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Cyclophosphamide: Mechanism of Action, Toxicokinetics, and Toxicity Profile

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Abstract

Cyclophosphamide is a widely used alkylating agent in the treatment of various malignancies and autoimmune disorders. It acts as a prodrug that is metabolized in the liver by the cytochrome P450 system to form active metabolites, including phosphoramide mustard, which interferes with DNA replication and induces cell death. Due to its broad antineoplastic activity, cyclophosphamide is commonly used in lymphoma, leukemia, and solid tumors. However, its clinical use is associated with several adverse effects, such as hepatotoxicity, nephrotoxicity, lung damage, neurotoxicity, cardiac toxicity reproductive toxicity and hemorrhagic cystitis. Understanding its mechanism of action and toxicity profile is essential for optimizing its therapeutic application.

Keywords: Cyclophosphamide, Toxicokinetics, Mode of Action, Toxicity

1. Introduction

Cyclophosphamide (CP), belonging to the nitrogen mustard class, is an example of chemotherapeutic drugs, which is a highly reactive cytotoxic bifunctional alkylating agent.

a. Chemical structure

The cytostatic alkylating drug cyclophosphamide (CP), also known as (RS)-N, N-bis (2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide, was synthesized from bis-b-chloroethylamine [1]. Its chemical formula is C7 H15Cl2 N2 O2 P [2].

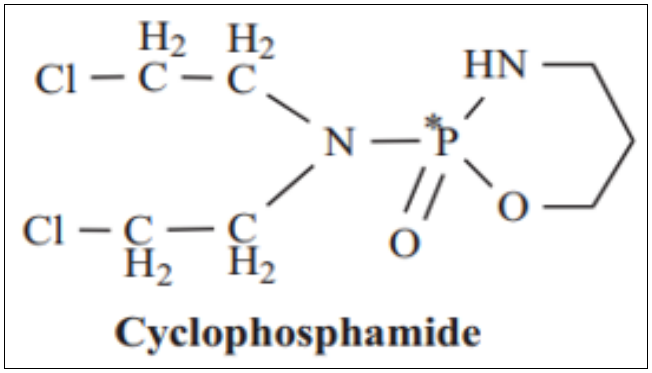

Fig 1: Chemical structure of cyclophosphamide [3]

b. Mode of action

Alkylating agents bind to biological molecules including DNA and proteins, degrading their structure and function. Covalently binding these chemicals to DNA causes methylation and replication mistakes resulted in aberrant mitosis, chromosomal breakage, and mutations [4].

Cyclophosphamide metabolism produces the active metabolites phosphorylamide and acrolein, cross-links are created by the phosphoramide metabolite at the guanine N-7 site inside and between adjacent DNA strands. These changes are irreversible

and ultimately result in programmed cell death [5]. If the alkylated DNA isn't restored, the tumor suppressor protein p53 is triggered, which stops the cell cycle and gives time for DNA repair. When DNA repair fails, p53 triggers apoptosis, also The hypothalamic-pituitary-adrenal (HPA) axis can trigger the apoptotic pathway by inhibition of anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2) and B cell leukemia-xL (Bcl-xL), along with the suppression of nuclear factor kappa B (NF-κB) activation, moreover, HPA enhances the phosphorylation of p38, c-Jun N-terminal kinase (JNK), and mitogen-activated protein kinases (MAPK) which accelerates cell death [6]. Although acrolein has no anticancer effect, it is the primary cause of hemorrhagic cystitis [7].

Cyclophosphamide has immunosuppressive properties and is selective for T cells in addition to its antimitotic and antineoplastic actions. CP is employed at high doses in malignant hematopoietic cell eradication therapy; however, lower doses are effective in selectively modulating regulatory T cells. It reduces the secretion of interferon-gamma and Interleukin (IL)-12 while enhancing the secretion of secreted by Type 2 T helper **(**Th2) cells, such as IL-4 and IL-10 in the cerebral spinal fluid and peripheral blood [8, 9].

