

# Clinical Pharmacology of Cyclophosphamide and Ifosfamide

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**Abstract:** The oxazaphosphorine cyclophosphamide (CPA) and ifosfamide (IFO) are two commonly used DNA-alkylating agents in cancer chemotherapy. This review highlights the pharmacokinetics and pharmacodynamics of the two important agents. As alkylating agents, CPA and IFO are usually combined with other anticancer drugs in the chemotherapy of solid tumors and hematological malignancies to obtain synergistic or additive anticancer effect due to complementary mechanism of action. Both compounds are prodrugs that are activated *via* 4-hydroxylation by cytochrome P450s such as CYP2B6 and CYP3A4 to generate alkylating nitrogen mustards (phosphoramidate mustard and ifosforamide mustard) and the byproduct acrolein. The resultant mustards can alkylate DNA to form DNA-DNA cross-links, leading to inhibition of DNA synthesis and cell apoptosis. Both CPA and IFO are also inactivated by *N*-dechloroethylation, resulting in *N*-dechloroethylated metabolites and the byproduct chloroacetaldehyde. Acrolein is the causative agent for hemorrhagic cystitis, whereas chloroacetaldehyde induces nephrotoxicity and neurotoxicity. Pharmacokinetics of CPA and IFO is markedly influenced by route of administration and duration of treatment, age, comedication, liver and renal function. Large interpatient variability in pharmacokinetics, clinical response rate and toxicity has been observed in cancer patients treated with CPA or IFO. Resistance to CPA or IFO occurs due to decreased activation by CYP3A4 and CYP2B6, increased deactivation of the agents, decreased entry into or increased efflux from tumor cells, increased cellular thiol level, increased DNA repair capacity, and/or deficient apoptotic response to DNA damage. A full understanding of factors affecting the pharmacokinetics, pharmacodynamics, toxicology and pharmacogenetics of CPA and IFO is important to optimize the dose and regimens of CPA and IFO in cancer chemotherapy.

**Key Words:** Cyclophosphamide, ifosfamide, metabolism, pharmacokinetics, pharmacodynamics, toxicity, resistance.

## 1. INTRODUCTION

The oxazaphosphorine cyclophosphamide (2-[bis-(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide, Cytoxan, CPA) and its structural isomer ifosfamide (N,3-(bis(2-chloroethyl)-tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine 2-oxide, Ifex or Holoxan, IFO) are two widely used DNA-alkylating agents in cancer chemotherapy (Fig. (1)). CPA was shown to have antitumor activity in murine models in the early 1940s [1] and shortly after it was introduced to clinical cancer chemotherapy. IFO was developed in the middle of the 1960s and introduced in clinical practice in the early 1970s [2-5]. CPA and IFO are used as a single agent, but more frequently, in combination with other anticancer agents in management of a wide spectrum of solid tumors and hematological malignancies. The combined use of CPA or IFO with other anticancer drugs in the chemotherapy is intended to obtain synergistic or additive anticancer effect resulting from complementary mechanisms of action. Recently, CPA given at low doses has gained increased interest as either an antiangiogenic or an immunostimulatory agent in combination with immunotherapies in the treatment of cancer [6-10]. CPA is also used for the mobilization of hematopoietic progenitor cells from the bone marrow into peripheral blood [11-16]. Moreover, CPA has been widely used as an immunosuppressive drug in combination with other immunosuppressants such as prednisone, mycophenolic acid, or azathioprine to treat some autoimmune diseases including systemic lupus erythematosus (SLE) [17-23] and rheumatoid arthritis [24-27].

Both compounds are administered as prodrugs that require activation by hepatic cytochrome P450 (CYP)-catalyzed 4-hydroxylation, yielding cytotoxic nitrogen mustards capable of reacting with DNA molecules and leading to cell death [28-30] and the byproduct acrolein. Both CPA and IFO are also inactivated by *N*-dechloroethylation, resulting in *N*-dechloroethylated metabolites and the byproduct chloroacetaldehyde (CAA). CAA can cause neurotoxicity and nephrotoxicity and acrolein induces urotoxicity. The resultant cytotoxic metabolites and toxic byproducts are detoxified by various aldehyde dehydrogenases (ALDHs) and by conjugation with glutathione (GSH) *via* GSH *S*-transferases (GSTs).

