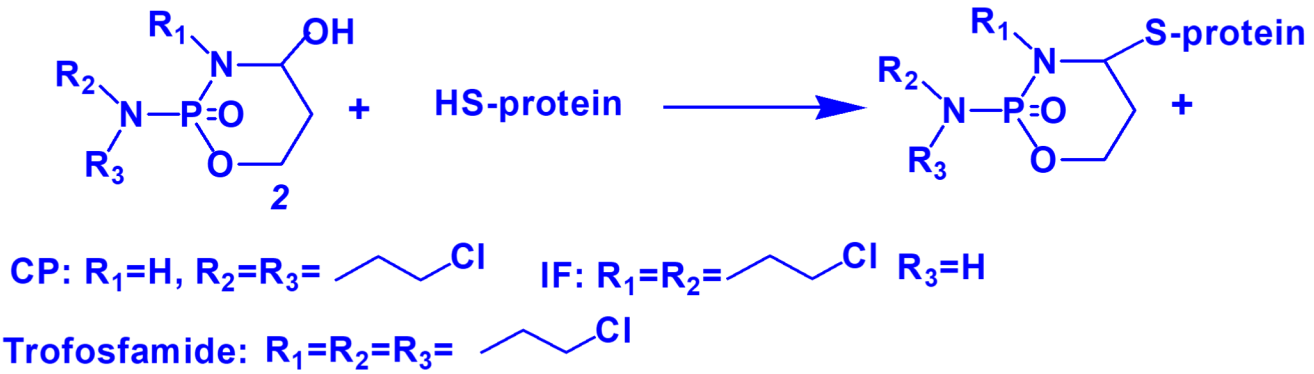


Fig.3

The reason for the toxicity of CPOH is the reaction with nucleophilic groups of macromolecules such as SH groups of proteins

Experimental evidence for the validity of the proposed mechanism of action, and the implications for the development of new cyclophosphamides

From the scheme for the mechanism of action (fig.2) it can be seen that cellular repair mechanisms reduce the apoptosis initiated by DNA damage. In the case of OX used in the clinic, the alkylating function consists of 2-chloroethyl groups (-CH_2CH_2Cl) which, create easily repairable DNA inter strand crosslinks and therefore have a low apoptotic yield. In contrast, alkylating functions in which the chlorine is substituted by a mesyl group generate intramolecular crosslinks that are poorly repairable and should result in a high apoptotic yield (http://www.atdbio.com/content/16/ Nucleic-acid-drug-interactions). In order to prove the suggested mechanism of action to be correct and to create new OX like compounds, it would be necessary to compare the effectiveness of different CP derivatives with 2-chloroethyl groups or 2-mesylethyl groups in the alkylating function.

As already mentioned, the pharmacologically active CP metabolite is OXALD. In the case of classic OX, the for-

mation of OXALD is only possible via toxic OXOH with formation of the toxic byproduct chloracetaldehyde. Therefore, it seems advantageous to experiment with thiazolidines or perhydrothiazines of OXALD, because these compounds spontaneously hydrolyze to OXALD by bypassing the toxic metabolites OXOH and chloroacetaldehyde [10].

The perhydrothiazines of IALD with 2-chloroethyl groups in the alkylating function called IAP and the perhydrothiazin with a 2-mesylethyl group in the alkylating function called SUM-IAP (formulas see fig.4) were synthesized and tested in CD2F1 mice bearing subcutaneously transplanted P388 tumors. IAP and SUM-IAP are 8-10 times less toxic than IFOH. IAP, which contains the same alkylating group as IF, is more potent than IF in the described tumor model [11].