Because of these effects, cyclophosphamide is regarded a beneficial adjunct to tumor vaccination protocols, post-transplant alloreactivity management, and the treatment of immune-mediated diseases and some types of vasculitis [10, 11, 12]. While the exact method by which cyclophosphamide performs its immunomodulatory impact is unknown, various studies have proposed a few possible mechanisms of action, these include eliminating regulatory T cells in naive or malignant host cells, inducing T cell growth factors such as type I interferons, and preconditioning host cells for donor T cells to reduce alloreactivity [8, 9, 12].

c. exposure

Cyclophosphamide is one of the most often used medicines in cancer chemotherapy. It is a cytotoxic alkylating drug having anticancer and immunosuppressive effects that is used to treat multiple myeloma, chronic and acute leukemia, solid malignancies, and lymphomas [10]. As an immune suppressor, CP has been widely used to treat severe autoimmune inflammatory diseases, including rheumatoid arthritis, systemic erythema associated with lupus and bone marrow transplant immune ablations [14].

d. Toxicokinetics profile of Cyclophosphamide:

Cyclophosphamide can be administered orally or intravenously on a range of regimens [12]. As a monohydrate, CP is soluble in water, normal saline or alcohol [3]. One hour after taking the medication orally, CP reaches its highest concentration due to good absorption. CP has an oral bioavailability of 85–100% [13] and the first-pass impact in the gut and liver causes a portion of the medication to be metabolized [3].

Cyclophosphamide metabolism:

Cyclophosphamide is an inert prodrug that needs to be activated chemically and enzymatically. It was discovered that this process takes place in the liver and is mediated by the cytochrome P450 system and involves oxidative hydroxylation of the C4 position in CP. The oxazaphosphorine ring undergoes hydroxylation to produce 4-hydroxycyclophosphamide (4OHCP), which coexists alongside aldophosphamide (ALDO), its tautomer [14]. Aldophosphamide, which enters the cell actively by glycoproteins or passive transport [15]. Aldehyde dehydrogenase transforms ALDO into the carboxyphosphamide (CARB). Carboxyphosphamide is protected by the antioxidant molecules glutathione [16, 17]. whereas 4OHCP is enzymatically transformed to 4ketocyclophosphamide [18]. There was an increase in the level of 4-hydroxycyclophosphamide and carboxyethyl phosphamide in the blood plasma of individuals treated with CP [19]. In cell culture tests, the portion of ALDO that was not eliminated to CARB is degraded by β-elimination of acrolein to PAM [18]. However, *in vivo*, phosphodiesterases (PDE) enzymatically break down ALDO to PAM and 3-hydroxypropanal (HPA) [20].

e. Cyclophosphamide toxicodynamic

Due to the cyclophosphamide's toxicities, there are several restrictions on using it, including gastrointestinal side effects, cardiac toxicity, gonadal toxicity, hepatotoxicity, and nephrotoxicity [21, 22]. CP caused toxicity in multiple organs in rats, including the testes, liver, lungs, spleen, and kidneys, compared to the control group [23].

2. Hepatotoxicity

Cyclophosphamide-treated rats show a notable increase in the activity and function of the liver biomarkers found in serum, including lactate dehydrogenase (LDH), gamma-glutamyl transferase (γGT), aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) [24].

By decreasing hepatic Glutathione **(**GSH**)** and simultaneously suppressing hepatic Glutathione Peroxidase **(**GSPx**)** , Glutathione Reductase **(**GSR**)** and Catalase **(**CAT**)** activities, it increased oxidative stress [24]. The diminished levels of these antioxidants may arise from their heightened ability to scavenge or inhibit CP-mediated free radicals as reactive nitrogen species (RNS**)** and reactive oxygen species (ROS) and/or CP reactive metabolites (acrolein, phosphoramide mustard, hydroperoxy cyclophosphamide, etc.) [25, 26]. It's interesting to note that CP caused a noticeable increase in the hepatic activity of Glutathione S-Transferase **(**GST**)** and Superoxide Dismutase **(**SOD) [24] As a kind of adaptation and defense against xenobiotic-mediated oxidative stress, cells exposed to xenobiotic intoxication have been shown in a number of models to occasionally up-regulate antioxidant enzymes like GST and SOD activities [27, 28].

Cyclophosphamide-induced hepatic lipid peroxidation by showing that CP injection significantly raised hepatic Malondialdehyde (MDA**)** and total (amino acids, peptides, proteins, and lipids) hydroperoxide (TROOH) levels [24]. The CP-intoxicated experimental animals elicited a pronounced increase in the levels of hepatic Nitric Oxide (NO) and Inducible Nitric Oxide Synthase (iNOS) [25].

