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Research Article

Mechanism of Action of Oxazaphosphorine Cytostatics Demonstrated by Regional Perfusion of the Tumor-Bearing Limb in Rats with 4-Hydroxycyclophosphamide

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Received: January 25, 2022; **Published:** March 29, 2022

Abstract

4-Hydroxycyclophosphamide (CPOH) and derivatives such as Mafosfamide, which hydrolyze spontaneously to 4-hydroxycyclophosphamide were developed to exploit the therapeutic potential of cyclophosphamide for the use in local therapy. Perfusion experiments on the isolated tumor-bearing limb in rat showed, however, that CPOH and derivatives are completely ineffective in local therapy in contrast to nitrogen mustard N-oxide or cisplatin.

The reason for this experimental result was a mystery until the discovery of the enzymatic cleavage of aldophosphamide with the formation of the proapoptotic cyclophosphamide metabolite 3-hydroxypropanal (HPA). HPA is an integral part of the mechanism of action of oxazaphosphorines, it intensifies the p 53 dependent apoptosis, which is initiated by DNA alkylation by the alkylating agent phosphoramide mustard, which is formed during the enzymatic cleavage of aldophosphamide together with HPA. With this knowledge and because the hemoglobin solution used for the regional perfusion of the tumor-bearing limb in rats did not contain any aldophosphamide-cleaving phosphodiesterases, it is understandable why the perfusion with CPOH and its derivatives in contrast to the perfusion with the alkylating substance N-oxide mustard was ineffective, and contemporary the failure of CPOH in the regional perfusion is proof of the correctness of the mechanism of action of oxazaphosphorine cytostatics presented.

Keywords: *Regional Perfusion of the Tumor Bearing Limb in Rats; Oxazaphosphorine Cytostatics; Mechanism of Action*

Introduction

Although oxazaphosphorine cytostatics (OX) such as Cyclophosphamide and Ifosfamide are among the oldest and most effective antineoplastic substances, its mechanism of action was unclear until a few years ago. The reason for this was that *in vitro* test results were transferred uncritically to *in vivo* conditions. After administration, OX are hydroxylated in the liver to OXOH by cytochrome c hydroxylases (Figure 1). OXOH form an equilibrium mixture with its tautomeric aldehydes (ALD). *In vivo*, ALD, the pharmacologically active OX metabolite, is substrate for three competing reactions. These are: Detoxification by aldehyde dehydrogenases to form the carboxylic acid (CARB); cleavage of ALD into the alkylating agent phosphoreamidemustarde (PM) and 3-hydroxypropanal (HPA) by phosphodiesterases in blood and in tissues [1] and finally the formation of PM through ß-elimination of acrolein. This latter reaction is catalyzed by phosphate or bicarbonate ions [2]. Due to the uncritical adoption of this reaction sequence determined in *in-vitro* experiments for *in vivo* conditions,

attempts to explain the mechanism of action of OX failed. Only after *in vivo* experiments on the metabolism of OX had shown that PM is not formed *in vivo* by ß-elimination of acrolein but by enzymatic cleavage of ALD by phosphodiesterases with the formation of the pro apoptotic aldehyde HPA, it was possible to develop a plausible model for the mechanism of action of OX. The fact that *in vivo* PM and HPA instead of PM and acrolein are formed is the key of understanding the comparatively high antitumor activity of OX and the starting point for improving the antineoplastic efficacy of OX [3]. The mechanism of action of alkylating agents is based on initiation of apoptosis by DNA alkylation. Unique for OX is its outstanding therapeutic effectiveness compared to other alkylating cytostatics. The discovery of pro-apoptotic HPA as an OX metabolite gives rise to the assumption that HPA, which occurs during the formation of the alkylating PM, is the cause of the comparatively high antineoplastic effectiveness of OX. This assumption is supported by the results of perfusion tests on the tumor-bearing limb in rats with 4-hydroxy-cyclophosphamide (CPOH), 4-S-ethanol-sulfido-cyclophosphamide (SECP), Mafosfamide (MF) and aldophosphamide-thiazolidine (THIA) and comparative whole-animal tests on the same tumor model with CP and THIA which are presented here (Formulas s. figure 1).

