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Review Article

3-Hydroxypropanal (Reuterin) the Key to Understanding the Mechanism of Action of Cyclophosphamide

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Abstract

Although cyclophosphamide (CP) has been clinically proven in the treatment of cancer for over 60 years, until recently nothing was known about its mechanism of action. The reason for this was that results from *in vitro* experiments were not critically questioned, but were uncritically transferred to *in vivo* conditions. *In vitro*, the formation of the alkylating metabolite phosphoreamide mustard (PAM) produces acrolein as a by-product, whereas *in vivo* the proapoptotic metabolite 3-hydroxypropanal (Reuterin) is formed, which enhances p53-controlled apoptosis caused by DNA alkylation by PAM. CP was found by a happy coincidence. It is not tailor-made for the mechanism of action. The present article describes the development of new cyclophosphamides adapted to the mechanism of action, which in animal experiments are less toxic and orders of magnitude more effective than the cyclophosphamides used clinically today.

Keywords: *Cyclophosphamide; Mechanism of Action; 3-Hydroxypropanal (Reuterin); Alkylating Function; Aldophosphamide-Thiazolidine-Perhydrothiazine; Apoptosis-Enhancer*

Introduction

Although the alkylating cytostatic cyclophosphamide (CP) has been used successfully in the clinic for more than 60 years as a cytostatic, its high therapeutic efficacy compared to other alkylating cytostatics for a long time was puzzling. This was due to the fact that the results of *in vitro* experiments with the cyclophosphamide metabolite 4-hydroxycyclophosphamide (CPOH) were uncritically transferred to *in vivo* conditions.

After injection, CP is hydroxylated in the liver to CPOH (See figure 1), which equilibrates with its tautomeric aldehyde, aldophosphamide (ALD). *In vitro*, the DNA-alkylating agent phosphoramide mustard (PAM) is formed from ALD by β-elimination of acrolein. This reaction was uncritically transferred to *in vivo* conditions and not questioned in more detail. Probably because the image of therapeutically effective PAM on the one hand and toxic acrolein, which can be rendered harmless with the antidote MESNA (mercaptoethane sulfonic acid), on the other, fitted well into the sales concept and was effective in advertising. This took away the chance to see the real reason why CP works so well and the opportunity to improve its effectiveness.

Overview of the CP-metabolism *in vivo*

In reality, after injection of CP, hydroxylation of CP to CPOH, and equilibration with ALD, PAM is not formed from ALD by β-elimination of acrolein but by enzymatic cleavage of ALD by phosphodiesterases (PDE) [1]. This reaction does not produce acrolein but 3-hydroxypropanal (HPA). The discovery of HPA as a CP metabolite initially seemed meaningless, since the same DNA-alkylating metabolite PAM is formed during the formation of acrolein *in vitro* and HPA *in vivo*. This view changed abruptly with the discovery of apoptosis and the associated biochemical reactions.