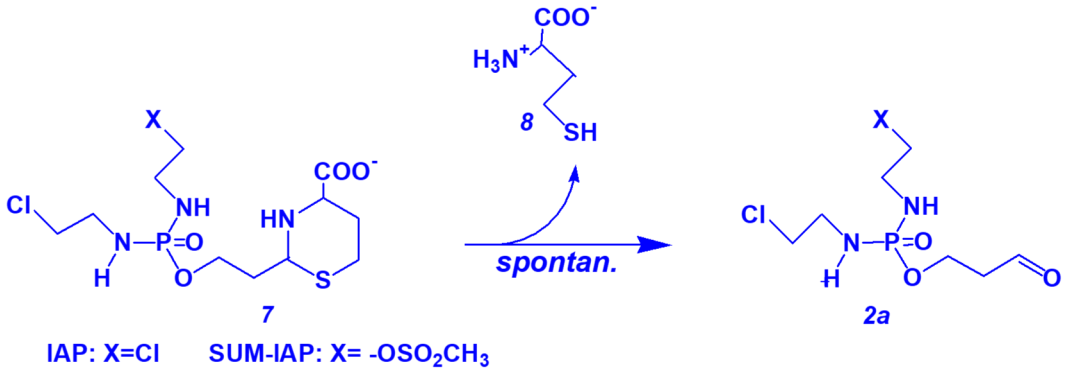


Fig.4

Hydrolysis of IAP and SUM-IAP (7) to IALD and SUM-IALD (2a) and homocysteine (8)

In order to determine whether the scheme formulated for the mechanism of action (Fig. 2) for oxazaphosphorine cytostatics is correct, therapy experiments with IAP and SUM-IAP were carried out.

CD2F1 mice bearing subcutaneously transplanted mouse leukemia cells were treated with either 0.8 mmol/kg (320mg/kg) IAP or 0.6 mmol/kg (266mg/kg) SUM-IAP on days 7-11 after tumor transplantation. Increase in life span of 60% was measured in the mice treated with IAP compared to the increase in life span of 285% in the animals treated with SUM-IAP [12].

In another experiment, the tumor growth curves were determined as a measure of the therapeutic effectiveness of IAP and SUM-IAP as shown in fig 5 (for details see legend fig 5). While only a slight delay in tumor growth can be measured after therapy with IAP, the tumor size is suppressed below the detection limit for 7 days after therapy with a lower dose SUM-IAP. After an interval of 7 days in which the tumor appeared to disappear, the tumor grew again. Re-therapy with SUM-IAP was less successful probably because tumor cells had become resistant to SUM-IAP.

Under the simplifying assumption that the measured tumor area is proportional to the tumor mass (correlation coefficient 0.93) and number of tumor cells and that the tumor cells are in the exponential growth phase, this and additional tumor growth curves were evaluated using the back extrapolation method according to Alexander and Mikulski [13]. From this experiments it can be concluded that there is a 10^4-10^5 times increase in anti-tumor activity when

one chlorine in the IAP molecule is substituted by a mesyl group in SUM-IAP [11]. This results demonstrate that the postulated mechanism of action for OX reflects the real conditions.

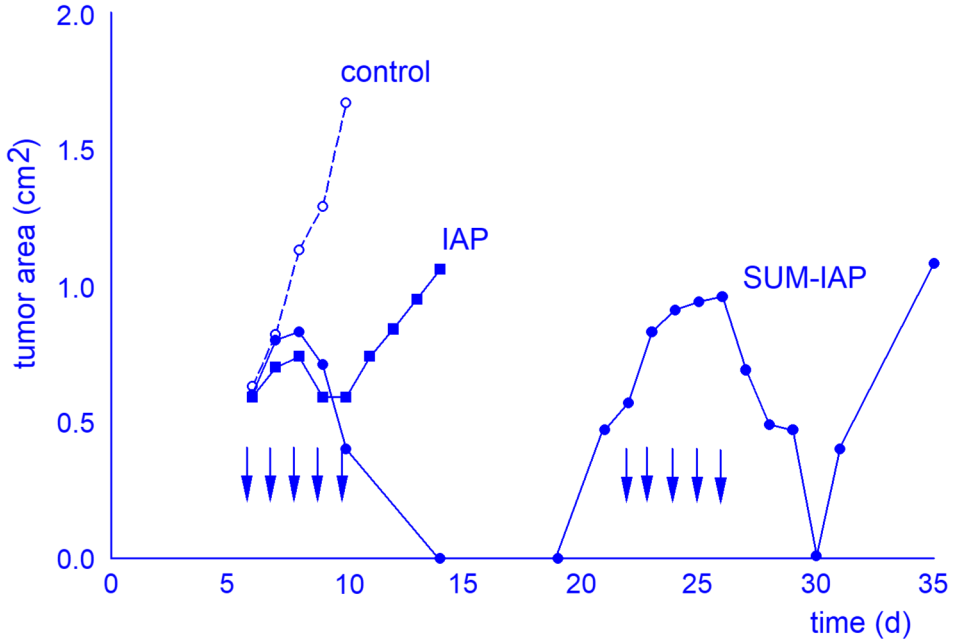
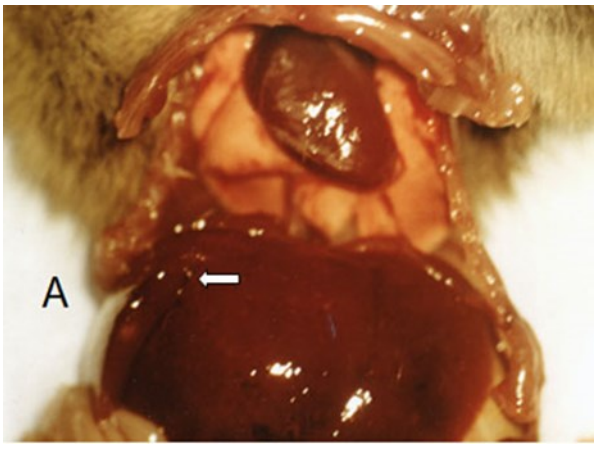