Summary on Materials and Methods

Mafosfamide (MF) and nitrogen mustard N-oxide (NMO) were provided by ASTA Factory, Germany, for synthesis of 4-S-ethanol-sulfido-cyclophosphamide (SECP) [4]. Aldophosphamide-thizolidine-4-carbonic-acid (THIA) was prepared by the reaction of CPOH with L cysteine in 0.07M phosphate buffer pH7, 37°C. With an excess of 10 mol%, L-cysteine quantitatively converts CPOH to THIA within 45 minutes.

Isolated perfusion of the tumor-bearing limb in rats

For details of the method see [5-8]. Experiments were carried out in male rats (body weight 400 - 500g). For the experiments, 0.2 ml ascites fluid, freshly taken from donor animals, containing approximately 10^6 Yoshida Ascites cells (gift from ASTA Factory, Germany) were transplanted into the m. gastrocnemius of the left hind leg, where they formed solid tumors. The perfusion experiments were carried out 3 days after tumor transplantation when tumor weight was approximately 1 - 3g. Rats were anesthetized either with Nembutal or Halothan. After insertion the venous - and arterial catheters via the v.- and a. epigastric superficialis, the extremity was decoupled from the circulatory system by a tourniquet. After perfusion and removal of catheters and tourniquet, the vasa epigastric were closed with a thermocautery and the skin incision was sutured.

For the perfusion, the 37°C warm, drug-containing perfusion solution, was pumped through the leg, which had been decoupled from the body's circulation. The perfusion rate was 0.5 ml/min. Perfusion solution was an oxygenated hemoglobin solution (Biotest, Frankfurt, Germany) containing 60% hemoglobin and glucose, Na^+, K^+, Ca^{2+}, Mg^+, Cl^-, HCO_3^-. The actual perfusion experiment consisted of 5 min pre-perfusion, 30 min drug perfusion and 10 min flush perfusion. In control experiments, no OHCP was fluorometrically [9] detectable in the contralateral extremity. The tumor weight was determined by palpatory comparison with model balls.

Evaluation of perfusion experiments

After tumor transplantation, untreated control animals and animals after control perfusion with hemoglobin solution without cytostatics die about 19 days after tumor transplantation. when tumor weight is 60 - 70g. In the perfusion experiments, the rats were killed when the tumor weight was approximately 20g. The test results were calculated as the standard growth delay (SGD). SGD = (T20 - TC)/TC. T20 = no. of days to reach a tumor weight of approximately 20g; TC = no. of days that untreated controls need to reach a tumor weight of approximately 20g.

Results

Therapy of the tumor-bearing limb in rats by regional perfusion

SECP and MF are oxazaphosphorine cytostatics which within minutes spontaneously hydrolyzes to 4-hydroxycyclophosphamide (Figure 1). Originally, they were developed to circumvent the hydroxylation of CP in the liver and to use the therapeutic potency of CP also for regional chemotherapy.

