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Short Communication

Conclusion From the Mechanism of Action for the Development of New Cyclophosphamides

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Introduction

Cyclophosphamide (CP) is one of the oldest, yet more than 65 years after its introduction into clinical practice, still indispensable drug for the treatment of cancer. It was originally developed to make nitrogen mustard (NM), which damages cells through DNA alkylation and with which therapeutic trials on cancer patients were already conducted in the 1940s [1], more tolerable. For this purpose, NM was to be incorporated into a non-toxic transport form and released within the tumor cell. This release was to be mediated by phosphamidase enzymes, which at that time were mistakenly believed to be present in increased activity in tumor cells [2]. The resulting substance synthesized according to this protocol, which showed the best efficacy in animal studies, was CP [3]. It quickly became apparent that CP is not converted into its active form within the tumor cell, but rather is hydroxylated in the liver by the cytochrome P450 enzyme system to 4-hydroxycyclophosphamide (CPOH) [4], which forms an equilibrium mixture with the tautomeric form aldophosphamide (ALD). Instead of NM, as originally intended, phosphoramide mustard (PAM) is released from ALD as a DNA alkylating agent. Due to its superior efficacy compared to other alkylating cytostatics, CP was added to the WHO's list of essential medicines in 1959, coinciding with its approval in the United States [5].

However, the question of why CP is more effective than other alkylating cytostatics such as melphalan, chlorambucil and estramustine remained unanswered for decades because the results of in vitro experiments regarding the formation of PAM from ALD were uncritically extrapolated to in vivo conditions. In vitro, PAM is formed from ALD by the β-elimination of acrolein. This reaction is catalysed by bicarbonate and phosphate ions [6], the concentrations of which are too low in vivo to form therapeutically effective concentrations of PAM. In vivo, in patients and animals, PAM is formed by enzymatic cleavage of ALD by phosphosterases into PAM and 3-hydroxypropanal (HPA), also known as reuterin [7]. While this had been known for some time, it was not considered important because the DNA-alkylating metabolite PAM was formed in both cases, and because toxic acrolein, supposedly detoxified by mesna [8] fit well into the existing concept. This changed when it was discovered that apoptosis, a universal process for maintaining cell homeostasis, is triggered by DNA damage and enhanced by HPA. With the discovery of HPA as a CP metabolite, it suddenly became clear why CP is more effective than similar alkylating cytostatics that act solely through DNA damage and the resulting p53-induced apoptosis. In CP, the focus of the effect is on apoptosis triggered by DNA damage, which is enhanced by HPA, because HPA inhibits the apoptosis-inhibiting proteins Bcl2 and Bcl-xL and the NFκB signalling pathway, and activates the MAP kinase pathway (Figure 1) [9].

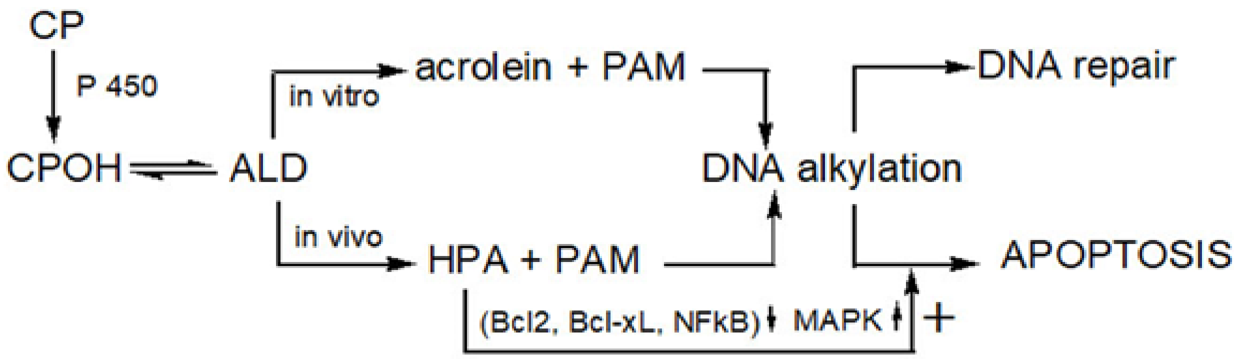

Figure 1: Hydroxylation of cyclophosphamide (CP) to 4-hydroxycyclophosphamide (CPOH) and formation of the DNA-alkylating metabolite phosphoramide mustard (PAM) from the pharmacologically active metabolite aldophosphamide (ALD). In vitro, PAM is formed from ALD by β-elimination of acrolein; in vivo, by enzymatic cleavage by phosphoesterases to form 3-hydroxypropanal (HPA). The scheme also applies to ifosfamide (IF); see text for details.

