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Review Article

The Mechanism of Action of Cyclophosphamide Points the Way for the Development of New Cyclophosphamide-Related Compounds

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Received: January 13, 2025; **Published:** January 29, 2025

Abstract

The mechanism of action of cyclophosphamide (CP), although successfully used in the clinic for more than 60 years, was unknown until a few years ago. The reason for this was that results from *in vitro* studies were uncritically transferred to *in vivo* conditions. *In vitro*, the alkylating metabolite phosphoramide mustard (PAM) is formed by β-elimination of acrolein from the CP metabolite aldophosphamide (ALD); *in vivo*, ALD is cleaved by phosphodiesterase with the formation of 3-hydroxypropanal (HPA) instead of acrolein. Three discoveries had to come together to elucidate the mechanism of action of CP and other oxazaphosphorine cytostatic (OX): namely that HPA is a CP metabolite, that HPA promotes p53-controlled apoptosis and that the cell death event following CP therapy is p53-controlled apoptosis triggered by DNA damage. Based on this, a simple model for the mechanism of action of OX was postulated. Based on this model and with the aim of reduced toxicity and improved antitumor efficacy, aldophosphamide thiazolidines and aldophosphamide perhydrothiazines were synthesized and tested in P388 tumor bearing mice. The toxicity of these compounds is 6-7 times lower in mice than the toxicity of the parent compounds CP and Ifosfamide (IF). Therapy experiments with p388 tumor bearing mice were carried out with SUM-IAP. SUM-IAP is an I-aldophosphamide perhydrothiazolidine with an alkylating function adapted to the mechanism of action whereby antitumor activity is increased 10^4 to 10^5 times. Formation of metastases resistant to SUM-IAP is prevented by combining SUM-IAP with cis platinum (CPT) and by combination with the apoptosis enhancer N-methylformamide (NMF). With SUM-IAP, the body's own immune defense against metastases can be activated by suppressing inhibitory regulatory T cells. All animals were cured in a corresponding experiment. In contrast to aldophosphamide perhydrothiazines, aldophosphamide thiazolidines crosses the blood-brain barrier and are candidates for the treatment of CNS tumors.

Keywords: *Mechanism of Action of Cyclophosphamide; Thiazolidines/Perhydrothiazines of Aldophosphamide; Apoptosis; Blood Brain Barrier; SUM-IAP; Anti-Metastatic Therapy*

Introduction

Cyclophosphamide (CP), a cytostatic drug that is indispensable for the treatment of cancer in the clinic, has a history of more than 60 years. For a long time, CP was simply classified as an alkylating drug. The parent substance of the substance group to which CP was classified is nitrogen mustard known as "HN2" originally produced as chemical warfare agent. The very toxic HN2 was one of the first chemotherapeutic agents for treatment of cancer [1]. In the 1950s it was assumed that the activity of phosphoamidases - enzymes that catalyze the cleavage of phosphorus-nitrogen bonds -, is greater in tumor cells than in normal cells [2]. Based on this (wrong) assumption the plan to reduce toxicity of HN2 was to synthesize a non-toxic prodrug that would be cleaved in the tumor cell by phosphoamidases -

into a phosphoric acid residue and HN2. Of the substances synthesized according to this specification, CP (*1*, figure 1) proved to be the most effective in animal experiments and was introduced into the clinic in Germany under the name "Endoxan" in 1958 [3]. Soon after its introduction into the clinic, it turned out that the idea underlying the development of CP was wrong. A non-toxic prodrug had been created, but not one that is cleaved in tumor cells by phosphoamidases into HN2 and a phosphoric acid residue, but a substance that is hydroxylated by cytochrome P450 enzymes [4] to 4-hydroxycyclophosphamide (CPOH, *2* figure 1)) from which the alkylating metabolite phosphoramide (PAM, *3* figure 1) is formed.

Until a few years ago the true mechanism of action of CP and other oxazaphosphorine cytostatic like Ifosfamide (IF) was unknown. One reason for this was that the results of *in vitro* tests were uncritically transferred to *in vivo* conditions, which meant that it was simply overlooked that CP and other OXs form a different by-product *in vivo* during degradation of CPOH to the alkylating agent PAM than during degradation *in vitro*. The by-product acrolein is formed *in vitro*, but 3-hydroxypropanal (HPA) is formed *in vivo* under the influence of esterases. For a long time this seemed irrelevant because in both cases the actual alkylating agent PAM, which was thought to inhibits cell growth through DNA alkylation, is formed. Only with advancing biochemical knowledge, more precisely with the realization that apoptosis is a universal mechanism for the regulation of cell homeostasis, did the discovery of HPA as CP metabolite gain significance for the elucidation of the mechanism of action of CP and other OX.

Three discoveries had to come together to elucidate the mechanism of action of OX. These are: The discovery of HPA as a CP metabolite [5], the discovery that HPA is a proapoptotic substance [6] and last but not least that the event leading to cell death after CP therapy is not DNA damage by alkylation but the apoptosis initiated by it [7].

In the following, a mechanism of action for OX based on these discoveries is presented and, using a model substance SUM-IAP adapted to this mechanism of action, it is shown how the OX currently used in the clinic can be improved by orders of magnitude and used for new indications such as immunological tumor therapy and therapy of CNS tumors.

Metabolism of CP

Representative of all OX, the metabolism of these substances is described below using CP as an example.

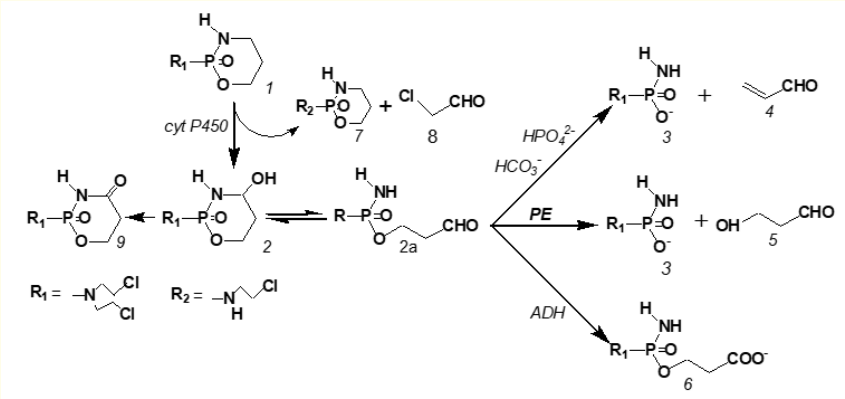


Figure 1: *Metabolism of CP. CP (1) is hydroxylated in vivo, to CPOH (2) by cyt P450 hydroxylases. CPOH equilibrates with its ring open tautomer ALD (2a). In vitro ALD becomes PAM (3) by ß-elimination of acrolein (4). This reaction is catalyzed by phosphate - and bicarbonate ions. In vivo, CPOH is detoxified to 4-ketocyclophosphamide (9) whereas ALD is cleaved by phosphoesterases into PAM (3) and 3-hydroxypropanal (5). This reaction competes in vivo with the formation of CARB (6) catalyzed by aldehyde dehydrogenases (ADH).*

The detection of CPOH (2 figure 1) after CP (1 figure 1) injection in animals and patients was initially difficult because no reference substance was available. Only after Peter had succeeded in producing CPOH by ozonisation of CP and subsequent reduction of the formed 4-hydroperoxycyclophosphamide to CPOH [8] it was possible to it investigate the chemical reactivity and properties of this substance in more detail and to detect CPOH in rat blood after CP injection [9].