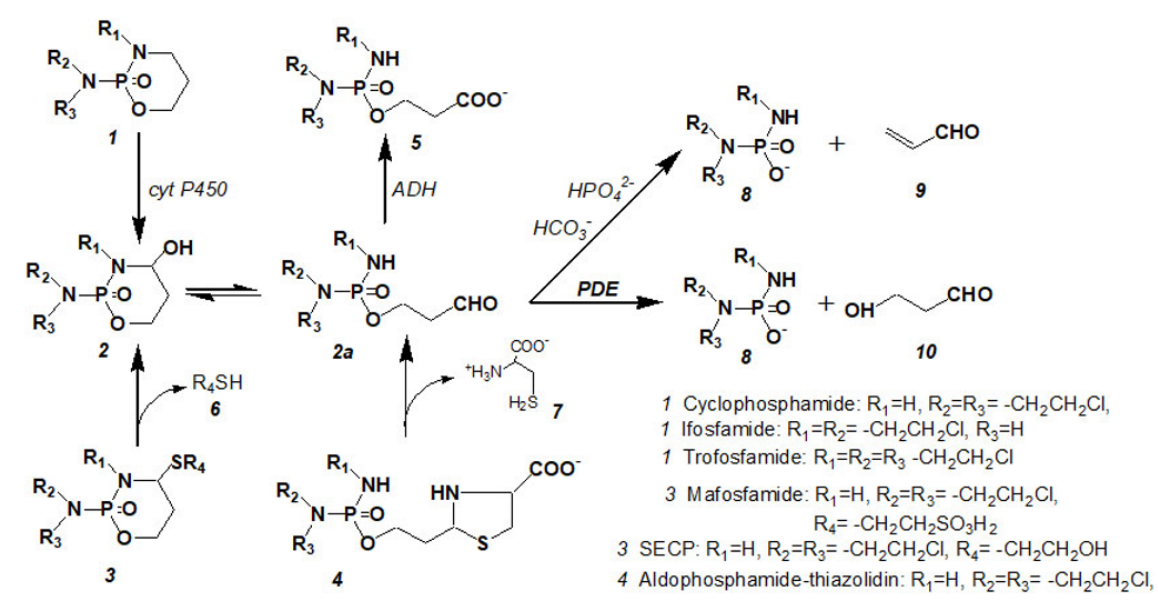

Figure 1: *Formation of 4-hydroxyoxazaphosphorines (OXOH, 2) and aldophosphamides (ALD, 2a) from oxazaphosphorine cytostatics (OX, 1) by Cyt p450 monooxygenases and hydrolysis of 4-SR-sulfido-oxazaphosphorines (MF and SECP, 3) and aldophosphamide-thiazolidines (THIA, 4) and main metabolic pathways of OX.*

After hydroxylation of OX by cytochrome P450 enzymes OXOH (2) forms an equilibrium mixture with its tautomeric aldehydes (ALD, 2a). ALD`s are the pharmacologically active metabolites. They are substrates for detoxification to carboxyphosphamides (CARB, 5) by aldehyde dehydrogenases (ADH), formation of phosphoramide mustards (PM, 8) by β-elimination of acrolein (9) and substrate for phosphodiesterases (PDE), which split ALD to PM (8) and 3-hydroxypropanal (HPA, 10).

Alternative ways to form OXOH (2) or ALD (2a) are the spontaneous hydrolysis of 4-SR-sulfido-cylophosphamides (3) with the formation of thiols (6) or the hydrolysis of aldophosphamide-thiazolidines (THIA, 4) with the formation of L-cysteine (7).

Figure 2 shows tumor growth curves after perfusion of the tumor bearing limb with hemoglobin solution which was 5 mM - the highest tolerable concentration - of MF. The therapeutic success is low. Compared to the tumor growth curves after perfusion without MF, only a marginal growth delay can be measured. Table 1 summarizes the results of further perfusion experiments. Only a slight growth delay was measured by perfusion with MF, SECP or the mother compound CPOH itself, while 2 out of 5 animals were cured by perfusion with cisplatin (CPT) and 5/6 animals were cured by perfusion with NMO.

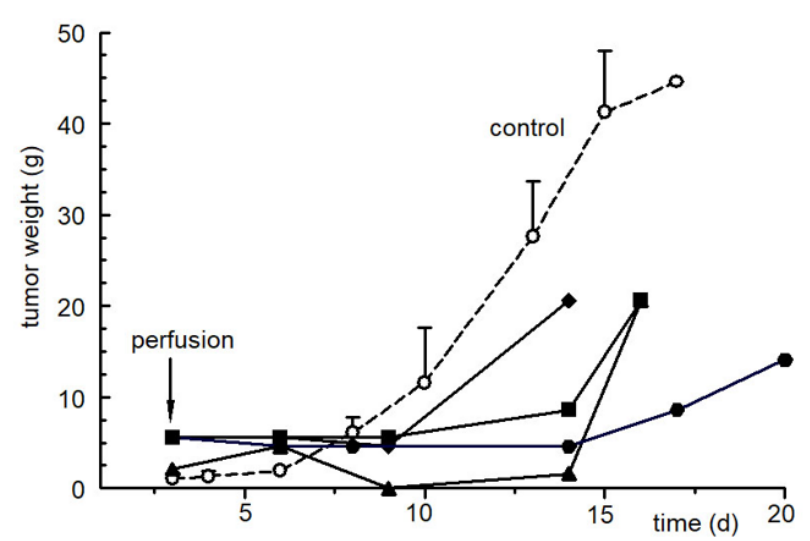

Figure 2: *Tumor growth curves of Yoshida-Ascites tumor cells transplanted into the m. gastrocnemius of male rats; 30 min regional perfusion with 5 µmol/ml MF, dissolved in oxygenated hemoglobin solution. Dotted line (control): Tumor growth in animals that were perfused with the hemoglobin solution without MF, mean ± standard deviation, n = 4.*