With the elucidation of the mechanism of action, the question arose: If no acrolein is formed in vivo, which CP metabolite is responsible for DNA toxicity, and how can the efficacy of mesna (2-mercaptoethanesulfonate sodium), which is supposedly neutralizing acrolein and used to prevent urinary tract toxicity, be explained? The explanation is simple: The toxic metabolite CPOH reacts with SH-containing compounds. If the reaction partner is a biomolecule such as an SH-containing membrane protein, CPOH is a toxin. However, if it reacts with a small molecule like mesna, it is neutralized and thus rendered harmless [10]. CP is a serendipitous result and not ideally suited to its mechanism of action. The main errors are the formation of toxic CPOH via the metabolic pathway to ALD and the competition between DNA repair and the initiation of p53-controlled apoptosis in response to DNA alkylation by PAM. Alkylating agents can alkylate DNA in two different ways: they can form inter strand and intra strand crosslinks. Inter strand crosslinks, such as those formed by the alkylating function of CP or IF, are readily repaired by the cell's DNA repair systems, whereas intra strand crosslinks are difficult or impossible to repair (http://www.atdbio.com/content/16/Nucleic-acid-drug-interactions) [11]. Therefore, novel cyclophosphamides adapted to the mechanism of action must meet two conditions: they must form ALD while bypassing toxic CPOH and possess an alkylating function that forms intra strand crosslinks. Such compounds include thiazolidines and perhydrothiazines of aldophosphamide with a modified alkylating function (Figure 2). These compounds hydrolyze to ALD directly, without the intermediate step of CPOH. They are 8-10 times less toxic than CPOH or IFOH, thus confirming the accuracy of considering CPOH the toxic CP metabolite [10].

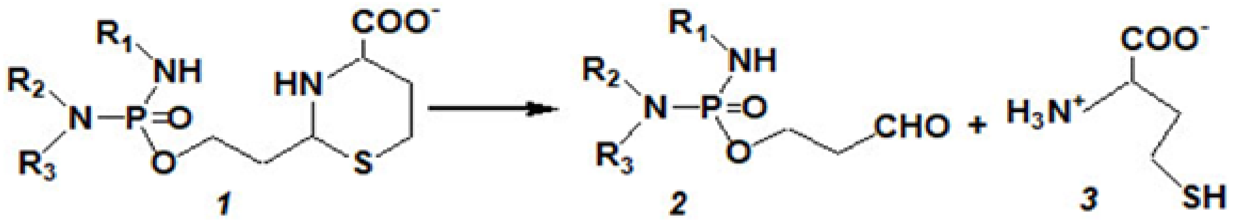

Figure 2: Hydrolysis of I-aldophosphamide perhydrothiazine (1, $R_1=R_2=-CH_2CH_2Cl$, $R_3=H$) to I-aldophosphamide (2) and homocysteine (3). IAP: I Aldophosphamide Perhydrothiazine,1, $R_1=R_2=-CH_2CH_2Cl$, $R_3=H$, SUM: Sulfonyl Methyl IAP,1, $R_1=-CH_2CH_2OSO_2CH_3$, $R_2=-CH_2CH_2Cl$, $R_3=H$

To verify the postulated mechanism of action, in vitro and in vivo experiments with IAP and SUM (formula see Figure 2) were conducted using P388 mouse leukemia cells. In cell culture experiments, IAP was significantly more cytotoxic than SUM. However, when the P388 cells were transplanted subcutaneously into CD2F1 mice, SUM was orders of magnitude more therapeutically effective than IAP. Thus, with the same molar dose that only achieved a marginal increase in lifespan with IAP, SUM reduced the subcutaneously growing tumor below the detection limit. Quantification of the therapeutic success according to Alexander and Mikulski [12] revealed a more than ten-thousand-fold increase in therapeutic success due to substitution of a chlorine atom in IAP with a mesyl group in SUM [13]. The reason for this enormous increase in efficacy is evidence for the correctness of the postulated mechanism of action in Fig. 1 because the chloroethyl groups in the alkylating function of IAP produce easily repairable DNA inter cross links and a low apoptosis yield, while the ethyl mesyl group in SUM achieves difficult-to-repair intra cross links and a high apoptosis yield [14].

CP is classified as an alkylating cytostatic agent, which either kills cells through DNA alkylation or induces apoptosis. This is only partially accurate, because CP carries two pharmacologically active metabolites, PAM and HPA, which complement each other. The activity of these metabolites determines whether the effect is shifted towards cytotoxicity through DNA alkylation or cell death through apoptosis. As already mentioned, CP is not tailored to its mechanism of action. It exerts its efficacy not only by initiating p53-controlled apoptosis, but through a combination of cytostasis and apoptosis. By altering the alkylating function in SUM, the efficacy is shifted towards apoptosis. This effect can be further enhanced by combining SUM with an apoptosis enhancer such as N-methyl-formamide, which induces apoptosis by cell cycle arrest in the G1 phase via the induction of the CDK2 inhibitor P27KIP1 [15,16] as the following experiment demonstrates.

Solid P388 tumor-bearing mice were treated with SUM (200 mg/kg, days 7-11 post-tumor transplantation). The increase in life span (ILS) was 170%. However, when the animals were subsequently treated with NMF (200 mg/kg, days 13-24) the animals were cured (ILS > 1000%). NMF alone was ineffective. Remarkably, apart from a short-term decrease in leukocyte count, no symptoms of toxicity were observed in this experiment [17]. All of this suggests that the mechanism of action of CP opens the door to a new class of cytostatic drugs, the apoptosis boosters for low toxicity chemotherapy.

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Conflict of Interest

No conflict of interest.

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