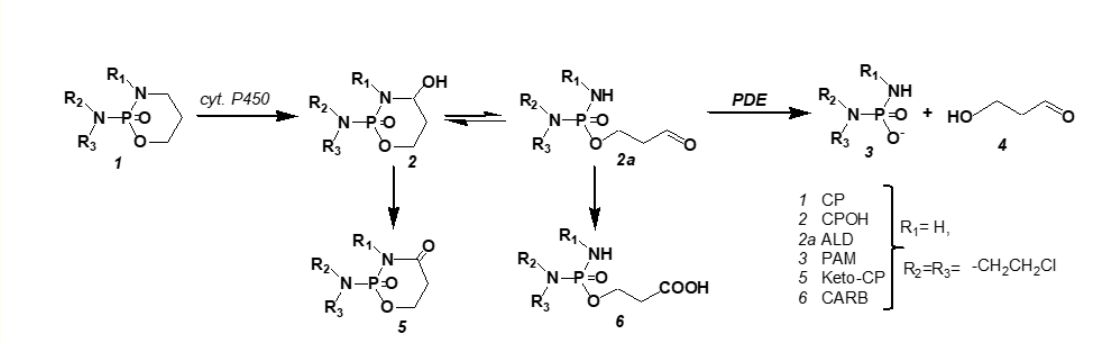

Figure 1: *Overview of the CP-metabolism in vivo. After injection, CP (1) is hydroxylated to CPOH (2) by cyt. P450 enzymes of the liver. CPOH forms an equilibrium with the tautomeric compound ALD (2a). ALD is the pharmacologically active metabolite. The bulk of CPOH and ALD is detoxified to the therapeutically inactive compounds 4-ketocyclophosphamide (keto-CP,5) and carboxyphosphamide (CARB,6). The remaining residue ALD is enzymatically by phosphodiesterases (PDE) degraded to the alkylating metabolite PAM (3) and the proapoptotic aldehyde HPA (4). After intravenous injection of 100 mg/kg CP in mice, 92% is hydroxylated to CPOH. 80% of this is detoxified to keto-CP and CARB [2].*

The mechanism of action of CP

HPA - an antibiotic produced by *Lactobacillus reuteri* and submitted to the culture medium - is also known as Reuterin. It has been shown to be active against bacteria viruses and fungi [3], it is used as food additive to prevent spoilage by growth of pathogens. Experiments by Iyer [4], who investigated the effects of the supernatant of *L. reuteri* cultures on tumor necrosis factor (TNF)-activated apoptosis signaling pathways in human leukemia cells, showed that HPA is an pro apoptotic aldehyde which stimulates apoptosis by inhibiting anti-apoptotic proteins Bcl-2 and Bcl-xL and the TNF dependent NF-κB activation.

Schwartz and Waxman [5] investigated the effect of CPOH on the caspase 8 (extrinsic) and caspase 9 (intrinsic) p 53 dependent pathways of apoptosis in 9L tumor cells. Contrary to other anticancer drugs like doxorubicin and cisplatin [6] in which activation of caspase 8 is the initial apoptotic event, after application of OHCP, activation of caspase 9 is the apoptosis-triggering event. This finding is in agreement with the report that caspase 8 specific inhibitors only block cisplatin but not CP induced apoptosis [7]. In 9L cells, overexpressing the anti-apoptotic Bcl-2 protein, Schwartz and Waxman could only determine the cytostatic effect after treatment with CPOH but no cytotoxic apoptosis. They state that the combination of CPOH induced DNA damage and Bcl-2 dependent cytotoxic response is necessary for cell death.

The discovery of HPA as a CP metabolite [1], the scientific work of Schwarz and Waxman who have demonstrated that the function of PAM is to induce cytotoxic p53 depended apoptosis by DNA alkylation and the results published by Iyer., *et al*. [4], showing that HPA is a proapoptotic aldehyde, lead to the scheme for the mechanism of action of CP, as shown in figure 2.