	Concentration (mM)	Long time survivors	Standard growth delay
4-hydroxy-cyclophosphamide (CPOH)	1	0/3	0.5
S-ethanol-sulfido-cyclophosphamide (SECP)	2	0/3	0.8
Mafosfamide (MF)	5	0/4	0.4
Nitrogen mustard N-oxide (NMO)	0.5	5/6	1.0
Cisplatin (CPT)	1.1	2/5	0.9

Table 1: *Regional perfusion of the tumor-bearing rat limb.10^6 Yoshida ascites tumor cells were transplanted into the m. gastrocnemius. 30 min perfusion 3 days after tumor transplantation. Survival time of control animals after perfusion with hemoglobin solution without test substance 18 - 20d. Animals with a survival time > 100 days after perfusion are considered to be long time survivors.*

Table 2 summarizes the results of the systemic therapy tests with CP in rats with tumor bearing limbs. The table shows that after intraperitoneal administration of 121 mg/kg CP 5/5 animals are cured (surviving time > 100d).

	Dose (mg/kg/mmol/kg)	Long time survivors	Standard growth delay
Cyclophosphamide (CP)	53/0.190	0/5	0.2
	81/0.290	0/5	1.0
	121/0.434	5/5	

Table 2: *Therapy of the tumor-bearing limb in rats with cyclophosphamide One intraperitoneal administration three days after tumor transplantation, long time survivors: survival time >100d.*

Summarizing: As a result of the experiments it can be stated that therapy by regional perfusion of the tumor-bearing limb in rats with still tolerable concentrations of CPOH, MF and SECP are ineffective in contrast to control perfusions with CPT and NMO and systemic therapy with CP. The high therapeutic index of OHCP formed *in vivo* from CP, does not come into operation in the perfusion experiments.

Mainly responsible for the toxicity of CP and other OX is CPOH and the hydroxylation product of other OX because it reacts with nucleophilic groups of macromolecules, e.g. free SH groups of proteins. The acute toxicity of CPOH in mice is 0.53 mmol/kg (143 mg/kg). This strong toxicity is drastically reduced if OXOH is bypassed in the formation of the pharmacologically active metabolite ALD. This is possible if, for example, instead of CPOH, the thiazolidine of aldophosphamide (THIA, 4 in figure 1) is used. THIA hydrolyzes spontaneously to aldophosphamide and L-cysteine bypassing CPOH. The acute toxicity of THIA in mice is approximately 10 times less than that of CPOH [10]. In order to test whether in the perfusions experiments better therapeutic success can be achieved by increasing the concentration of the pharmacologic active metabolite ALD, comparative perfusion experiments and systemic therapy experiments with THIA were carried out in rats with tumor bearing limbs. The results of these tests are summarized in table 3.

Therapy of the tumor bearing limb in rats with THIA by			
Perfusion (mM)	**Intraperitoneally administration (mg/kg/µmol/kg)**	**LTS**	**SGD**
20		0/5	0.0
	215/0.566	1/5	1.5
	464/1.221	0/5	2.6
	681/1.792	2/2	

Table 3: *Therapy of the tumor bearing limb in rats with THIA by regional perfusion and intraperitoneal administration, LTS (long time survivors) surviving time > 100d, SGD (standard growth delay) SGD = $(T_{20} - T_C)/T_C$; T_{20} = no. of days to reach a tumor weight of approximately 20g; T_C = no. of days that untreated controls need to reach a tumor weight of approximately 20g.*

In further experiments was checked whether the poor results of the regional perfusion with SECP and MF compared to the results of perfusion experiments with NMO or CPT are only due to the fact that no CPOH, SECP or MF gets into the tissue of the perfused limb. Experiments were carried out in which the SECP concentration in the perfusion solution leaving the limb was measured. The total amount of SECP penetrated into the tissue was calculated from the difference between the areas under concentration curves of the perfusion solution entering the limb and leaving the limb. The difference between the areas under the curves (auc) in the perfusion experiments with 5 mM MF was ~37 mM minutes and is about 12 times greater than the auc for CPOH after intraperitoneal injection of 121 mg/kg CP - a CP dose which cures 5 of 5 treated animals (Table 2). The auc in this experiment was calculated to be ~3.1 mM min (Figure 3). The experiment shows that a 12 fold increase in the amount of the pharmacologic active metabolite ALD that acts on the tumor tissue does not lead to better therapeutic success in this tumor model.