Fig.5

Tumor growth curves of subcutaneously transplanted P388 tumors in female CD2F1 mice following therapy with 0.5 mmol/kg (200mg/kg) IAP and 0.3 mmol/kg (133mg/kg) SUM-IAP s.c. on days 7 – 11 and days 22 - 26 (arrows), mean of 3 animals, control: tumor growth curves of untreated animals.

Metastasis formation after SUM-IAP therapy in mice

Although the antitumor activity of SUM-IAP compared to IAP against the described tumor model was increased 10^4-10^5-fold by optimizing the alkylating function with regard to apoptosis initiation, the animals were not cured (surviving time >100d) by treatment with SUM-IAP alone The animals died due to growth of metastases between day 40 and day 60. Repeated treatment of the growing metastases with SUM-IAP was unsuccessful, due to the initial treatment the tumors had become resistant to SUM-IAP. Postmortem examination of sacrificed mice showed that the liver is the starting point for the formation of metastases as shown in fig.6.



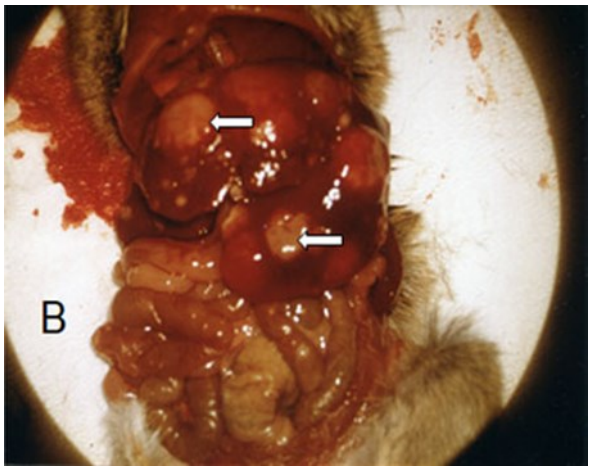


Fig. 6

Metastases (white arrows) in the abdominal cavity of mice 25 (A) and 50 (B) days after therapy with SUM-IAP. After subcutaneous transplantation of 10^6 P388 tumor cells into female CD2F1 mice, the animals were treated on days 7 to 11 with 266 mg / kg SUM-IAP. On days 25 and 50, the animals were sacrificed and examined for metastases in the abdomen.

25 days after the start of therapy, small metastases can be seen on the liver (fig.6 A). 50 days after the start of therapy, the entire abdomen is covered with metastases that kill the animal (fig.6 B). In short, this was the situation in almost all experiments with SUM-IAP alone: Eradication of the primary tumor but death of the animals due to metastases approximately between 30 and 60 days after start of therapy. The cause of the formation of metastases in the liver is very likely the detoxification of OX ALD to ineffective carboxylic acid by aldehyde dehydrogenases in the liver.