Moreover, Cyclophosphamide is associated with enhanced synthesis of inflammatory mediators generated by damaged cells or immune cell-induced leukocyte infiltration at the injury site [29]. ROS enhances expression of inflammatory mediators and Nuclear Factor –kappa **(**NF-κB**)** signaling pathway [30]. NF-κB is one of the most important transcription factors involved in the regulation of genes related to inflammation, cell division and survival [31]. CP

induced a significant increase in NF-κB, Interleukin-6(IL-6), and Tumor Necrosis Factor alpha (TNF α) levels, with a reduction in Interleukin-10 (IL-10) level [32].

Also, Cyclophosphamide treatment resulted in a large decrease in the anti-apoptotic hepatic B-cell lymphoma 2 (Bcl-2) level but a considerable increase in the levels of pro-apoptotic factors caspase-3 and Bcl-2-associated X protein (Bax) [33, 34]. CP activates the apoptotic and autophagic pathways by elevating the expression of cysteine aspartate-specific protease-3 and the levels of light chain 3B (LC3B), while also enhancing the expression of 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage [31].

Additionally, Cyclophosphamide induced marked hepatic damage, characterized by extensive mononuclear cell infiltration, loss of cellular boundaries, hepatocellular necrosis, and substantial cytoplasmic depletion in hepatocytes [35].

3. Nephrotoxicity

An additional side effect of CP is nephrotoxicity, which is demonstrated by elevated blood urea nitrogen (BUN) and serum creatinine levels [36].

Cyclophosphamide intoxication induced histopathological alteration as Bowman's capsule degradation, tubular edema, and necrosis in the kidney [37]. Also showed expanding Bowman's capsule, interstitial hemorrhage, and necrosis with loss of nuclei and shrinkage glomeruli [36]. There was a decrease in the number of renal glomeruli and an increase in the amount of interstitial tissue [38].

These adverse consequences may result from oxidative stress and the production of extremely reactive electrophiles during the metabolism of CP, which further deteriorates target tissues and cell membranes [39].

After CP breaks down into its active metabolites, it produces hazardous reactive oxygen species, which causes seriously injured cells to generate more pro-inflammatory cytokines like NF-κB [40]. Elevated concentrations of these cytokines additionally repress PPAR-γ expression, hence impacting other physiological processes controlled by PPAR-γ [40].

Rats given cyclophosphamide showed a decline in antioxidant components like GSH, CAT, and SOD activities in renal tissue [41] with an increase in MDA level and a decrease Nrf2 expression, which was accompanied by elimination of NF-κB repression, pro-inflammatory cytokine TNF-α is upregulated, while anti-inflammatory cytokine IL-10 is suppressed in renal tissue [36].

In the kidney, Cp poisoning induced a decline in Bcl-2, while elevation the expression of the apoptotic regulating protein Bax. Cell death results from the activation of caspases triggered by Bax [37].

4. Neurotoxicity

The brain undergoes oxidative stress when CP is administered [42]. Because CP has a detrimental effect on hippocampus neurogenesis, it causes cognitive and psychosocial deficits, particularly on the hippocampal-dependent memory task [43, 44]. Throughout maturity, the hippocampal neurogenesis persists [43]. In addition to causing neuronal damage, CP can cause disturbance of the cholinergic pathway [45].

Rat hippocampus Acetylcholinesterase (AChE) activity is elevated in the brain by CP-mediated oxidative stress [46]. Here, CP's elevated AChE activity may reduce cholinergic signaling, which could result in memory loss and cognitive impairment [47].

Rats subjected to CP revealed sections stained with H&E that either indicated necrosis or degeneration of the hippocampal neurocytes, which include the granule cells of the dentate gyrus and the pyramidal cells of the hippocampal proper. There was a notable decrease in the thickness of the granule cell and pyramidal cell layers [48, 49].

The immunohistochemical section revealed that many apoptotic neurons with positive responses to the apoptosis marker p53 and decreased positive responses to the anti-apoptotic marker Bcl2 [49].

Also, CP administration induced an increase in expression of Glial Fibrillary Acidic Protein (GFAP) indicated that CP caused astrocyte activation. This could be an attempt to compensate for damage to the neurons [48]. Because GFAP increases the structural stability of astrocyte processes, it is recognized to be crucial for modifying astrocyte shape and movement [50]. It was discovered that brain damage, resulting from chemical agents, illnesses, or trauma, caused astrogliosis. Rapid Glial Fibrillary Acidic Protein (GFAP) production in astrogliosis can be seen by immunostaining with GFAP antibodies [51].