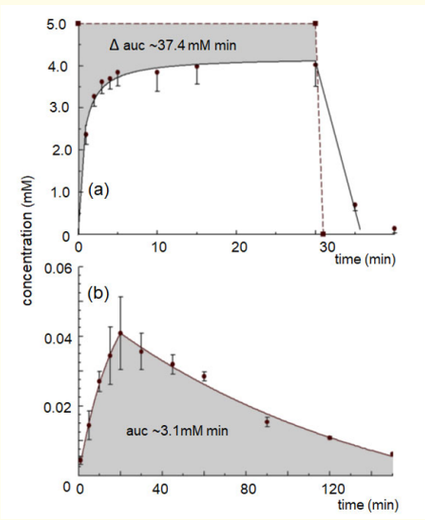

Figure 3: *Concentration time curves of a. MF concentration in the perfusate entering the limb (dotted line) and in the effusat leaving the limb (effusat, solid line). The difference between the two areas under the curves, that is the area marked in gray, is a measure of the amount of MF that has penetrated the leg tissue, mean ± standard deviation, n = 4. b. Concentration of CPOH in the rat blood after intraperitoneally application of 150 mg/kg CP, mean ± standard deviation, n = 4.*

Discussion

In order to understand the mechanism of action of OX, in addition to the discovery of the enzymatic cleavage of ALD with the formation of the alkylating agent and HPA, two further discoveries described in the scientific literature are necessary. These are the results of the experiments by Schwartz and Waxman [14] and by Iyer., *et al* [15]. Schwartz and Waxman have shown that CPOH only inhibits, but does not kill, the growth of cells in which the ability to apoptosis is switched off by overexpression of anti-apoptotic Bcl protein. Iyer., *et al*. have investigated the biological effects of HPA, which is known as Reuterin because it is produced by *Lactobacillus reuteri* and submitted to the culture medium [16]. They found that Reuterin (HPA) promotes apoptosis by inhibiting anti-apoptotic proteins Bcl2 and BcxL and inhibiting NF ϰB activation but enhancing mitogen-activated protein kinase (MAPK).

The discovery of HPA as an OX metabolite and the results of the investigations by Schwartz and Waxman and Iyer., *et al*. lead to the already in detail published scheme [17] for the mechanism of action of OX shown in figure 4. As shown below, this scheme is confirmed by the results of the perfusion experiments.

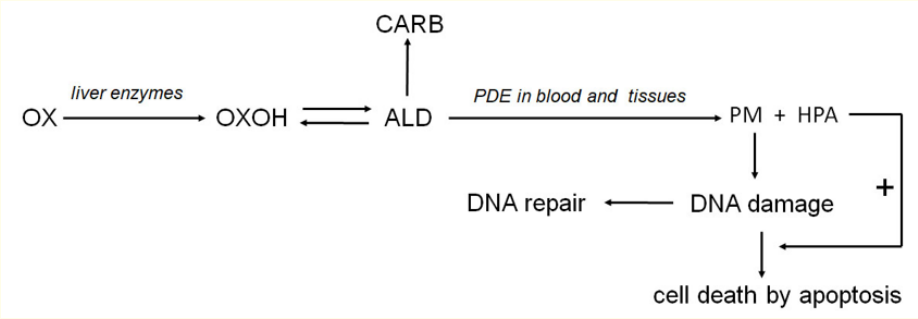

Figure 4: *Scheme for the mechanism of action of oxazaphosphorine cytostatics (OX). After hydroxylation of OX in the liver to OXOH, the tautomeric aldehyde (ALD) is spontaneously formed. ALD is either detoxified into the therapeutically ineffective carboxylic acid (CARB) or is split into the alkylating metabolite phosphoreamide mustard (PM) and 3-hydroxypropanal (HPA) by phosphodiesterases (PDE) in blood and tissues. PM alkylates the DNA of cells. DNA damage is either repaired or initiates apoptosis, which - and this is unique for OX - is intensified by HPA.*

As a result of the experiments with CP and CPOH on the tumor-bearing limb in rats, it can be stated that the pharmacologically active CP metabolite CPOH fails in regional perfusion. While all treated rats are cured by systemic administration of CP, only a marginal growth delay can be measured after regional perfusion with CPOH, MF, SECP or THIA and this although the amount of CPOH - measured on the area under the time concentration curve - is about 12 times greater in the perfusion experiments than after intraperitoneal application of CP (Figure 3). The slight growth delay after perfusion with CPOH or compounds that dissociate to CPOH is very likely due to the decomposition of CPOH to PM and acrolein, catalyzed by the bicarbonate ions in the perfusion medium.