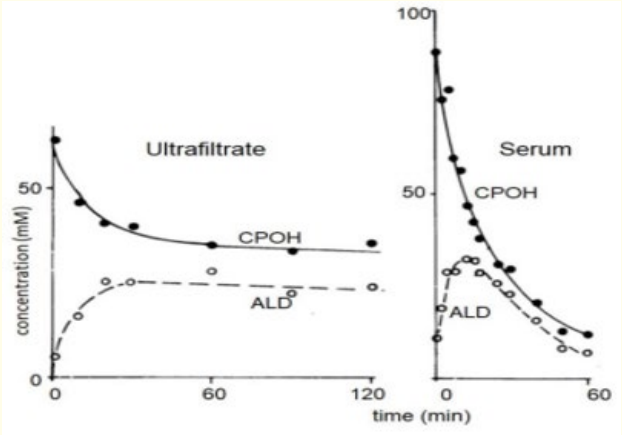


Figure 2: *Decomposition of CPOH/ALDO in protein free serum ultrafiltrate and rat serum. Decomposition of and formation of ALD from CPOH in protein-free rat serum ultrafiltrate (left) and rat serum (right), pH 7, 37°C, chromatographic determination of CPOH and ALD [10]. The figure is taken from reference [13] and adapted to this article, for details of the methodology see there.*

In vitro investigations with CPOH (s. figure 2) showed that CPOH forms an equilibrium mixture with its tautomeric aldophosphamide (ALD *2a* figure 1). The investigations further showed that *in vitro* ALD is decomposed to the DNA-alkylating CP metabolite PAM by ß-elimination of acrolein (*4* figure 1) [10]. *In vivo* after formation from CP a small proportion of CPOH is enzymatically detoxified to non-cytotoxic 4-ketocyclophosphamide (*9* figure 1). The bulk of ALD formed from CPOH is detoxified by aldehyde dehydrogenases (ADH) to inert carboxyphosphamide (CARB *6* figure 1). A toxic byproduct of the cyt. P450 hydroxylation reaction is toxic chloroacetaldehyde (*8* figure 1) produced by side chain hydroxylation. In this reaction dechloro cyclophosphamide (*7* figure 1) is formed [11]. In contrast to the decay in protein-free rat serum ultrafiltrate, the decay in rat serum is very rapid. In rat serum ultrafiltrate, the decay half-life of CPOH is more than 20 hours; In rat serum, however, only 20 minutes indicating that the decomposition in rat serum is enzyme catalyzed (s. figure 2). The ALD-cleaving enzyme was identified as phosphoesterases [12,13].

Reaction products of the enzymatic cleavage of ALD

Figure 3 shows the record of HPLC runs 10 and 90 min after start of incubation of CPOH/ALD in rat serum. After protein precipitation samples were treated with 2,4-dinitrophenylhydrazine. The hydrazones of the aldehydes in the incubation samples were analyzed by HPLC. In both the samples 3-hydroxypropionaldehyde (HPA) is detectable - much more after 90 min than after 10 min - indicating that ALD is the substrate for the splitting by phosphoesterases. Based on these findings the enzymatic decomposition reaction of ALD is according to the reaction sequence marked by PE in figure 1. Acrolein is detectable right from the start of the incubation and it probably is a byproduct produced during the reaction of CPOH/ALD with 2.4-dinitrophenylhydrazine.

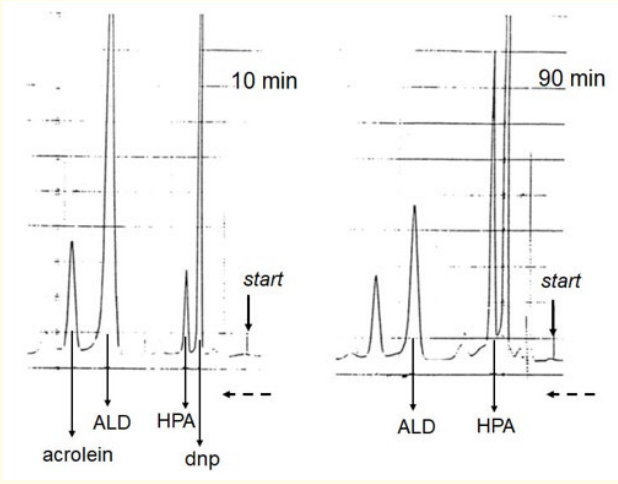


Figure 3: *Time dependent formation of HPA from CPOH/ALD. HPLC analysis of aliquots of CPOH incubated in rat serum (pH7, 37°C) for 10 min or 90 min). Protein precipitation and reaction of the aldehydes with 2,4 dinitrophenylhydrazine. dnp: 2,4 dinitrophenylhydrazine, HPA hydrazone of 3-hydroxypropanol, ALD: hydrazone of aldophosphamide, Acrolein: hydrazone of acrolein. Arrows indicate start of HPLC run. The figure is taken from reference [5] and adapted to this article, for details of the methodology see there.*

The byproduct of decomposition of ALD to PAM *in vivo* is not acrolein but 3-hydroxypropanal (HPA, *5* figure 1). As is shown in figure 3.

CPOH: Formation *in vivo* and identification as the CP metabolite required for the outstanding therapeutic efficacy of CP

Figure 4 shows the blood level curves of CP and CPOH in the blood of female NMRI mice after intravenous injection of 100 mg/kg CP. CP is eliminated from the blood with a half-life of 6 minutes. This corresponds to the formation of CPOH, for which a half-life of 5 minutes was calculated. From the areas under the blood concentration curves and the values for total body clearance determined in separate experiments, it can be calculated that 92% of the injected CP is hydroxylated to CPOH, of which 81% is detoxified to therapeutically ineffective 4-ketocyclophosphamide and CARB, so that ultimately only 19% of the CPOH formed is available for therapeutic reactions.

