

## Perspective

# 3-Hydroxypropanal (Reuterin) the Overlooked Cyclophosphamide Metabolite

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## INTRODUCTION

Cyclophosphamide (CP) is one of the oldest anticancer drugs, but one that is also highly effective by today's standards. The theoretical idea for the development of CP was to administer a nontoxic prodrug that liberates in tumor tissue nitrogen mustard which damages DNA by alkylation. At the time, when CP was developed, it was believed that enzymes that catalyze splitting of phosphorus–nitrogen bonds (phosphoramidases) were preferentially found in tumor tissue [1,2]. This was the basis for the development of pro drugs from which alkylating nitrogen mustard should be split off in the target tissue "tumor". Of the substances synthesized based on this theoretical requirement, CP proved to be the most effective in animal experiments. The theoretical concept was wrong, but the result was a very effective anticancer drug.

### Brief history of the metabolism of CP

Very soon it turned out that CP is completely ineffective against tumor cells in vitro. Substances that kill tumor cells in vitro are only detectable in the blood after injection of CP. From the stable but therapeutically ineffective CP metabolites 4-ketocyclophosphamide (keto-CP) and carboxyphosphamide (CARB) found in the blood after CP injection, it was concluded that CP is hydroxylated by liver cytochrome P450 enzymes to form 4-hydroxycyclophosphamide (CPOH), which forms an equilibrium with its tautomer aldophosphamide (ALD) [3]. In vitro experiments in phosphate buffer solution with synthetically prepared CPOH showed that the alkylating agent was phosphoramidate mustard (PAM) formed by  $\beta$ -elimination of acrolein from ALD [4]. PAM was assumed to kill cells by DNA alkylation. The detection of CPOH (Figure 1) after CP (Figure 1) injection was initially difficult because no reference substance was available. Only after a synthesis for CPOH was developed [5], it was possible to prove CPOH in blood by thin layer chromatography and mass spectrometry [6,7], after CP injection. This was the state of knowledge about the metabolism of CP that had been frozen for decades and on which hypotheses about the mechanism of action and possibilities for improving the therapeutic effectiveness of CP were based.

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The two reactions competing for ALD, detoxification to CARB by aldehyde dehydrogenases (ALDH) and formation of PAM from ALD by  $\beta$ -elimination of acrolein have long been the focus of interest because they explain the different sensitivity of different tissues to CP. Cells with high ALDH activity such as intestinal mucosal cells, hematopoietic stem cells or occult tumor cells in bone marrow transplants [8], that can be eradicated with congeners of CP such as Mafosfamide (MF), which spontaneously hydrolyses to CPOH have low activity of ALDH. But attempts to explain thereby the tumor specificity of CP [8], were not convincing because by co-treatment with inhibitors of ALDH CP resistant tumors did not become CP sensible.

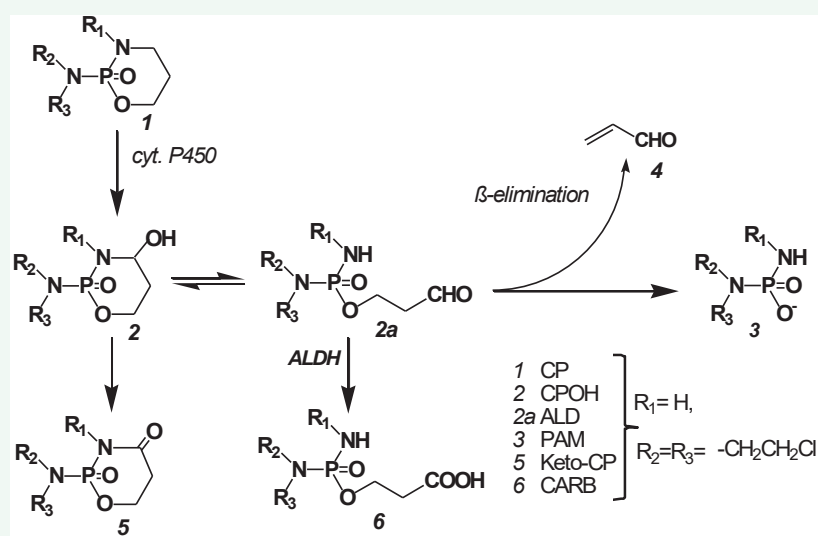
### Historical overview of the metabolism of CP

After application of CP [1], in animals and patients, the stable but therapeutically ineffective metabolites ketocyclophosphamide [2], and CARB [6], were detected in the blood and urine. It was concluded that CP is hydroxylated by liver cytochrome P450 enzymes to give CPOH [2], which is in equilibrium with its tautomer ALD (2a). In vitro experiments with synthetic CPOH in buffer solutions showed that the alkylating agent PAM [3], is formed from ALD by  $\beta$ -elimination of acrolein [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 [9].