Cyclophosphamide dramatically lowered cerebral Nrf2, GSH, and the activity of the GSH-dependent antioxidant enzyme GPx. Thus, changes in Nrf2 and GSH metabolism may cause CP to impair cerebral antioxidant equilibrium. The oxidative action increases the formation of ROS, which may have exceeded the SOD and CAT actions [47].

5. Reproductive Toxicity

Testicular tissue is prone to oxidative stress damage more quickly than other tissues due to its high rate of mitochondrial oxygen consumption, accelerated rate of cell division, and proportionately higher levels of unsaturated fatty acids. In addition, the low oxygen pressure and weak testicular artery create fierce cell competition for oxygen [52].

Cyclophosphamide treatment induced testicular toxicity, characterized by a significant reduction in the percentages of sperm motility, sperm vitality and normal sperms, but increased the percentages of sperms with abnormal head, abnormal tail, spermatic droplets and tailless head [53].

It's reported that injection of CP caused dysregulation of the steroidogenesis system, as evidenced by a sharp decline in the CP group's serum concentrations of Luteinizing Hormone (LH) and testosterone [1].

Leydig cells include two primary enzyme groups involved in the steroidogenesis pathway: hydroxysteroid dehydrogenases (HSDs) and cytochrome P450 (CYPs) [54]. The process of steroidogenesis begins with the trafficking of cholesterol via the inner mitochondrial membrane-produced steroidogenic acute regulatory protein (StAR), which is then converted to pregnenolone by the enzyme cytochrome P450, family 11 (CYP11) [55]. CP induced downregulation of androgenic-related gene expression, such as HSD, CYP11, and StAR, was found to be associated with a decrease in plasma testosterone levels [56].

cyclophosphamide can lower serum testosterone levels by inhibiting the activity of the17β-hydroxysteroid dehydrogenase **(**17β-HSD**)** enzyme, which catalyzes the conversion of androstenedione to testosterone and is essential for the biological activity of steroid hormones like estrogen and androgen [57].

Cyclophosphamide induced histological alterations include a decrease in the height of the germinal epithelium, the length and diameter of the seminiferous tubules, a decrease in the mean total volume of the testes and their constituent parts, and the quantity of spermatogenic cells [58], also reduced daily sperm production as well as sertoli and Leydig cells [59].

This reduction in spermatogenic cells, which has been related to the indirect impairment of spermatogenesis, gonadotropin production, and testosterone levels, is most likely the cause of the lower testicular weight in CP-treated rats [60].

Testicular oxidative stress plays a significant role in the pathophysiology of male infertility because it can alter the testicular microvasculature and hormonal signaling patterns, which can increase germ cell death and ultimately decrease spermatogenesis [61]. CP had an impact on the testicular redox state as it induced an increase in testicular NO and MDA levels. Additionally, it reduced GSH and Total Antioxidant Capacity (TAC) levels [56].

Moreover, cyclophosphamide's active metabolite acrolein stimulates NF-kB and Activator Protein-1(AP-1), two transcription factors. Upon entering the cell nucleus, NF-kB triggers the transcription of pro-inflammatory cytokines such as TNF-α and IL-1B, hence elevating apoptosis. Furthermore, cyclophosphamide reduces the Nrf2 factor, which is essential for cell survival in the presence of oxidative stress [58].

Moreover, CP induced alteration in the female reproductive system as evidenced by CP treatment caused ovarian failure or impaired infertility in cancer patients, Due to apoptotic alterations in granulosa and theca cells, followed by follicle loss [62]. It is well recognized that CP can use a variety of mechanisms, such as oxidative stress, inflammation, and apoptosis, to cause ovarian toxicity and severe ovarian damage, which can lead to premature ovarian failure (POF) [63].

6. Cardiac Toxicity

It was discovered that cyclophosphamide caused immediate heart injury, including severe inflammation and necrosis, hemorrhage and fibrosis [64]. Also, A decrease in heart rate, depression of the PR segment, elevation of the ST segment, and abnormal electrocardiogram (ECG) are indicative of CP-induced cardiotoxicity [36]. Excessive therapeutic dosages of CP resulted in a deadly cardiotoxicity that exhibits a range of myopericarditis symptoms and indicators. This could induce fatal complications like cardiac tamponade, arrhythmias, and congestive heart failure [65].