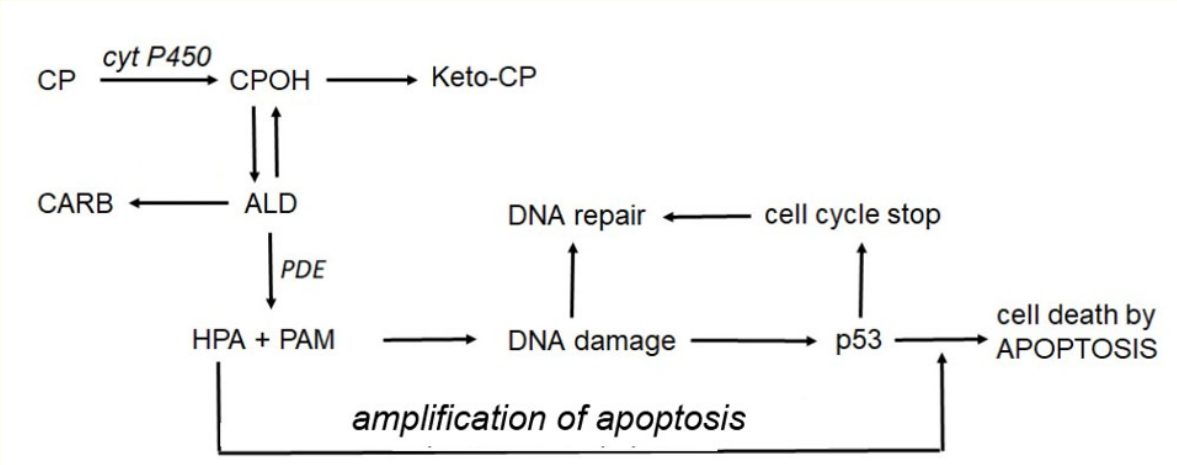

Figure 2: *Mechanism of action of CP. CP is hydroxylated by P450 enzymes in the liver to CPOH. CPOH is in equilibrium with its tautomere ALD. CPOH is oxidized to Keto-CP. The bulk of ALD is oxidized by aldehydedehydrogenases to CARB. Keto-CP and CARB are non-toxic, therapeutically ineffective metabolites. The amount of ALD not oxidized, is decomposed by phosphodiesterase (PDE) to the alkylating PAM and proapoptotic HPA. 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 CP - enhanced by HPA.*

CP metabolites responsible for toxicity

According to prevailing opinion, acrolein is the cause of the dose-limiting urotoxicity that is successfully treated with mesna, which forms thioethers with acrolein [8]. The treatment with thiol compounds to reduce cyclophosphamide toxicity is successful although - as shown in the previous chapter - no acrolein is formed *in vivo* after CP administration. This raises the question of the mechanism of action of thiol compounds in reducing cyclophosphamide toxicity. Acute toxicity tests in mice show that considering that 80% of the intraperitoneally applied CPOH is detoxified by first pass effect to non-toxic and therapeutically irrelevant keto-CP and CARB [2], CPOH is about 7 - 8 times more toxic than PAM [9]. This shows that Mesna does not detoxify acrolein but CPOH - as shown in figure 3.

Experimental evidence for the validity of the proposed mechanism of action, and the cause of toxicity as well as the implications for the development of new cyclophosphamides are shown below.

Development of new cyclophosphamides adapted to the mechanism of action

From the scheme for the mechanism of action (Figure 2) it can be seen that cellular repair mechanisms reduce the apoptosis initiated by DNA damage. In the case of CP and ifosfamide (IF), the alkylating function consists of 2-chloroethyl groups ($-CH_2CH_2Cl$) which, in contrast to 2-mesylethyl groups ($-CH_2CH_2OSO_2CH_3$), create easily repairable DNA interstrand crosslinks (http://www.atdbio.com/content/16/

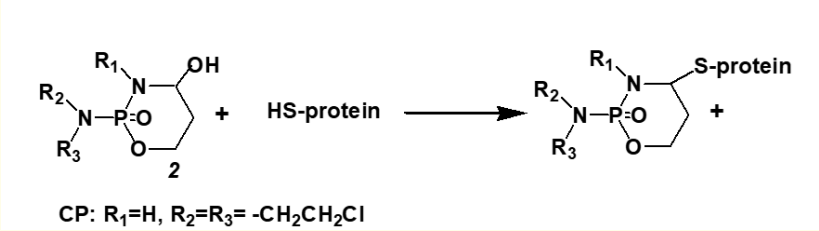

Figure 3: *The reason for the toxicity of CPOH is the reaction with nucleophilic groups of proteins, e.g. SH groups.*

Nucleic-acid-drug-interactions). In order to prove the suggested mechanism of action to be correct, it would be necessary to compare the effectiveness of different CP derivatives with 2-chloroethyl groups or 2-mesylethyl groups in the alkylating function. The pharmacologically active CP metabolite is ALD. In the case of CP or IF, the formation of ALD is only possible via toxic CPOH or toxic 4-hydroxy-IF (IFOH). Therefore, a way was sought to form ALD or I-aldophosphamide (I-ALD) bypassing CPOH or IFOH *in vivo*. The solution of the problem is to use the thiazolidines or perhydrothiazines from ALD or I-ALD. These aldophosphamide derivatives hydrolyze spontaneously, bypassing CPOH or IFOH to ALD or I-ALD. The LD_{50} of the thiazolidine of ALD (TIA, figure 4, 8 n = 1) was determined after a single intraperitoneal injection in mice with 5.89 mmol/kg (2162 mg/kg), while for CPOH in 2 experiments LD_{50} values of 0.53 mmol/kg (143 mg/kg) and 0.65 mmol/kg (175 mg/kg) were measured [9]. If CPOH is bypassed during the formation of ALD, the toxicity drops by a factor of about 10. These experimental result confirm the statement that CPOH is the metabolite responsible for non-alkylation toxicity of CP.