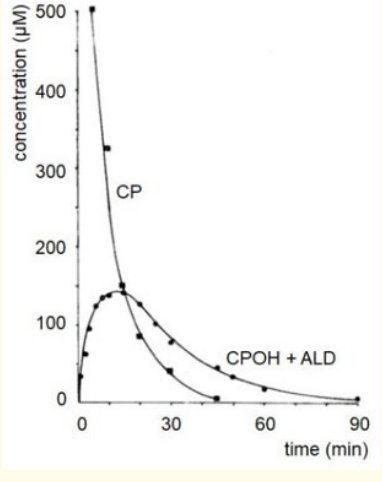


Figure 4: *Blood level of CP and CPOH + ALD in female NMRI mice after intravenous injection of 100 mg/kg CP. Mean values of 5 mice, flourometric CPOH/ALD determination, chromatographic CP determination [10]. The figure is taken from reference [14] and adapted to this article, for details of the methodology see there.*

Since CPOH/ALD is metabolized in the liver to the therapeutically ineffective 4-ketocyclophosphamide and CARB, only a small part of the injected CPOH reaches the bloodstream (first-pass effect) and develops its therapeutic effect.

A comparison of the therapeutic efficacy of CPOH after intraperitoneal and intravenous injection into human mammary carcinoma-bearing female nu/nu mice shows in figure 5 that the first-pass effect after intraperitoneal injection renders the main amount of CPOH ineffective.

The figure shows the tumor growth curves following therapy with CPOH after intraperitoneal injection (Figure 5A) and intravenous injection (Figure 5B). While a dose-dependent growth retardation can be measured after intravenous injection, which leads to growth arrest after administration of 46.4 mg/kg (Figure 5B, tumor growth curve 2), this dose is almost ineffective after intraperitoneal injection.

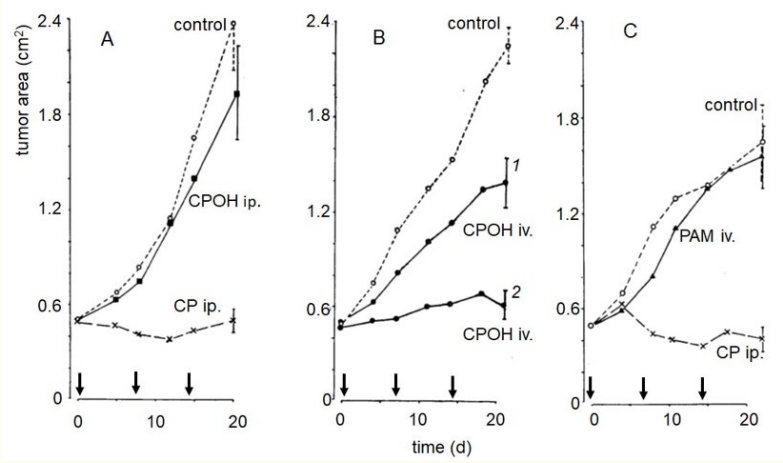


Figure 5: *Tumor growth curves of a human breast carcinoma hetero transplanted on female nu/nu mice after treatment with intraperitoneally administered CPOH 31.6 mg/kg, (graph A), intravenously administered CPOH (graph B curve 1: 17.8 mg/kg, curve 2: 31.6 mg/kg) and intravenously administered PAM 46 mg/kg (graph C) The administered doses of CPOH and PAM correspond to 20% of the LD50. CP: 100 mg/kg, control: control group without treatment, mean values from 5 - 10 individual measurements, standard deviation of the last measurement. The figure is taken from reference [15] and adapted to this article, for details of the methodology see there.*

An important result of the therapy tests shown in figure 5, is the complete therapeutic failure of PAM. While CP completely stops tumor growth and CPOH achieves a therapeutic success similar to CP after intravenous injection (Figure 5A), PAM is completely ineffective after both intravenous and intraperitoneal injection (the latter is not shown). This result shows that CPOH and not PAM is the carrier of the therapeutic efficacy of CP. This result confirms the statements of Brock and Brock and Hohorst, who determined the therapeutic indices of CP metabolites.

The therapeutic index, the ratio from the amount of a therapeutic agent that causes toxicity to the amount that causes the therapeutic effect measured in Yoshida ascites sarcoma-bearing rats is according to Brock [16] and Brock and Hohorst [17], 120 for CPOH but only 3.5 for PAM and 2.5 for the mother substance nitrogen mustard. This result indicates that DNA alkylations by PAM released from ALD *in vivo* are much more efficient than DNA alkylations produced by bare PAM injected.

The question of why this is the case is answered below with the formulation of a scheme for the mechanism of action of OX.

Mechanism of action of CP and other OX

The mechanism of action for CP and other OX formulated below is based on the following experimental findings described above:

1. ALD is the carrier of the therapeutic efficacy of CP and other OX.
2. The formation of PAM from ALD *in vivo* does not produce acrolein but HPA.
3. HPA is a pro-apoptotic OX metabolite that activates p53-driven apoptosis.
4. The event leading to cell death after OX therapy is p53 driven apoptosis initiated by DNA alkylation by PA.

These experimental findings lead to the mechanism of action formulated in figure 6.

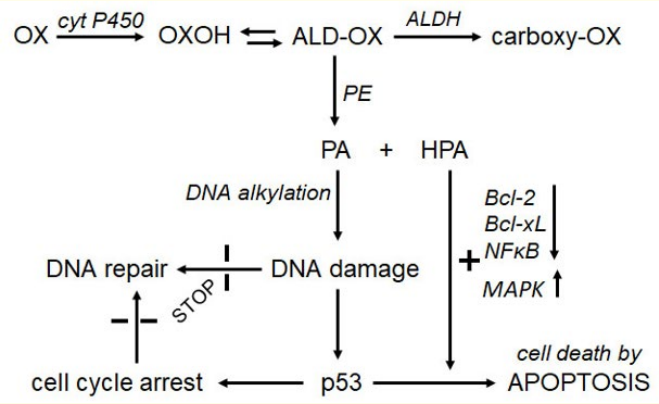


Figure 6: *Mechanism of action of OX based on literature data [3-5].*

Oxazaphosphorine cytostatics (OX) like CP IF, are hydroxylated by P450 enzymes in the liver to the corresponding hydroxyl derivatives (OXOH), which are in equilibrium with its aldophosphamide tautomers (ALD-OX). The ALD-OX compounds are the pharmacologic active metabolites. The bulk of it is oxidized by aldehyde dehydrogenases to the nontoxic carboxy compounds. The amount of ALD-OX not oxidized, is decomposed by phosphoesterases (PE) to the alkylating phosphoramide (PA (PAM, I-PAM)) and HPA. The PA derivatives damage 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, enhanced by HPA which supports apoptosis by inhibiting the antiapoptotic proteins Bcl-2 and Bcl-xL, and by inhibiting NF-κB activation. Additional HPA promotes apoptosis by enhancing mitogen-activated protein kinase (MAPK) activities by enhancing JNK and p38 phosphorylations.