The old metabolite scheme of CP solidified over time because it was the basis for the very successful introduction of mercaptoethanesulfonic acid (Mesna) as an antidote to CP -and Ifosfamide (IF) toxicity. According to the old notion of CP metabolism, acrolein was the main cause of CP and IF dose limiting hemorrhagic cystitis, which is successfully treated with 2-mercaptoethanesulphonate (MESNA), It was believed that Mesna exert their toxicity-reducing effect by forming inert thioethers with acrolein [10].

Studies of the breakdown of CPOH in rat serum and protein-free rat serum ultrafiltrate demonstrated that CPOH breaks down more than 100 times faster in rat serum than in rat serum ultrafiltrate. Phosphodiesterases (PDE) of the snake venom phosphodiesterase type (EC 3.1.4.1) were identified as the enzymes



**Figure 1** Historical overview of the metabolism of CP.

responsible for this decomposition. [11,12]. This was nothing exciting per se, but the whole thing became exciting when the reaction products of the decomposition of the tautomers CPOH/ALDO were identified. The reaction products are not acrolein and PAM but PAM and 3-hydroxypropanal (HPA) [13]. However, at the time HPA was discovered as a CP metabolite it was assumed that HPA was only an insignificant intermediate product on the metabolic pathway to PAM, the discovery was not taken seriously. This is probably also why HPA did not fit into the worldview in which acrolein is the cause of hemorrhagic cystitis. The real cause of hemorrhagic cystitis after CP and IF application is CPOH (IFOH) itself, which reacts with nucleophilic groups, e.g., SH groups of membrane proteins and is neutralized by Mesna [14].

### The real metabolism of CP

PAM [3] is formed from ALD (2a) by enzymatic cleavage of ALD by phosphodiesterases (PDE). This creates HPA [7].

### Implications of the discovery of HPA as a CP metabolite for CP's mechanism of action

After the discovery of apoptosis, the view of the mechanism of action of alkylating substances changed. It has become apparent that any substance that damages DNA thereby initiates apoptosis.

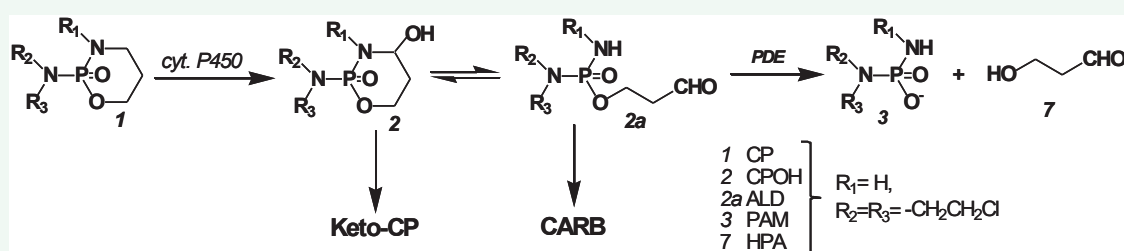
Schwartz and Waxman [15], investigated the effect of MF on the caspase 8 (extrinsic) and caspase 9 (intrinsic) dependent pathways of apoptosis in 9L tumor cells and 9L tumor cells transduced with CP hydroxylating CYP2B6. Contrary to other anticancer drugs like doxorubicin [16], and cisplatin [17], in which activation of caspase 8 is the initial apoptotic event, after application of MF, activation of caspase 9 was detectable before the activation of caspase 8. In addition caspase 9 was activated to a greater extent than caspase 8, indicating the p53 mediated apoptotic pathway, in response to upstream DNA damage, is triggered by CPOH released in minutes from MF. Seki et al came to the same conclusion, showing that caspase 8 specific inhibitors only block cisplatin but not CP induced [16].

In the functional mechanism of alkylating agents, Schwartz and Waxman [15], distinguish between a cytostatic and a cytotoxic effect. In 9L cells, overexpressing the anti-apoptotic Bcl-2 protein, they could only determine the cytostatic effect after treatment with MF 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 mentioned reports clearly demonstrate that the functional mechanism of OHCP is bimodal: First occurs the cytostatic DNA damage by PAM and, secondly, the cytotoxic p53 mediated apoptosis is observed. The second step is critical because it can be manipulated by factors that affect the pathway of apoptosis. Such a factor is very likely HPA, one of the two reaction products of the enzymatic decomposition of OHCP.