Immunologic anti metastatic therapy with SUM-IAP ?

The following discovery was made in an attempt to prevent metastasis formation by increasing the dose. Therapy of P388 tumor-bearing mice with a high dose of 666 mg/kg on days 7 and 8 after tumor transplantation led to the well-known result, namely metastasis formation and death of the animals 30 days after the start of therapy. However, if the treatment with SUM-IAP was repeated after a recovery period of 7 days, all animals survived the observation period of 100 days and can be considered cured (for details see legend Fig. 7). Fig. 7 shows the course of the concentration of leukocytes in the blood of the mice. After a short-term decrease after SUM-IAP injection, there is a short-term increase on day 30 to 4 to 5 times the baseline value before the start of therapy.

It is well known that CP has an immune-stimulating effect at low doses. This is due to special sensitivity of T cell inhibiting regulatory T cells (Treg) to CPOH. It is shown in scientific literature that the high sensitivity is due to induction of apoptosis by CPOH and the decreased ability Treg to repair damaged DNA [14]. From the result of this experiment it was concluded that the animals did not survive because the formation of metastases (resistant to SUM-IAP) was prevented by the cytotoxic effect of the second application of SUM-IAP, but by cytotoxic T cells which could unfold their effects freely because inhibitory Tregs were switched off by the second therapy cycle with SUM-IAP.

Thus, SUM-IAP attacks tumor cells in two different ways: By boosting the DNA alkylation-mediated apoptosis by HPA and further by apoptotic inhibition of Treg by which it is made possible that cytotoxic T cells can attack tumor cells.