A measure of the therapeutic quality of a drug is the therapeutic index, that is the quotient from the lethal dose to the therapeutically effective dose. According to Brock [12] and Brock and Hohorst [13], the therapeutic index - measured in Yoshida ascites tumor bearing rats - is less than 5 for simple alkylating antitumor agents such as PM, NMO and nitrogen mustard but greater than 100 for CPOH. In the perfusion experiments the therapeutic quality of CPOH obviously does not come into operation. Why? According to the scheme for the mechanism of action for OX [17] (See figure 4), the pharmacologically active metabolite is aldooxazaphosphorine (2a, figure 1). For the

development of OX effectiveness it is absolutely necessary that this metabolite is cleaved by phosphodiesterases into the alkylating agent PM and the proapoptotic HPA. However, the formation of HPA was not possible in the perfusion experiments because the hemoglobin solution used for the perfusion did not contain any aldophosphamide-cleaving enzymes, but HCO_3^- ions which catalyze the ß-elimination of acrolein from ALD. Thus, the ineffectiveness of the therapy experiments by local perfusion confirms the presented scheme for the mechanism of action of OX.

Attempts to develop improved OX have aimed in the past to increase the concentration of PM in tumor cells. One example is Glufosfamide, which was developed with the idea of using the increased glucose requirement of tumor cells to enrich isophosphoramide mustard, the alkylating function of Ifosfamide, in tumor cells through glycosidic coupling to glucose. This strategy proved unsuccessful. It was wrong because it is not the alkylation of the DNA that leads to cell death, but the apoptosis triggered by the DNA alkylation. The scheme in figure 4 shows a simple way of improving OX. DNA damage is repaired quickly and effectively by the cell's own repair mechanisms. It can be assumed that only a small part of the DNA damage caused by the alkylating function of OX is "translated" into apoptosis. Therefore, it makes sense either to prevent DNA repair or to create irreparable DNA damage. The common OX contains 2-chloroethyl groups as an alkylating function, which generate easily repairable DNA inter strand crosslinks and thus lead to a small yield of apoptosis. Apoptosis can be greatly increased if in the alkylating function a chlorine is substituted by a mesyl group, which makes intra strand crosslinks which are difficult to repair [18,19]. An OX in which this principle is implemented is the substance SUM-IAP (mesyl-I-aldophosphamide-perhydrazine), which is orders of magnitude more effective in mice with subcutaneously transplanted P388 tumors than the corresponding control substance, which contains a 2-chloroethyl group instead of the mesyl-ethyl group [17].

Conclusion

The experiments have shown that the CP metabolite CPOH, which is said to belong to the group of alkylating cytostatics, is completely ineffective in local therapy in contrast to directly alkylating NMO or CPT. This despite having a therapeutic index about 20 times higher than NMO. This is because the alkylating CP metabolite PM does not kill cells by DNA alkylation. DNA damage is only the trigger for the cell death event by 53 mediated apoptosis. This also applies to a lesser extent to all alkylating substances, but - as the experiments have shown - is absolute for OX. Without HPA-enhanced apoptosis, OX are ineffective. They do not belong to the alkylating cytostatics, but form a separate group. They are apoptosis boosters.

Acknowledgements

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

Conflicts of Interests

There are no conflicts of interest.

Bibliography

1. Voelcker G. "Enzyme Catalyzed Decomposition of 4-Hydroxycyclophosphamide". *The Open Conference Proceeding Journal* 8 (2017): 3.
2. Low JE., *et al*. "Conversion of 4-hydroperoxycyclophosphamide and 4-hydroxycyclophosphamide to phosphoramide mustard and acrolein mediated by bifunctional catalysis". *Cancer Research* 42.3 (1982): 830-837.
3. Voelcker G. "Influence of the alkylating function of aldo-Ifosfamide on the anti-tumor activity". *Anticancer Drugs* 29.1 (2018): 75-79.
4. Peter G., *et al.* "Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives". *Cancer Treatment Reviews* 60.4 (1976): 429-435.