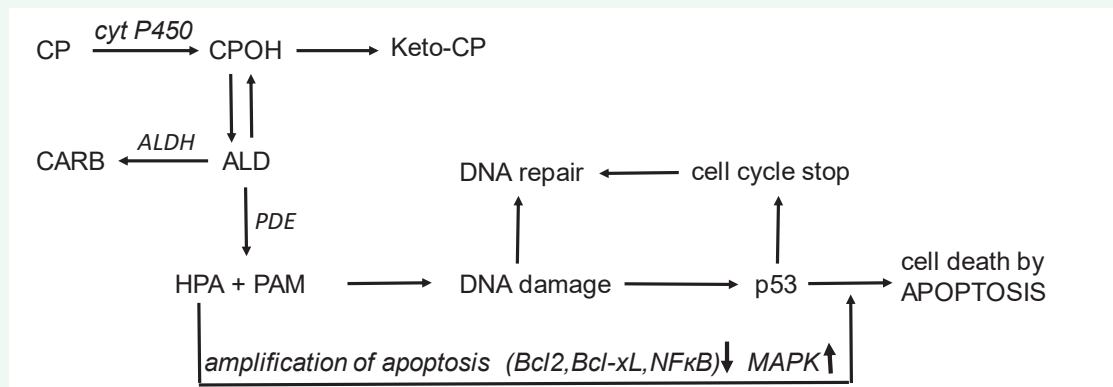
HPA, also known as reuterin, is produced by *Lactobacillus reuteri* and submitted to the culture medium. It has been shown to be active against bacteria viruses and fungi [18], it is used as food additive to prevent spoilage by growth of pathogens. Experiments by Iyer [19], who investigated the effects of the supernatant of *L. reuteri* cultures (LR) on tumor necrosis factor (TNF)-activated apoptosis signaling pathways in human leukemia cells, showed that reuterin inhibits (i) the formation of the anti-apoptotic proteins Bcl-2 and Bcl-xL and (ii) the TNF dependent NF- $\kappa$ B activation by inhibition of the translocation of the p65 subunit of NF- $\kappa$ B into the nucleus. The experiments of Iyer et al. further showed that the degradation of I $\kappa$ B $\alpha$  by lack of the ubiquitination of I $\kappa$ B $\alpha$  is suppressed by the supernatant of LR. Thus, by the inhibition of the nuclear translocation of NF- $\kappa$ B apoptosis is enhanced [19].

In summary, the scientific literature gives the following picture:

1. The event leading to cell death after CP application is not DNA damage but apoptosis triggered by DNA damage [5].
2. The CP metabolite HPA is an apoptosis enhancer. This leads to the action scheme of CP and other OX shown in (Figure 3).



**Figure 2** The real metabolism of CP.



**Figure 3** Mechanism of action of CP and other OX.

## Mechanism of action of CP and other OX

CP (and other OX) is hydroxylated by P450 enzymes in the liver to CPOH from which a small part is oxidized to keto-CP. CPOH is in equilibrium with its tautomer ALD. ALD is the pharmacologically active metabolite. The bulk of it is oxidized by ALDH to the nontoxic CARB. 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 and other OX - enhanced by HPA which supports apoptosis by inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-xL, and by inhibiting NF- $\kappa$ B activation. Additional HPA promotes apoptosis by enhancing mitogen-activated protein kinase (MAPK) activities by enhancing JNK and p38 phosphorylations.

## EXPERIMENTAL EVIDENCE FOR PRESENTED MECHANISM OF ACTION OF CP AND OTHER OX

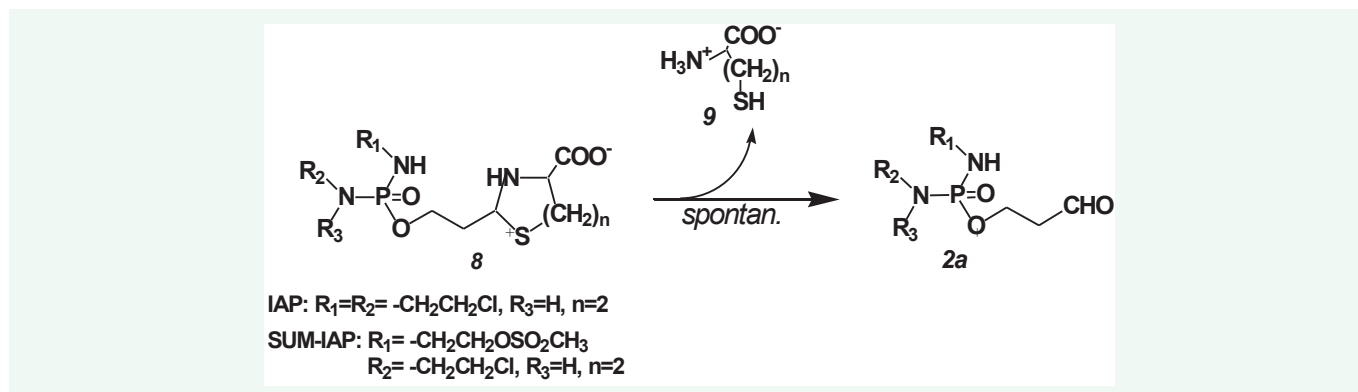
In experiments on regional perfusion of the tumor-bearing limb in rats, CPOH proved to be completely ineffective in contrast to systemic therapy with CP [20, 21]. This was despite the fact that the CPOH concentration in the perfusate (oxygenated hemoglobin solution) at the point of entry into the rat leg (a. epigastric superficialis) was about 100 times higher than after CP application. The amount of CPOH taken up by the tissue was 12 times greater in the perfusion experiments than after systemic therapy with CP [22].