Since DNA damage is repaired very quickly by the cell's own repair mechanisms, it follows from this scheme that the effectiveness of OX can be increased by preventing DNA repair (indicated by "STOP" in the scheme figure 6).

Toxicity of CP

According to the prevailing opinion, the alkylating agent PAM is formed from ALD by spontaneous β-elimination of acrolein which in addition to the reactions caused by the alkylating agent PAM and chloroacetaldehyde (*8* figure 1) are considered to be causes of cyclophosphamide toxicity. The supposed toxicity due to acrolein is suppressed by the addition of thiol compounds such as MESNA, which is assumed to form inert thioether with acrolein [18]. The treatment with thiol compounds to reduce cyclophosphamide toxicity is successful

although no acrolein is formed *in vivo* after CP administration. This raises the question of the cause of that part of cyclophosphamide toxicity not caused by alkylation and the mechanism of action of thiol compounds in reducing cyclophosphamide toxicity.

In acute toxicity tests in mice following intraperitoneal injection, the LD50 of 0.53 mmol/kg (143 mg/kg) was determined for CPOH. and 0.81 mmol/kg (183 mg/kg) for PAM [19]. The result shows that CPOH not only generates its toxicity as a carrier of PAM but is toxic itself. As already mentioned (See figure 5), most of the CPOH applied intraperitoneally is detoxified to CARB by the first pass effect, so that the true toxicity of CPOH is much greater than indicated by the toxicity test. A further indication that CPOH does not develop its toxicity as a carrier of PAM but is toxic itself are the manifestations of intoxication: After application of CPOH animals die within 24h after drug application; but after administration of PAM mice die between day 4 and day 6 after drug application. Immediately after PAM injection no poisoning is observed, but after injection of CPOH severe signs of local toxicity like pink coloration of superficially perfused parts of the body and adhesions of the peritoneum which indicate that the mice have suffered from peritonitis were observed. The cause of the pronounced local toxicity is the reactivity of the hemiaminal group of the oxazaphosphorine ring of CPOH with nucleophiles such as free thiol groups of membrane proteins as shown in figure 7.

From the fact that after injection of CP no acrolein is formed but HPA it can be concluded that, the toxic CP metabolite neutralized by the thiol compound MESNA is not acrolein but CPOH, which is responsible for most not by the alkylating function-caused toxicity.

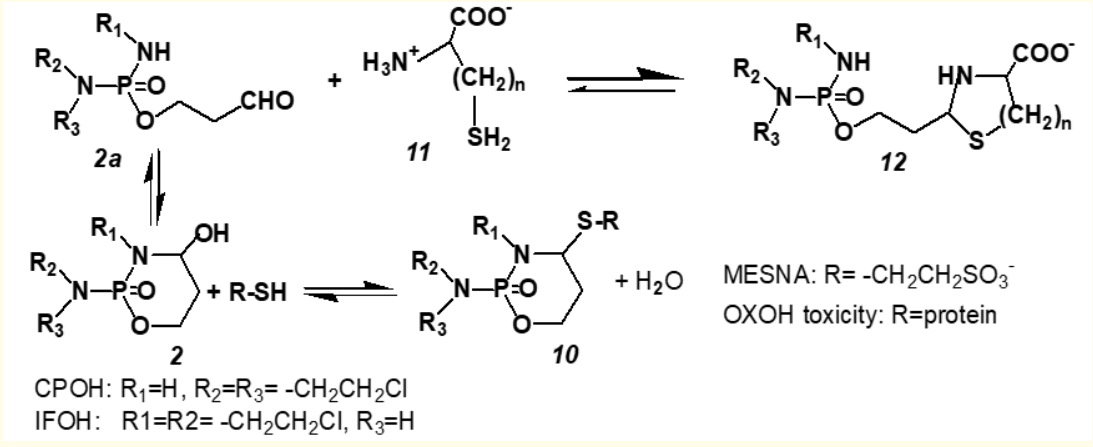


Figure 7: *Reaction of the tautomer pair OXOH/ALD-OX with thiol compounds.*

OXOH (2) forms sulfido-cyclophosphamide compounds (10) with compounds such as MESNA ($HSCH_2CH_2SO_3$) and protein SH groups. ALD-OX (2a) forms stable thiazolidines (12, n=1) or perhydrothiazines (12, n=2) with β- or γ -aminothiols such as cysteine (11, n=1) or homocysteine (11, n=2), which hydrolyze to ALD-OX and the thiol in aqueous solution with half-lives of more than 10 hours.

The reaction of CPOH (2) with low molecular weight thiol compounds such as MESNA reduces the toxicity of CPOH. However, the reaction with thiols is also the cause of CPOH toxicity when CPOH reacts with SH-containing biomolecules such as membrane proteins.

Interim results

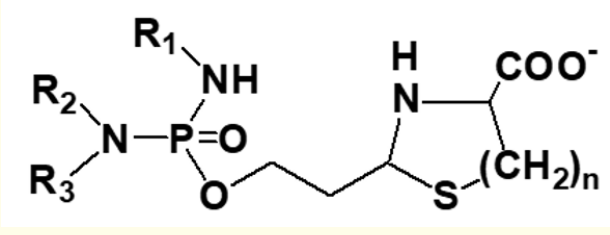
Important conclusions from the proposed mechanism of action of OX and toxicity tests are: The pharmacologically active metabolite of OX is ALD-OX. The metabolites responsible for the toxicity are mainly OXOH and chloroacetaldehyde (*8* figure 1). The latter is believed to have nephrotoxic and neurotoxic side effects [20]. It follows that in the development of new OX, ALD-OX must be formed bypassing the hydroxylation reaction in the liver. Candidates for these new OX are thiazolidines (TIA) and perhydrothiazines (ALP) of ALD-OX (*12*, figure 7) which are formed in the reaction of ALD-OX with β - or γ-aminothiols such as cysteine or homocysteine (figure 7). These compounds are stable and hydrolyze to ALD-OX under physiological pH and temperature conditions, bypassing toxic CPOH and toxic chloroacetaldehyde.