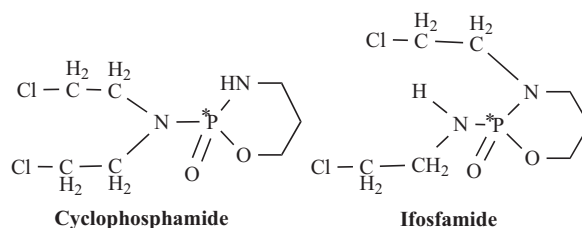


Fig. (1). Chemical structures of cyclophosphamide and ifosfamide.

Considerable interpatient variability in the pharmacokinetics of CPA and IFO has been observed. Pharmacokinetics of the two agents is markedly influenced by route of administration and duration of treatment, age, coadministered drugs, genetic factor, liver and renal function. As non-specific alkylating agents, both CPA and IFO have severe host toxicities [31]. Therefore, supportive therapies including stem cells, hematopoietic growth factors [e.g. granulocyte colony-stimulating factor (G-CSF) or granulocyte-macro-

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phage colony-stimulating factor (GM-CSF)], and the sulfhydryl compounds [e.g. mesna and amifostine (WR-2721)], are usually combined to protect the key organs of the host. The protective mechanism of mesna and WR-1065 [the active metabolite of WR-2721] involves the thiolate anions that participate in chemical reactions similar to those with GSH [32]. Although the metabolism and mechanism of action of CPA and IFO are similar, there are marked differences in metabolite formation, pharmacokinetics and toxicity profiles of these two agents. Although CPA is the most widely used alkylating agent, IFO appears more effective in a wide range of malignant diseases. Therefore, a better understanding of the pharmacological profile of CPA and IFO is important for their optimal use in cancer chemotherapy. This review highlights the clinical pharmacokinetics and pharmacodynamics of these two agents.

## 2. CLINICAL PHARMACOKINETICS OF CYCLOPHOSPHAMIDE AND IFOSFAMIDE

### 2.1. Absorption

Both CPA and IFO are soluble in water, saline or alcohol as a monohydrate and can be readily administered orally. Oral administration of CPA is usually given at low doses (75–200 mg/day) when used as an immunosuppressive agent and as a component in the CMF (CPA, methotrexate and fluorouracil) regimen of breast cancer [33, 34]. CPA is well absorbed and the peak concentration appears 1 hour following oral drug administration. The oral bioavailability of CPA is 85–100% [33–37], and a fraction of drug is metabolized due to the first-pass effect in the liver and gut. At higher doses (0.7 g/m<sup>2</sup>), CPA has an 87.7% oral bioavailability [38].

Similarly, oral IFO is rapidly absorbed through gastrointestinal tract with an oral bioavailability of almost 100% [39–45]. The maximum concentration is reached within 1 to 2 hours after oral administration of IFO [39, 46, 47]. However, oral administered IFO resulted in unacceptable incidence of neurotoxicity [39, 48], which probably resulted from a shift in metabolic pathways towards dechloroethylation with increased formation of neurotoxic CAA compared with after intravenous administration [39, 44].

### 2.2. Distribution

After oral or intravenous administration, CPA is rapidly distributed throughout the body with ~20% (0–30%) plasma protein binding, whereas the ability of protein binding is higher for its metabolite, 4-hydroxy-CPA (<67%) [49]. CPA is not structurally modified by blood plasma. The volume of distribution ( $V_d$ ) of CPA is increased in obese patients, leading to an increased elimination half-life ( $t_{1/2\beta}$ ) of CPA [50]. Several studies suggested that CPA entered into cerebrospinal fluid through blood-brain barrier (BBB) with varying cerebrospinal fluid to plasma ratios from 0.2 to 4 [51–53]. The active metabolites of CPA have limited penetration into the brain due to their increased polarity and higher plasma protein binding [53]. This may contribute to the lack of neurotoxicity associated with the intravenous administration of CPA.

The distribution of IFO is more extensive with lower plasma protein binding compared with CPA [54]. Some

studies in obese patients showed a longer  $t_{1/2\beta}$  for IFO due to the increased  $V_d$  in obese patients [55]. Like CPA, IFO and its active metabolite 4-hydroxy-IFO can pass the BBB and reach cerebrospinal fluid [52, 56]. The concentrations of IFO in cerebrospinal fluid are almost as high as those in plasma and the majority of metabolites of IFO can be measured in cerebrospinal fluid [52, 56]. This may be associated with the neurotoxicity commonly encountered in cancer patients treated with IFO.