In perfusion experiments with nitrogen mustard N-oxide, which has a comparable effect to PAM, and in experiments with cisplatin, the animals were cured. The reason for the failure of the perfusion experiments with CPOH was clearly that the perfusion solution did not contain ALD-splitting PDE.

CP was "found" by a happy coincidence. It is not tailor-made for the reaction scheme in Figure 3. Disadvantages are the individually varying hydroxylation by cyt. p450 enzymes in the liver, the formation of toxic CPOH en route to the pharmacologically active metabolite ALD, and the lack of adaptation of the alkylating function for DNA damage that initiates optimal apoptosis exploitation. To correct the first two CP "design errors" mentioned, thiazolidine and perhydrothiazines were synthesized from aldophosphamide and I-aldophosphamide, which hydrolyze spontaneously, bypassing toxic CPOH (IFOH) to ALD (IALD) [23] (Figure 4).

Spontaneous hydrolysis of thiazolidines ( $\delta$  n=1) and perhydrothiazines ( $\delta$  n=2) to ALD (2a,  $R_1=H$ ,  $R_2=R_3 = -CH_2CH_2Cl$ ) and I-aldophosphamide (2a,  $R_1=R_2 = -CH_2CH_2Cl$ ,  $R_3=H$ ) and L-cys (9 n=1) or homocysteine (9 n=2).

These compounds are nine fold lower toxic than CPOH and more effective than the parent compounds CP and IF against subcutaneously transplanted P388 tumors in mice [23]. A special feature of the thiazolidine compounds is that the thiazolidine ring gives them the ability to cross the blood-brain barrier [24]. Because the perhydrothiazine of I-aldophosphamide (Figure 4),  $R_1=R_2 = -CH_2CH_2Cl$ ,  $R_3=H$ , n=2) is easy to synthesize and easy to handle, further experiments with this compound (I-aldophosphamide perhydrothiazine, IAP) were carried out.



**Figure 4** Spontaneous hydrolysis of thiazolidines ( $\beta$   $n=1$ ) and perhydrothiazines ( $\beta$   $n=2$ ) from aldophosphamide ( $\beta$   $R_1=H$ ,  $R_2=R_3= -CH_2CH_2Cl$ ) and l-aldophosphamide ( $\beta$   $R_1=R_2= -CH_2CH_2Cl$ ,  $R_3=H$ ) to ALD ( $2a$ ) and L-cys ( $9$   $n=1$ ) or homocysteine ( $9$   $n=2$ ).

Another construction flaw of CP - the lack of adaptation to the initiation of apoptosis - can be corrected as follows: According to the scheme (Fig.3), the apoptosis yield is greater, the lower the amount of alkylated DNA that is repaired by the cell's own repair mechanisms. According to Povirk and Shuker [25], chlorethyl groups as found in IAP form interstrand cross links. whereas methane sulfonate groups form intrastrand cross-links [22]. In contrast to interstrand cross-links, intrastrand cross-links are poorly repaired

(<http://www.atdbio.com/content/16/Nucleic-acid-drug-interactions>).

If in the IAP molecule (Figure 4) in the residue  $R_1$  ( $-CH_2CH_2Cl$ ) the chlorine is substituted by a sulfonyl methyl group ( $R_1$  is now  $-CH_2CH_2OSO_2CH_3$ ), a perhydrothiazine derivative called SUM-IAP is obtained. SUM-IAP is in cell culture experiments with P388 mice leukemia cells significantly less cytotoxic than IAP but against the same cells when growing as solid tumors in mice  $10^4$ - $10^5$  times more effective than IAP [26].

These experimental results - the failure of CPOH in regional perfusion of the tumor-bearing limb in rats and the enormous increase in antitumor effect when repair of the alkylated DNA is prevented - are a strong indication that the scheme in (Figure 3) is correct.

One can discuss whether HPA, the long-neglected CP metabolite, makes CP, long classified as an alkylating cytostatic, to a new class of cytostatics, the class of apoptosis boosters.

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