To test whether these compounds are suitable as cytostatics, TIA and ALP were synthesized and tested in animal experiments. The acute toxicity in mice when administered intraperitoneally was approximately 5 mmol/kg (1900 mg/kg) for ALP and 6 mmol/kg (2160 mg/kg) for TIA [19,21]. Compared to CPOH these compounds are 7- 9 times less toxic. This confirms the result of the toxicity tests, according to which CPOH is the metabolite responsible for the toxicity of CP.

In therapy experiments in which mice bearing subcutaneously transplanted P388 mouse leukemia cells an increase in life span of 113% and 170% was measured when animals were treated with approximately 5% of the LD 50 of TIA (100 mg/kg) or 10% LD50 of ALP (200 mg/kg) on days 1-5 after tumor transplantation. These experiments show that the TIA and ALP have antitumor activity.

Antitumor activity of thiazolidines and perhydrothiazines of ALD-OX

Table 1 and figure 8 shows a selection of the results of therapy experiments with thiazolidines and perhydrothiazines of ALD-OX.



	R_1	R_2	R_3	ILS[1] (%)	LTS[2] (%)	ΔG[3] (g)
n =1						
10	H	$-CH_2CH_3$	$-CH_2CH_3$	8	0/5	+1
11 (TIA)	H	$-CH_2CH_2Cl$	$-CH_2CH_2Cl$	148	0/5	0
12	$-CH_2CH_2Cl$	$-CH_2CH_2Cl$	H	94	0/5	0
13	$-CH_2CH_2OSO_2CH_3$	$-CH_2CH_2Cl$	H	180	1/5	0
14	H	$-CH_2CH_2Br$	$-CH_2CH_2Br$	197	0/5	-2
15	$-CH_2CH_2Br$	$-CH_2CH_2Br$	H	222	1/5	0
n=2						
20	H	$-CH_2CH_3$	$-CH_2CH_3$	6	0/5	0
21 (ALP)	H	$-CH_2CH_2Cl$	$-CH_2CH_2Cl$	235	1/5	-3
22 (IAP)	$-CH_2CH_2Cl$	$-CH_2CH_2Cl$	H	160	0/5	-1
23 (SUM-IAP)	$-CH_2CH_2OSO_2CH_3$	$-CH_2CH_2Cl$	H	237	2/5	0
24	H	$-CH_2CH_2Br$	$-CH_2CH_2Br$	6	0/5	+3
25	$-CH_2CH_2Br$	$-CH_2CH_2Br$	H	320	2/5	-1

Table 1: *Antitumor activity and toxicity of ALD-OX-thiazolidines (TIA, n=1) and ALD-OX-perhydrothiazines (ALP, n = 2).*

TIA: Subcutaneous Administration of 0.26 mmol/kg (corresponding to 100 mg/kg compound 12), days 1 - 5. ALP: Subcutaneous Administration of 0.51 mmol/kg (corresponding to 200 mg/kg compound 22) days 1 - 5, female CD2F1 mice, intraperitoneal transplantation of 10^6 P388 mouse leukemia cells on d 0.

1: ILS increase in life span, 2: LTS long time survivors (surviving time >100d), 3: ΔG difference in body weight at d 0 and the lowest body weight after drug administration. The table is taken from reference [22] and adapted to this article, for details of the methodology see there.

The table shows the best therapeutic efficacy (long time survivor: LTS) with low toxicity (difference in body weight before and after therapy: ΔG) for the substances in which a chlorine of the alkylating function is substituted either by bromine or by a mesyl group. The brominated compounds were toxic when dose was increased and were not further investigated. Of the remaining compounds the best results are achieved with the mesyl compounds 13 and 23. In therapy tests with these compounds in advanced solid P388 tumor bearing mice no signs of toxicity such as hair loss or weight loss were observed.

Figure 8 shows the tumor growth curves of another therapy test with thiazolidines and perhydrothiazines of ALD-OX. The ALD-OX thiazolidines and ALD-OX perhydrothiazines with the alkylating function of CP (compounds 11, 21) lead to a stronger tumor growth retardation than the corresponding compounds with the alkylating function of IF (compounds 12 and 22). However, this is insignificant when comparing the two compounds 22 (I-aldophosphamide-perhydrothiazines, abbreviated IAP) and 23 (sulfonyl-methyl-I-aldophosphamide-perhydrothiazines, abbreviated SUM-IAP): By substituting a chlorine of the alkylating function of IAP with a methylsulfonyl group, a marginally effective substance becomes a substance that shrinks the subcutaneously transplanted P388 tumor below the detection limit. Unfortunately, the success of the therapy is not long-lasting; the tumor can be measured again after 4 days. A second cycle of therapy to treat the regrowing tumor was ineffective, probably because the remaining tumor cells had become resistant to SUM-IAP.

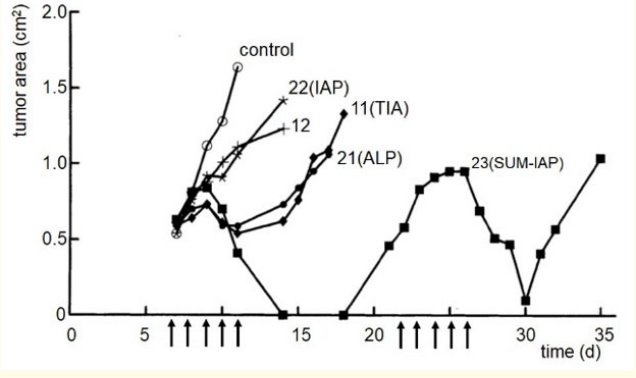


Figure 8: *Tumor growth curves following subcutaneous transplantation of 10^6 mouse leukemia cells into female CD2F1 mice and therapy with the compounds (formulas see table 1) indicated. Daily subcutaneous application on day 7-11 and 22-26 (arrows). Daily dosages were equimolar to 111 mg/kg compound 12, mean values from 3 animals, control: tumor bearing mice without therapy. The figure is taken from reference [22] and adapted to this article, for details of the methodology see there.*

These results and the fact that synthesis and handling of the perhydrothiazines derivate contrary to synthesis and handling of the thiazolidines is hassle free gave rise to further investigations with the perhydrothiazines IAP and SUM-IAP of I-aldophosphamide.

In this context, a comparison of the toxicity and anti-tumor activity of IAP which has the alkylating function of IF with the toxicity and anti-tumor effect of IF itself is of interest: One day after subcutaneous transplantation of 10^6 P388 tumor cells mice were treated with equitoxic doses of IAP and IF. The treatment with IF only resulted in an increase in life span, while treatment with IAP cured the animals [23].