CPA and IFO and their active metabolites are extensively bound by erythrocytes, which may serve as transporters of activated CPA and IFO metabolites [57–61]. The protoxic metabolite of CPA, 4-hydroxy-CPA, is trapped intracellular and transported to tumor tissues. For IFO, it is believed that the cytotoxic ifosforamide mustard is the major transport form of IFO by erythrocytes, because the antitumor activity and selectivity of extracellular delivered ifosforamide mustard were comparable with those of 4-hydroxy-IFO [62]. The erythrocytes from IFO-treated patients contained 77% of the total contents of ifosforamide mustard in whole blood [58]. These findings indicate that monitoring of erythrocyte concentrations of active CPA and IFO metabolites is of importance, as the data can have a predictive value for therapeutic efficacy.

### 2.3. Transport

There are increasing data on the transport of CPA and IFO and their metabolites across cellular membrane. Both CPA and IFO are highly hydrophilic and do not diffuse readily through the lipid bilayer of cells. Similarly, the phosphoramidate mustard and ifosforamide mustard, the respective cytotoxic but unstable metabolites of CPA and IFO, bear a negative charge with  $pK_a$  of 4.5–4.8 at physiological pH and are thus relatively difficult to pass the cellular membrane. 4-OH-CPA and 4-OH-IFO are the corresponding circulating metabolites that enter tumor cells to form ultimate cytotoxic phosphoramidate mustard, ifosforamide mustard and the byproduct acrolein. It appears that the 4-OH-CPA, 4-OH-IFO, and acrolein can readily cross the cell membrane by passive diffusion *in vitro*. However, active transport cannot be excluded for CPA, IFO and their metabolites based on resistance and transport studies.

The GSH conjugate of imino-CPA (GSCY) has been found to be actively excreted into the bile *via* the multidrug resistance associated protein (MRP2) in hepatocytes [63]. It is well known that MRP2 exports organic anions, including glucuronide, glutathione, and sulphate conjugates into bile [64, 65]. The MRP2-mediated biliary excretion of GSCY appears to compete with oxidative pathway of 4-OH-CPA to non-toxic *O*-carboxyethylcyclophosphoramidate mustard (carboxyphosphamide, CEPM) and  $\beta$ -elimination to form acrolein and phosphoramidate mustard, as reduced MRP2 activity resulted in greater hepatic exposure to GSCY and less formation of acrolein and phosphoramidate mustard [63]. In addition, a recent *in vitro* study at our laboratory found that overexpression of MRP4 in the human hepatoma cell line HepG2 cells conferred significant resistance to CPA and IFO [66]. Preincubation of MK-571, celecoxib, or DL-buthionine-(*S,R*)-sulphoximine (BSO) significantly reversed