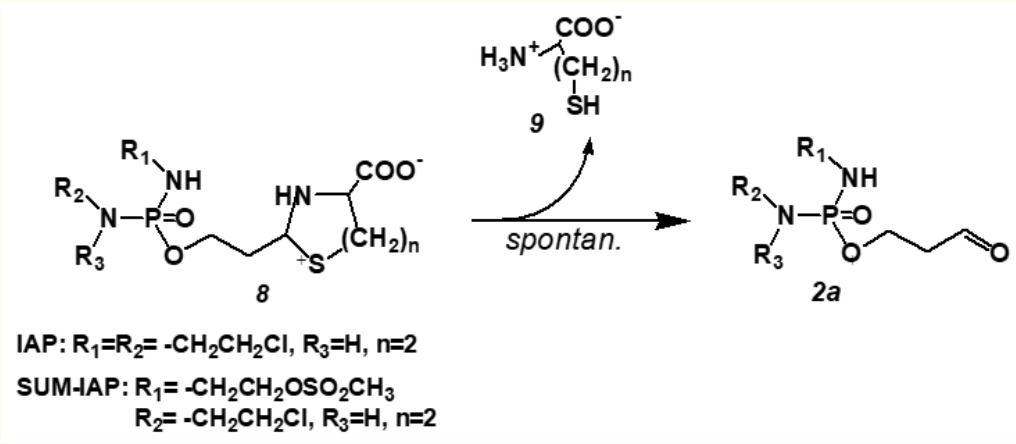

Figure 4: *Hydrolysis of ALD and I-ALD thiazolidines (8 n = 1) and ALD and I-ALD perhydrothiazines (8 n = 2) to ALD or I-ALD (2a) and cysteine (9 n = 1) or homocysteine (9 n = 2).*

To answer the question whether HPA-assisted apoptosis can be increased by preventing repair of DNA damage caused by PAM or I-PAM, the perhydrothiazines of I-ALD were synthesized and tested in therapy experiments on CD2F1 mice bearing solidly growing ad-

vanced P388 mouse leukemia cells. The experiments were carried out with the I-aldophosphamide perhydrothiazines IAP and SUM-IAP (formula see figure 4). IAP contains the alkylating function of IF, which creates easily repairable DNA interstrand cross links. In the SUM-IAP, a 2-chloroethyl group of the alkylating function is replaced by a 2-mesylethyl group, which creates poorly repairable DNA intrastrand cross links [10,11]. CD2F1 mice bearing subcutaneously transplanted mouse leukemia cells were treated with either 0.8 mmol/kg (320 mg/kg) IAP or 0.59 mmol/kg (266 mg/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 order to roughly quantify the improved therapeutic efficacy of SUM-IAP due to modifying the alkylating function by a mesyl group, further therapy experiments were carried out. Measure of effectiveness of therapy was the measured tumor area, which correlates well with the tumor mass after excision. (correlation coefficient 0.93). Under the simplifying assumption that the tumor area is proportional to the tumor mass and number of tumor cells and that the tumor cells are in the exponential growth phase, the 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 [14]. From this result it can be concluded that in accordance with the postulated mechanism of action for CP and other cyclophosphamides such as IF, the apoptosis triggered by DNA alkylation can be increased by orders of magnitude, if the DNA damage cannot or can only poorly be repaired.

Conclusion

This article describes the mechanism of action of CP and the possibility of developing new, more effective and less toxic anticancer drugs based on this. CP is classified in the group of alkylating cytostatics. However, this article clearly shows that this is wrong. The alkylating part of the overall effect only has the function of triggering p53-controlled apoptosis. It does not depend on the degree of DNA damage. By incubation with 5 µM IAP about 25 times more P388 mouse leukemia cells die due to DNA damage than by incubation with 5 µM SUM-IAP. But when the same cells grow as subcutaneously transplanted cells in CD2F1 mice, then SUM-IAP is 10^4 - 10^5 times more potent than IAP [14]. The result of this experiment shows that DNA damage is only a signal generator for lethal apoptosis and that in the development of new cyclophosphamides, in addition to avoiding the formation of toxic metabolites, it is important to keep the DNA damage that triggers apoptosis as low as possible.

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