In order to roughly quantify the improved therapeutic efficacy of SUM-IAP due to modifying the alkylating function by a mesyl group the measured tumor areas which correlates well with the tumor mass after excision (correlation coefficient 0.93) were evaluated using the back extrapolation method according to Alexander and Mikulski [24]. From these 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 methylsulfonyl group in SUM-IAP.

Mechanism of action-based explanation for the outstanding antitumor efficacy of OX, in which a 2-chloro-ethyl group is substituted by a 2-mesyl-ethyl group in the alkylating function

In a further experiment, the cytotoxicity of IAP and SUM-IAP against the same P388 mice leukemia cells used in the animal experiments was examined in cell culture experiments. The result was surprising. SUM-IAP, which was 10^4-10^5 times more cytotoxic than IAP in the therapy experiments in P388 tumor-bearing mice, was significantly less effective against the same cells in the cell culture experiments. The *in vitro* concentration/response curves show that IAP is approximately 1.5 time more cytotoxic against P388 mice leukemia cells *in vitro* than SUM-IAP [23].

The effect reversal of IAP and SUM-IAP *in vitro* and *in vivo* was a mystery and unsolvable until the discovery that the event leading to cell death after CP therapy is not DNA damage but the p53 controlled apoptosis initiated by the DNA damage. *In-vitro*, the decay products of IAP or SUM-IAP are IPAM or SUM-IPAM and acrolein. *In-vivo*, however, the decay is catalyzed by PE; the decay products are the proapoptotic HPA and IPAM or SUM-IPAM. From this, it can be assumed that the *in-vitro* results indicate non apoptotic cell death and the *in-vivo* results indicate DNA damage-induced apoptotic cell death. Substitution of the chloroethyl group in the IAP molecule by a mesyl-ethyl-group in the SUM-IAP molecule, which has order of magnitude higher antitumor activity has major effects on the type of alkylation. Chloroethyl groups as found in IAP form inter strand crosslinks that are repaired very efficiently, whereas methane-sulfonate-groups present in the SUM-IAP molecule form intra strand cross-links [25,26] which are poorly repaired. (http://www.atdbio.com/content/16/Nucleic-acid-drug-interactions). If in the mechanism of action of alkylating agents the purpose of DNA damage is to trigger cytotoxic apoptosis, then it is more advantageous to render mild poorly repairable but long-lasting DNA damage because the poorly repairable DNA damage forces more cells to undergo cytotoxic apoptosis. Obviously, SUM-IAP produces more poorly repairable long-lasting intra strand cross-links than IAP.

As an interim result, it should be noted that the therapy experiments with IAP with the alkylating function of IF, which causes easily repairable DNA inter strand damage, and the therapy tests with SUM-IAP, whose alkylating function causes poorly repairable DNA intra strand links, confirm the correctness of the presented mechanism of action of CP and other OX cytostatics.

Aldophosphamide thiazolidine (TIA) crosses the blood brain barrier

High doses of TIA (formula tab. 1) administered intraperitoneally to mice were tolerated without manifestations of intoxication. After intravenous injection of the same doses, the animals developed convulsions and died shortly after injection. After addition of 10 mol% cysteine to the injection solution, high doses of TIA were tolerated by all animals without toxic side effects even after intravenous injection. The symptoms of intoxication after intravenous injection of TIA were interpreted as poisoning of the central nervous system (CNC).

To verify the correctness of this assumption, the organ distribution of TIA was measured 20 hours after intraperitoneal injection of 1000 mg/kg TIA in mice in the 105000g supernatants of homogenized organs. With the exception of erythrocytes and serum, TIA was detected in all organs including the brain [27].

The suppression of toxic central nervous side effects by cysteine prompted Jungkamp to investigate the transport of ^{35}S TIA through cell membranes of Ehrlich ascites cells (EAT) [28]. Jungkamp's experiments showed that TIA enters the cells through a strophanthin-inhibitable Na^+ cotransport, which is inhibited by L-cysteine; conversely, the transport of L-cysteine into EAT is also inhibited by TIA. This transport into EAT is bound to the thiazolidine ring of the TIA molecule because the perhydrothiazine derivative ALP, which contains the perhydrothiazine ring, instead the thiazolidine ring does not inhibit the influx of L-cysteine into EAT. From the results of Jungkamp's experiments it can be concluded that TIA transports the pharmacologically active metabolite of oxazaphosphorine, i.e. ALD, into the CNS. This shows a way to treat CNS tumors with oxazaphosphorin cytostatics with an alkylating function optimized for the mechanism of action, e.g. a SUM-IAP derivative with a thiazolidine ring (compound 13 table 1).

Oxazaphosphorines-specific therapeutic problems

The pharmacologically active OX metabolite is the aldehyde (*2a* figure 1). *In vivo* the aldehyde is substrate for 2 competing enzymatic reactions: The enzymatic cleavage into PAM and HPA (*3* and *5* figure 1) by phosphoesterases and the oxidation to the therapeutically ineffective carboxylic acid (*6* figure 1) catalyzed by ADH. After intravenous injection of 100 mg/kg CP in mice, about 80% of the CPOH/ALD mixture formed is rendered ineffective by this reaction [14].

Different aldehyde dehydrogenase activities of individual tissues are the reason for their organ specificity towards CP. Cells with low ALD activity such as occult tumor cells in bone marrow transplants can be destroyed by Mafosfamide, which spontaneously hydrolyzes to CPOH. Different ADH activities in tumor cells and normal cells have been used in the past to explain the mechanism of action of CP [29].

ADH activity is high in the liver. During therapy with OX, the liver becomes a functional compartment with a low ALD concentration and thus the starting organ for the formation of metastases [30]. In therapy tests with SUM-IAP, the primary tumor was eradicated, but the animals died 30 to 60 days after tumor transplantation due to the growth of the metastases formed in the liver. The aim of the experiments described below was to prevent the formation of metastases after therapy with SUM-IAP. For this purpose, SUM-IAP was combined with cis platinum (CPT) and the apoptosis enhancer N-methylformamide (NMF). CPT was chosen because it is not detoxified in the liver and - as postulated for SUM-IAP - inhibits cell growth by cross-linking of two guanine bases of the same DNA strand.

Antimetastatic therapy experiments with SUM-IAP in combination with CPT and NMF

Table 2 shows the results of therapy tests with SUM-IAP and SUM-IAP in combination with CPT in CD2F1 mice bearing solid growing P388 mouse leukemia cells: 266 mg/kg SUM-IAP were administered on day 7-11 and day 21-25. One of four animals survived (surviving time >100d). The other animals died from metastases in the region of the lymph nodes of forelegs and hind legs between day 52 and day 70. In the same experiments with two additional. applications of CPT (1.8 mg/kg on day 13 and day 27), however, four of five animals were cured. Increasing the CPT dose to 3.6 mg/kg did not result in additional therapeutic success. On the contrary, the combination of substances was toxic. In contrast to monotherapy with SUM-IAP, the animals lost 18% of body weight. One animal died due to toxicity of the substance combination. The therapeutic effect of cisplatin alone was marginal. 3.6 mg/kg ip. on days 7 and 10 after tumor transplantation resulted in an increase in life span of 76% only [31].