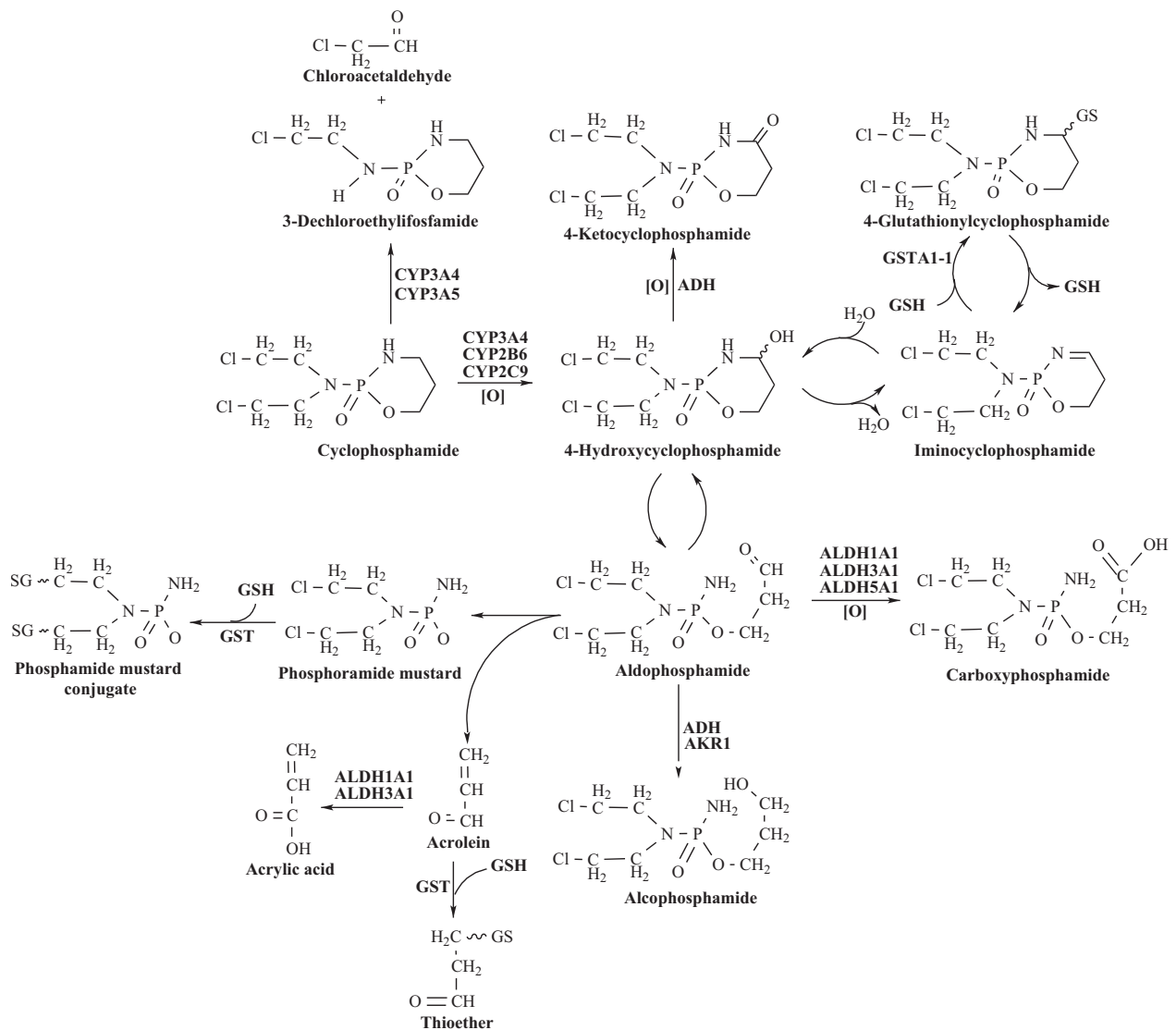
the resistance. MK-571, celecoxib and BSO are all known MRP4 inhibitors [65, 67]. These results indicate that both CPA and IFO are potential substrates for MRP4.

However, it appears that P-glycoprotein (PgP) plays a little role in the transport of CPA and IFO and their metabolites based on *in vitro* resistance studies. Exposing tumor cells to CPA, IFO or 4-OH-CPA generated significant acquired resistance. Unlike those natural product anticancer drugs, resistance to CPA and IFO is not associated with the multidrug resistance (MDR) phenotype or markedly elevated

expression of PgP, although cross-resistance with cisplatin and other alkylating agents has been observed [68-70]. This so-called MDR phenomenon is not seen with CPA and IFO. This indicates that the two oxazaphosphorines are not typical substrates for PgP.

#### 2.4. Metabolism

The metabolic pathways of CPA in humans are shown in Fig. (2). As a prodrug, CPA is converted to active alkylating mustard by the liver in the body [71]. The initial activation