Furthermore, as demonstrated by an elevated 8-OHdG concentration in the cardiac tissue, cyclophosphamide causes cardiac oxidative DNA damage, which accounts for the aberrant ECG alterations [51].

The observed increase in serum Cardiac Troponin I (cTnI) levels, LDH, and Creatine Kinase–MB (CK-MB) activity suggests myocardial injury after CP. In addition, it indicated that the heart of rats treated with CP had impaired myocardial membrane stability, integrity, and function due to increased lipid peroxidation and poor antioxidant defense. This led to the leakage of CK-MB, LDH, and cTnI, which raised the levels of these substances in the blood [36].

Histological analysis of cardiac samples revealed evidence of myocardial necrosis [64], wavy myocardiac fibers with loss of cardiac cell components and focal fatty alterations [36].

7. Bladder Toxicity

The bladder is filled with acrolein metabolites [67], causing urotoxicity through direct interaction with the uroepithelium [68]. According to research on both humans and animals, hemorrhagic cystitis is a dangerous adverse effect of using CP [69, 70]. Hemorrhagic cystitis, which involves bleeding of the bladder mucosa due to a disseminated inflammation in the bladder, is typically caused by oxazaphosphorine chemotherapy such as CP and ifosfamide [68].

Also, Hemorrhagic cystitis is indicated by edema, the formation of blood clots, and injury to the bladder urothelium [71]. Patients with extensive bladder hemorrhage who received a high dosage of intravenous CP were reported to have a death risk of 2% to 4% [68]. Furthermore, CP-induced cystitis causes an overactive bladder, resulting in urothelium damage and severe inflammation [72].

Several studies on animal models of CP-induced urinary bladder injury have found that CP injection increased bladder lipid peroxidation (LPO) and decreased bladder GSH [73].

Inflammation is linked to the pathogenesis of CP-induced hemorrhagic cystitis [73].

Acrolein activated NF-kB, leading to the generation of inflammatory cytokines such as TNF-α, IL-6, along with ROS [75]. In bladder tissue treated with CP, Cyclooxygenase-2 (COX-2) mRNA expression is elevated. COX-2 is a pro-inflammatory enzyme increased by cytokines such as TNF-α and has a role in the pathophysiology of CP-induced hemorrhagic cystitis [72].

8. Lung Toxicity

Cyclophosphamide showed a considerable decrease in body weight along with an increase in lung/body weight ratio [76]. This drop in body weight may be attributed to CP's harmful and degenerative effects [77]. However, the biochemical and histological findings indicate that the higher lung/body weight ratio may be caused by edema, fibrillation, or enhanced collagen formation [78].

In bronchoalveolar lavage fluid, CP raised the levels of total protein and LDH [76]. Elevated LDH and total protein levels signify microvascular leakage, airway cell influx, and damage to the airways and/or alveoli [79]. Also, the amount of neutrophils, lymphocytes, eosinophils, and basophils in bronchoalveolar lavage fluid was significantly increased by CP-injection. This impact is linked to the inflammatory cascades that occur following CP treatment [76].

In lung homogenates, CP caused a clear state of oxidative stress, as evidenced by a considerable increase in MDA levels and an obvious decrease in GSH and SOD levels [76].

Significantly elevated NO levels were found in CP intoxication. This rise in NO levels could result from iNOS overexpression [80]. The liberated nitric oxide may react with superoxide anions to generate peroxynitrite [81] and activate NF-κB to promote the generation of pro-inflammatory cytokines [82].

9. Conclusion

Because of its wide therapeutic efficacy, cyclophosphamide continues to be a mainstay in the treatment of numerous cancers and autoimmune diseases. Its active metabolites, which disrupt DNA replication and cause cell death, are

largely responsible for its therapeutic efficacy. However, cautious clinical monitoring is required due to the pharmacokinetic complexity and dose-dependent toxicity of cyclophosphamide. To maximize therapeutic results, a thorough grasp of its pharmacokinetics, toxicity profile, and mode of action is crucial. Strategies to increase efficacy while reducing side effects should be the main focus of future study.

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