SUM-IAP (mg/kg/schedule)	CPT (mg/kg/schedule)	LTS[1]	ILS[2] (%)
266/ d7-11, d21-25	-----	1/4	370
266/ d7-11, d21-25	1.8/ d13,27	4/5	-----
266/ d7-11, d21-25	3.6/ d13,27	4/5	-----
-----	3.6/ d7,10	0/4	76

Table 2: *Therapy experiments with SUM-IAP, CPT and combinations of SUM-IAP and CPT, female CD2F1 mice bearing subcutaneously transplanted P388 mice leukemia cells.*

[1]Long time survivors, surviving time > 100 days.

[2]Increase in life span, surviving time of untreated control animals 11 days after tumor transplantation.

The data for the table are taken from reference [31] and adapted to this article, for details of the methodology see there.

The experiment shows that the combination of SUM-IAP with a second cytostatic agent that is not detoxified in the liver can partially reverse the formation of metastases, but it also shows that the toxicities of both substances are additive.

Table 3 shows the results of therapy experiments with SUM-IAP in combination with NMF. The animals were treated either with SUM-IAP alone or with SUM-IAP followed by NMF administration. The animals treated with SUM-IAP alone showed the usual result: Eradication of the primary tumor, but death of the animals 29 to 56 days after tumor transplantation due to formation of metastases. However, if the animals were additionally treated 12 times with 200 mg/kg NMF daily after SUM-IAP therapy, 4 out of 5 animals survived free of metastases (surviving time >100d).

In contrast to the combination with CPT, the combination with NMF is absolutely toxicity-free. Body weight increased steadily during and after treatment up to a final weight of 28 - 29g [31]. The animal that died on day 50 after tumor transplantation was free of metastases. The cause of death was renewed growth of the primary tumor.

SUM-IAP (mg/kg/schedule)	NMF (mg/kg/schedule)	LTS	ILS (%)
266/ d7-11	----	0/5	170
266/ d7-11	130/ d13-24	0/5	130
266/ d7-11	200/ d13-24	4/5	-----
-----	200/ d8-12	0/5	12

Table 3: *Therapy with SUM-IAP, NMF and combinations of SUM-IAP and NMF, female CD2F1 mice bearing subcutaneously transplanted P388 mice leukemia cells.*

[1]Long time survivors, surviving time > 100 days.

[2]Increase in life span, surviving time of untreated control animals 11 days after tumor transplantation.

The data for the table are taken from reference [31] and adapted to this article, for details of the methodology see there.

Immunological anti-metastatic therapy with SUM-IAP

In a further experiment to prevent the formation of metastases by treatment with high doses of SUM-IAP 666 mg/kg SUM-IAP were administered on days 7 and 8. The primary tumor was reduced below the detection limit from day 14 to day 21, but from day 21 onwards the primary tumor was measurable again in all animals. All animals developed metastases and died on days 30 and 31.

But when after a recovery break of 5 days following the SUM-IAP injections the animals were treated again with 666 mg/kg SUM-IAP on days 14 and 15, all animals were cured (survival time >100 d). The course of leukocyte concentration in the blood was surprising in this experiment. Following the leukocyte depression immediately after the last SUM-IAP injection, there was an excessive regeneration to 2-6 times the initial value. The highest measured leukocyte concentration 15 days after the last SUM-IAP injection was 24753 ± 7290 leukocytes per µl blood compared to 6009 ± 1315 (mean ± SD) before the first SUM-IAP injection.

The strong increase in the leukocyte count after 2 therapy cycles with SUM-IAP is an indication that the prevention of metastasis formation and thus the healing of the animals is not due to the antitumor effect of SUM-IAP, but to immunological effects caused by SUM-IAP.

Discussion and Conclusion

CP is an accidental discovery and poorly adapted to its mechanism of action. This is not surprising, because at the time CP was developed it was only known that cancer cells proliferate faster than normal cells. Biochemical peculiarities of cancer cells such as altered signal transduction, altered gene expression or apoptosis induction as a target for the treatment of cancer had yet to be discovered. Although CP, like chlorambucil, melphalan or estramustine, is a derivative of nitrogen mustard and damages cells through DNA alkylation, it is “something special” due to its comparatively high antitumor efficacy and therefore has been included in the list of essential drugs by the WHO [32].

All attempts to explain why CP exceeds other alkylating cytostatics in therapeutic effectiveness were unconvincing and not suitable for improving CP. Attempts to improve the efficacy of CP failed due to the assumption that cell death is exclusively a consequence of DNA damage by PAM. Consequently, attempts to improve CP were limited to getting as much PAM as possible into the tumor cell, as shown by the development of Glufosfamide (β-D-glucose-isophosphoramide mustard), which was developed with the intention of using the increased glucose requirement of tumor cells for the accumulation of IPAM in tumor cells [33]. Glufosfamide was incomprehensibly

developed although - as already mentioned - Brock and Hohorst had already shown in the 1970s that the high therapeutic quotient of CP compared to PAM is linked to the presence of the CP metabolite pair CPOH/ALD [16,17]. It was not possible to understand this with the biochemical knowledge available long before the turn of the millennium.

Only with the discovery of HPA as a CP metabolite, with the discovery that HPA enhances p53-controlled apoptosis and finally with the discovery that the event leading to cell death after CP therapy is p53-controlled apoptosis, was it possible to explain why the high therapeutic quotient is linked to the presence of CPOH/ALD - more precisely to ALD. ALD is the metabolite that makes CP special in the group of alkylating cytostatics, because it is decomposed in the two cell-killing metabolites namely DNA alkylating PAM and apoptosis-enhancing HPA. Accordingly, the following reaction sequence results for the mechanism of action of CP and other OX: ALD is cleaved into PAM and HPA, PAM alkylates the DNA; the DNA alkylation initiates p53 controlled apoptosis, which is enhanced by HPA. This mechanism gives rise to two requirements for the development of new, better CP-related substances, namely avoidance of the formation of toxic chloroacetaldehyde and toxic CPOH and apoptosis efficient DNA alkylation, e.g. DNA damage that cannot or can only be poorly repaired by the cell's own repair mechanisms. The model substance SUM-IAP fulfills both requirements. SUM-IAP hydrolyzes spontaneously to ALD, bypassing CPOH, and with its modified alkylating function it produces DNA damage that cannot be repaired. The greatly reduced toxicity of SUM-IAP and the orders of magnitude increase in antitumor activity in the P388 tumor model in mice (see figure 8) impressively confirm the correctness of the scheme presented in figure 6 and 9 for the mechanism of action for SUM-IAP and further confirms the correctness of the conclusions for the development of new CP-related substances.