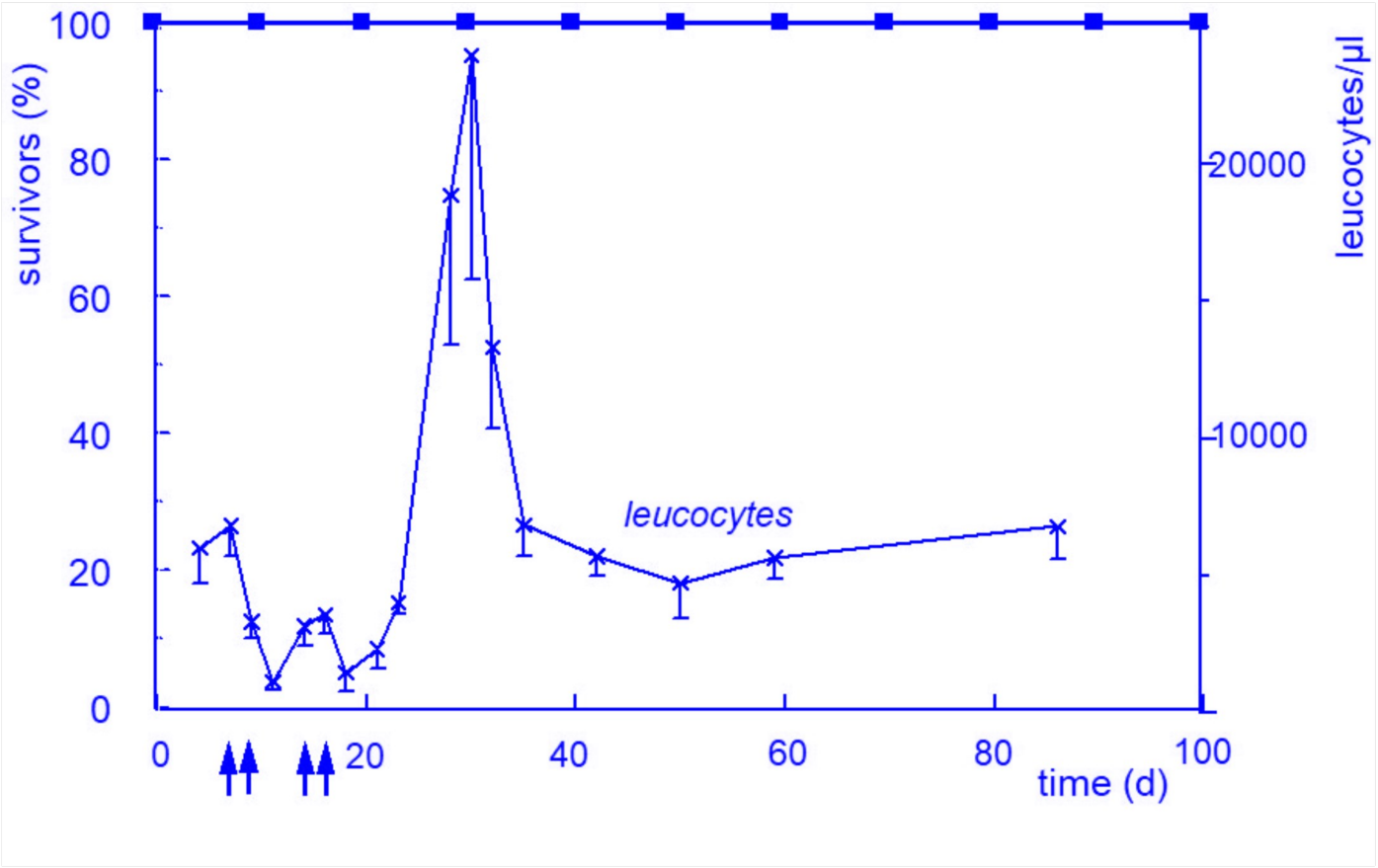


Fig. 7

Blood leukocytes (x) and survival (▪) of female P388 tumor bearing CD2F1 mice after therapy with SUM-IAP (666mg/kg subcutaneouly) d7.8 and d14.15 (arrows), mean –SD, n=5

Anti metastatic therapy with SUM-IAP in combination with the apoptosis booster N-methylformamide (NMF)

NMF has been synthesized in 1953 as a solvent for the parenteral administration of water-insoluble substances in experimental chemotherapy. Since NMF was found to be active against S-180-Sarcoma in mice [15] clinical trials have been carried out but were canceled because signs of hepatotoxicity were observed.

Kalyany et al. [16] investigated the interaction of NMF with superoxide dismutase 1 (SOD 1) Their findings show that NMF causes loss of enzymatic activity due to perturbation of secondary structure of SOD 1. SOD 1 deficiency induces O_2^--accumulation in the cytoplasm and mitochondria which increases loss of mitochondrial membrane potential and DNA-damage-mediated p53 apoptose. From this it can be concluded that NMF is a booster of the p53 dependent apoptosis. NMF was chosen for combination therapy with SUM-IAP because anti metastatic activity of NMF was demonstrated [17].

In a therapy experiment (see fig.8), mice with subcutaneously growing P388 tumors were treated with 266mg/kg SUM-IAP on days 7-11. All animals developed visible metastases in the region of lymph nodes of forelegs until day 29. Postmortem examination showed liver metastases in all animals. A further therapy test with the same dose and schedule of SUM-IAP application but with additional applications of 200 mg/kg NMF (12 injections on day 13 to 24) was carried out. The mice were cured. In the animals, neither visible external metastases nor liver metastases or metastases anywhere else in the abdominal range were found after postmortem examination [18]

The other most remarkable result in the experiment in which SUM-IAP was combined with NMF is lack of any sign of toxicity except a short, but reversible decrease in the number of leukocytes after SUM-IAP injection. The body weight of animal increased steadily during additional therapy with NMF. In control experiments with NMF alone, no or only marginal antitumor activity (ILS 12 %) was detected.