5. Laber P. Die regionale Perfusion des Tumor tragenden Rattenbeins mit stabilisiertem, aktivierten Cyclophosphamid (4-(S-ethanol)-sulfido-Cyclophosphamid; Die Entwicklung eines Perfusionsmodell zur Überprüfung der Eignung zur regionalen Tumortherapie an der hinteren Rattenextremität. Inauguraldissertation zur Erlangung des Doktorgrades der Zahnmedizin des Fachbereichs Humanmedizin der Johann Wolfgang Goethe-Universität Frankfurt am Main (1985).

6. Rockinger H. Die regionale Perfusion des Tumor tragenden Rattenbeins mit stabilisiertem, aktivierten Cyclophosphamid (4-(S-ethanol)-sulfido-Cyclophosphamid; Regionale Perfusion mit stabilisiertem aktiviertem Cyclophosphamid zur Therapie des Yoshida Sarkoms an der hinteren Rattenextremität. Inauguraldissertation zur Erlangung des Doktorgrades der Zahnmedizin des Fachbereichs Humanmedizin der Johann Wolfgang Goethe-Universität Frankfurt am Main (1985).

7. Erven G. Vergleichende Untersuchungen über die Antitumorwirkung von stabilisierten, aktivierten Cyclophosphamid und cis Platin am Modell des tumortragenden Rattenbeins durch regionale Perfusion. Inauguraldissertation zur Erlangung des Doktorgrades beim Fachbereich Veterenärmedizin der Justus-Liebig-Universität Gießen (1986).

8. Skupin W. Die regionale Perfusion des tumortragenden Rattenbeins mit stabilisierten, aktivierten Cyclophosphamiden in Kombination mit Protektorthiolen. Inauguraldissertation zur Erlangung des Doktorgrades beim Fachbereich Veterenärmedizin der Justus-Liebig-Universität Gießen (1990).

9. Voelcker G., *et al.* "Fluorometric determination of "activated" cyclophosphamide and ifosfamide in blood". *Journal of Cancer Research and Clinical Oncology* 93.3 (1979): 233-240.

10. Voelcker G. "Causes and possibilities to circumvent cyclophosphamide toxicity". *Anti Cancer Drugs* 31 (2020): 617-622.

11. Dost F. "Grundlagen der Pharmakokinetik G Thieme Stuttgart" (1968).

12. Brock N. "Comparative pharmacologic study *in vitro* and *in vivo* with cyclophosphamide (NSC-26271), cyclophosphamide metabolites, and plain nitrogen mustard compounds". *Cancer Treatment Reviews* 60 (1976): 301-308.

13. Brock N and Hohorst HJ. "The problem of specificity and selectivity of alkylating cytostatics: studies on N-2-chlorethylamido-oxazaophosphorines". *Z Krebsforsch* 88 (1977): 185-215.

14. Schwartz PS and Waxman DJ. "Cyclophosphamide induces caspase 9-dependent apoptosis in 9L tumor cells". *Molecular Pharmacology* 60 (2001): 1268-1279.

15. Iyer C., *et al.* "Probiotic Lactobacillus reuteri promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kappa B and MAPK signalling". *Cellular Microbiology* (2008).

16. Talarico TL and Dobrogosz WJ. "Chemical characterization of an antimicrobial substance produced by Lactobacillus reuteri". *Antimicrobial Agents and Chemotherapy* 33 (1989): 674-679.

17. Voelcker. "Oxazaphosphorine cytostatics: from serendipity to rational drug design". *Anti-Cancer Drugs* 30 (2019): 435-440.

18. Povirk LF and Shuker DE. "DNA damage and mutagenesis induced by nitrogen mustards". *Mutation Research* 318 (1994): 205-226.

19. Iwamoto T., *et al.* "DNA intrastrand cross- at 5′-GA-3′ sequence formed by busulfan and its role in the cytotoxic effect". *Cancer Science* 95 (2004): 454-458.

Volume 10 Issue 4 April 2022