The tumor model used is a reflection of reality namely established tumors that form SUM-IAP-resistant metastases after successful treatment. The aim of the experiments was to cure the P388 tumor-bearing animals by preventing metastasis formation. Since the detoxification of the pharmacologically active metabolite SUM-aldophosphamide in the liver was recognized as the starting point for the formation of metastases, SUM-IAP was combined with CPT. The results of these experiments (See table 2) were not satisfactory. Although the survival rate was increased, the combination was toxic as measured by the body weight of the animals. Additional DNA damage through intramolecular DNA strand cross-linking therefore does not increase the apoptosis yield. The situation is quite different in the therapy experiments in which SUM-IAP was combined with NMF. Although only half the SUM-IAP dose was administered as in the combination trial with CPT, 4/5 animals survived without any signs of impaired well-being due to the substance combination.

There is little recent information about the mechanism of action of NMF in the scientific literature. However, based on the results of the therapy trials with the apoptosis enhancer SUM-IAP presented here, it can be assumed that NMF enhances the apoptosis initiated by SUM-IAP through an as yet unknown biochemical process. The studies of Kalyany., *et al*. [34] provide evidence for this. Their findings show that NMF causes perturbation of secondary structure of SOD 1 and thereby loss of enzymatic activity of SOD1. SOD1 deficiency induces O^{2-} accumulation which increases loss of mitochondrial membrane potential and DNA-damage-mediated p53 directed apoptosis [35].

It has long been known from clinical observations that some tumors can regress after bacterial infections. Based on observations about the influence "which violent erysipelas have on the regression of tumors", W. Busch undertook a specific attempt to cure a patient "with a huge sarcoma of the neck glands" in 1865. The patient's skin over the tumor was injured with a branding iron. In the course of the promptly occurring skin infection by streptococci, which was accompanied by fever, the tumor regressed within 2 weeks [36].

Further attempts to regress tumors through artificial infections were described in 1893 by Coley [37] who injected devitalized bacteria into tumor patients. These experiments were subsequently forgotten. The reason for this was probably that a more reliable treatment method was available with the development of radiotherapy. Another reason may have been that tumor therapy by stimulating the immune system was not comprehensible in animal experiments because the prerequisite for this, namely the detection of tumor-specific antigens that are recognized as foreign by the immune system of the host organism, was not possible with the experimental animals available at

the time. The difficulty was not to distinguish between immune reactions to transplanted normal mouse tissue and tumor tissue from the same animal. It was only after Gross proved the existence of tumor-specific antigens by transplantation experiments in syngeneic mice in 1943 [38] that experiments on tumor therapy using the body's own immunological mechanisms were resumed.

Only today, more than 150 years after Busch's "healing attempt", we are approaching the goal of immunological tumor therapy. Substances like SUM-IAP can help achieve the goal. This assumption is supported by the finding that in animal models the metronomic administration of CP selectively reduces the number of circulating regulatory T cells (Treg) and thus reduces their immunosuppressive activity [39]. The biochemical reason for this immunostimulatory effect is the reduced DNA repair capacity of Treg compared to cytotoxic T cells (Tc) directed against tumor cells [40]. Therefore, Treg are more susceptible to apoptosis after DNA damage than Tc, which are no longer prevented from attacking tumor cells by Treg´s. Cyclophosphamide-related substances such as SUM-IAP offer the possibility of selectively inhibiting inhibitory Treg´s and thus helping the immune system to destroy tumor cells.

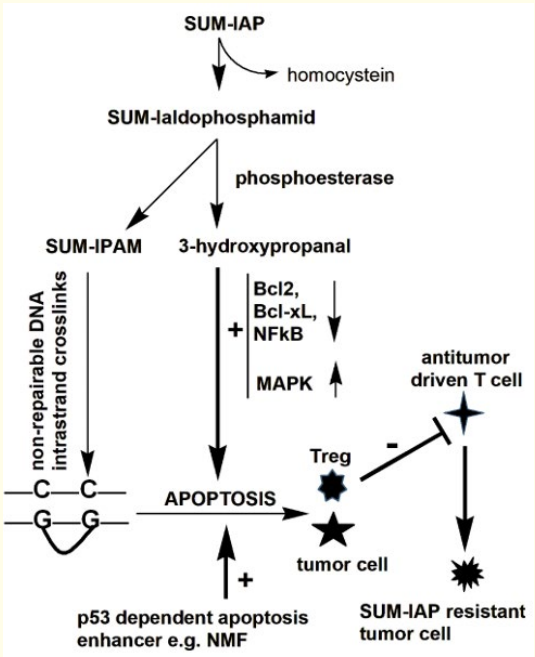


Figure 9: *Overview of the mechanism of action of SUM-IAP.*

SUM-IAP hydrolyzes with the elimination of homocysteine to SUM-i-aldophosphamide, which is cleaved by phosphoesterases into alkylating SUM-IPAM and proapoptotic 3-hydroxypropanal (HPA). SUM-IPAM alkylates the DNA to form unrepairable intrastrand crosslinks. DNA damage triggers p53-controlled apoptosis. Whereby tumor cells and inhibitory regulatory T cells (Treg) are eliminated. By eliminating inhibitory Tregs, cytotoxic T cells directed against tumor cells can attack tumor cells that have become resistant to SUM-IAP.

As figure 9 shows, all pharmacological effects caused by SUM-IAP are due to the substance's ability to enhance p53 directed apoptosis. Tumor cells in which this system is damaged or no longer present cannot be treated with SUM-IAP. Another disadvantage is that not only fast-growing tumor cells are attacked, but all fast-growing cells such as cells of the hematopoietic system. Protection against the cell-killing effectiveness of SUM-IAP is a well-functioning DNA repair system. The major disadvantage of alkylating cytostatics, the lack of tumor

specificity, is not eliminated by SUM-IAP. However, SUM-IAP is indirectly tumor-specific. Tumors exploit multiple escape mechanisms to evade immune recognition. One of these escape mechanisms is the suppression of tumor-specific Tc by Treg´s, which is prevented by apoptotic cell death of Treg´s caused by SUM-IAP.

Funding Support

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

Conflict of Interest

The author declares no conflict of interests.

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Volume 13 Issue 2 February 2025
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