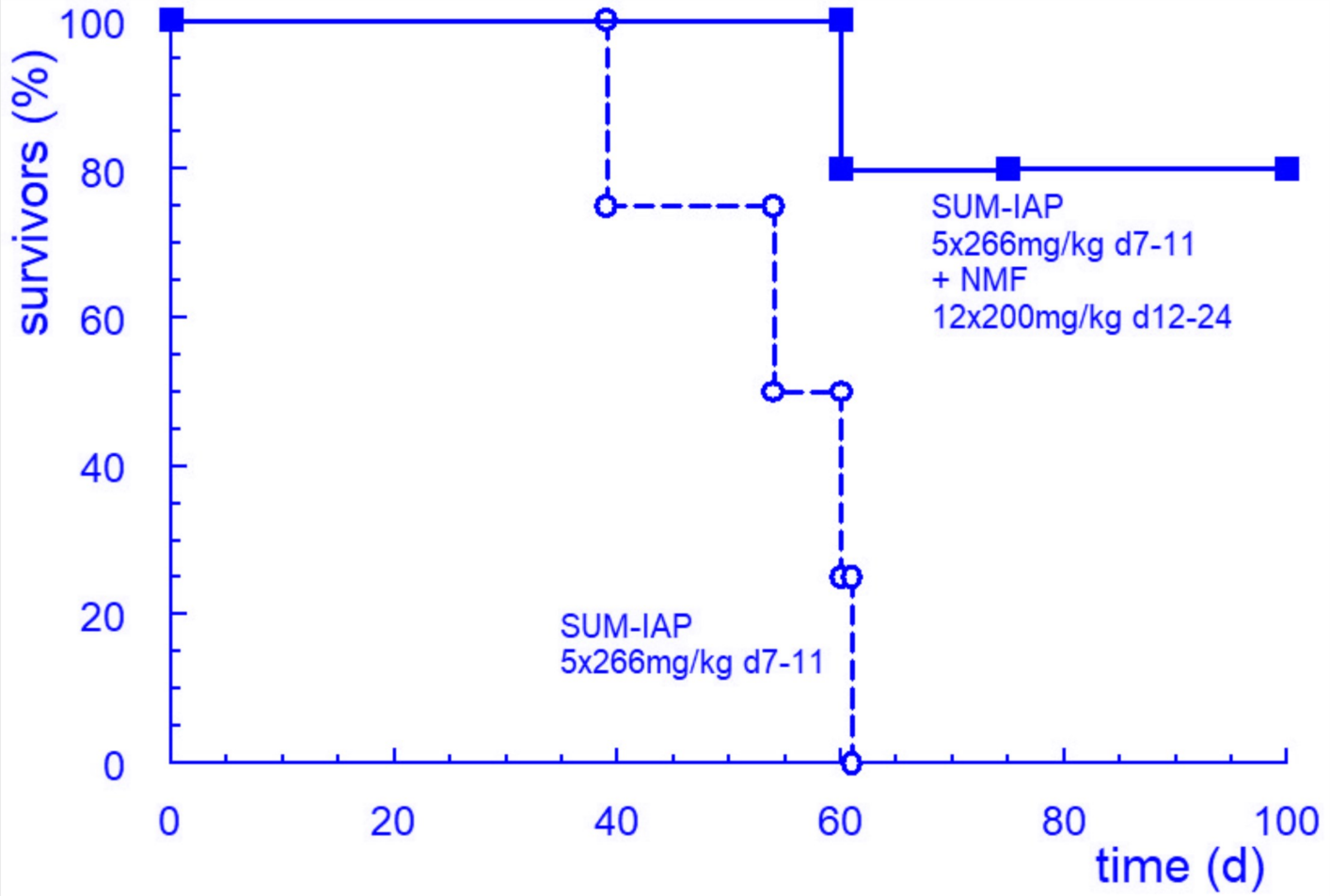


Fig. 8

Survival curves of CD2F1 mice with subcutaneously growing P388 tumors after therapy with SUM-IAP (0) and SUM-IAP in combination with N-methylformamide (NMF ■) SUM-IAP 5x266mg/kg s.c. d7-11, NMF 12x200mg/kg i.p. d12-24. Untreated, tumor-bearing controls died between days 11 and 13

Anti metastatic therapy with SUM-IAP in combination with cisplatin (CPT)

As already mentioned the reason for formation of liver metastases is the high activity of aldehyde dehydrogenases in the liver which detoxify the pharmacologic active metabolite SUM-Ialdophosphamide to the therapeutically ineffective carboxylic acid. This was the reason to combine SUM-IAP with CPT which is not detoxified in the liver. 4 out of 5 of the mice treated with 266 mg/kg SUM-IAP on days 7-11 and 21-25 and additionally with 1.8 CPT on days 12 and 26 survived the observation period of 100 days. But mice of a control group which were only treated with SUM-IAP died between day 52 and 70 due to growth of metastasis. CPT alone was only marginally effective at the dose administered, (Increase in life span of 67%) [18]. In contrast to the experiments with SUM-IAP alone and in combination with NMF, the combination of SUM-IAP with 3.6 mg/kg CPT was toxic as indicated by body weight loss of >25% in the mice after therapy.

Discussion

Antineoplastic substances can roughly be divided into 2 groups. In those whose mechanism of action is based solely on damaging fast-growing cells more than normal cells and those that interfere with tumor-specific biochemical processes. The first group includes alkylating agents that block cell growth by damaging DNA. Because of its development as a modified nitrogene mustard derivative, cyclophosphamide is assigned to category 1, although it shows superior antitumor activity compared to the parent substance nitrogene mustard itself or derivatives thereof such as Chlorambucil, Melaphalan or Estramustine.

For a long time the only measurable difference between CP and the nitrogene mustard derivatives mentioned was the end product of the metabolism. CP does not produce the alkylating agent nitrogen mustard, but PAM. Due to ignorance of the true mechanism of action, the formation of PAM has been considered to be the cause of CP's outstanding antitumor activity. Based on this assumption, attempts to improve the anti-tumor activity of CP were made either by modifying the alkylating function, which led to the development of Ifosfamide, or by concentration the alkylating agent in tumor cells, as was tried in the development of Glufosfamide (β-D-glucose-isophosphoreamide mustard) which had been synthesized with the intention to use the higher glucose requirement of tumor cells for an accumulation of IPAM in the tumor cells. Only with the discovery of apoptosis and the apoptosis enhancer HPA as a CP metabolite it was possible to understand the true mechanism of action of CP and other oxazaphosphorines.

CP was found by serendipity and is not tailor-made for its mechanism of action, it needs improvement. The alkylating function produces easily repairable DNA damage that does not allow a large apoptosis yield. This is improved in the model substance SUM-IAP. SUM-IAP spontaneously hydrolyzes, bypassing toxic IFOH and chloroacetaldehyde. The alkylating function in SUM-IAP is developed to produce low-toxicity and poorly repairable or irreparably DNA damage. The result is that toxicity of SUM-IAP is one seventh to one eighth lower than that of CPOH and antitumor effect orders of magnitude greater.

Despite this increase in effectiveness, healing of the mice with SUM-IAP alone was initially not possible because

the liver, as a functional compartment with a high rate of detoxification of OXALD, was the starting point for the formation of metastases. The mice were only able to survive in combination with CPT, which, however, is toxic. In contrast to the combination with CPT, the combination with the apoptosis enhancer NMF which also prevented the formation of metastases is absolutely non-toxic. The experiment with NMF shows that the initiation of p53-based apoptosis by irreversible DNA damage by SUM-IAP is not only enhanced by HPA but probably by any apoptosis enhancer.

In addition to fast-growing tumor cells, CP and other alkylating agents inhibit the growth of all fast-growing cells. This lack of tumor specificity and high systemic toxicity limit the use of this class of substances. In the case of SUM-IAP systemic toxicity is reduced to a minimum as mentioned above. The lack of tumor specificity of alkylating anticancer drugs is not eliminated by SUM-IAP, but it has an indirect tumor-specific effect. Many tumor cells express tumor-specific antigens which are recognized by cytotoxic T cells but do not proceed to attack the tumor cell because the cytotoxic T cell is locked by Treg. Since the inhibition of cytotoxic T cells directed against tumor cells can be eliminated by apoptotic cell death of Tregs by SUM-IAP thus the nonspecific acting alkylating agent becomes tumor-specific. This is nothing special in itself. CP does the same in low, non-cytotoxic doses. What is special about SUM-IAP, however, is that in high doses, it simultaneously eradicates the tumor cell by DNA damage -initiated apoptosis and by activation of tumor-directed cytotoxic T cells by inhibiting Treg.

Acknowledgements

This study was funded by the Bundesministerium fur Forschung und Technologie.

Conflicts of interest

There are no conflicts of interest.

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