**Fig. (2).** Metabolism of cyclophosphamide (CPA). CPA is activated by hepatic CYP2B6, CYP3A4 and CYP2C9 to form 4-hydroxycyclophosphamide (4-OH-CPA), which enters blood and is transported to tumor cells by erythrocytes. 4-OH-CPA is in equilibrium with its tautomer aldophosphamide that can decompose by  $\beta$ -elimination to form cytotoxic phosphoramidate mustard and the byproduct acrolein. Acrolein is detoxified by conjugation with glutathione (GSH). Alternatively, 4-hydroxycyclophosphamide is detoxified to form *O*-carboxyethylcyclophosphamide mustard (CEPM, namely, carboxyphosphamide) primarily by aldehyde dehydrogenase (ALDH1A1) and, to much lesser extent, by ALDH3A1 and ALDH5A1. It is also oxidized by alcohol dehydrogenase (ADH) to form 4-ketocyclophosphamide. Furthermore, 4-OH-CPA undergoes reversible dehydration to form iminocyclophosphamide that is further conjugated with intracellular GSH, giving rise to 4-glutathionylcyclophosphamide (GSCY), a substrate for multidrug resistance associated protein 2. Moreover, CPA can be converted to 3-dechloroethylifosfamide by CYP3A4-catalyzed *N*-dechloroethylation with the production of the byproduct chloroacetaldehyde (CAA).

of CPA is 4-hydroxylation at C<sup>4</sup> of oxazaphosphorine ring to form 4-OH-CPA [72]. Multiple CYP enzymes including CYP2B6, CYP2C9 and CYP3A4 in the liver are responsible for CPA 4-hydroxylation [29, 73-75]. CYP2B6 is the major contributor (a mean of ~45% of total metabolism) for the activation of CPA with the highest intrinsic clearance *in vitro* and *in vivo*, compared with 25% and 12% for CYP3A4 and CYP2C9, respectively [29, 75, 76]. Other CYPs including CYP2A6, CYP2C8 and CYP2C19 also make a minor contribution to CPA 4-hydroxylation [73]. By contrast, the inactivation pathway of CPA involves minor (~10%) side chain oxidation (*N*-dechloroethylation) primarily by CYP3A4/3A5 and, to a minor extent, by CYP2B6 to generate 3-dechloroethyl-IFO and the neurotoxic and nephrotoxic byproduct CAA [74].

4-OH-CPA is a major circulating metabolite of CPA that enters tumor cells and decomposes through its tautomer aldophosphamide (an aldehyde intermediate) by spontaneous  $\beta$ -elimination to form ultimate cytotoxic phosphoramidate mustard (*N,N*-bis-2-(2-chloroethyl)phosphorodiamidic acid) and an equimolar amount of the byproduct acrolein (a highly electrophilic  $\alpha,\beta$ -unsaturated aldehyde) [77-79]. Alternatively, aldophosphamide can be oxidized by alcohol dehydrogenase (ADH) and aldo-keto reductase (AKR1) to generate alcohosphamide. Alternatively, 4-OH-CPA is detoxified to CEPM by cytosolic ALDH1A1, and to a much lesser extent, by ALDH3A1 and ALDH5A1 [80-89]. ALDH catalyzes the conversion of a broad range of aldehydes to the corresponding acid *via* NAD<sup>+</sup>-dependent irreversible reaction. Furthermore, 4-OH-CPA is oxidized by ADH to non-toxic 4-keto-CPA [87, 88, 90-93], but to a much lesser extent compared with CEPM formation. Moreover, 4-OH-CPA undergoes reversible dehydration to form iminocyclophosphamide that is further conjugated with intracellular GSH by GSTA1, A2, M1, and P1, giving rise to non-toxic GSCY [94, 95].

The resultant phosphoramidate mustard is a bifunctional alkylator of DNA and the ultimate cytotoxic metabolite of CPA [96]. The alkylation involves generation of the intermediate phosphoramidate aziridinium ion through an intramolecular nucleophilic attack (cyclization reaction) of the nitrogen on the  $\beta$ -carbon of a chloroethyl chain [97]. Cellular thiols (e.g., GSH) and other nucleophiles react rapidly with phosphoramidate aziridinium ions, resulting in thioether products [98]. CEPM is one of the major chemically stable metabolites of CPA, which are easily detected in patient plasma and urine [99]. However, acrolein is a highly reactive aldehyde that covalently binds to cellular macromolecules and subsequently disrupts the function and causes organ toxicity [100, 101]. It is detoxified by conjugation with GSH *via* GSTs in hepatocytes [102] and this may cause intracellular GSH depletion and injuries of the hepatocytes [103]. Reaction of GSH with acrolein is *via* nucleophilic addition at the  $\beta$ -carbon atom, generating stable thioether compounds [104, 105].

The metabolism of IFO is similar to that of CPA, but there are some differences in the extent of formation of certain metabolites, because IFO differs structurally from CPA in the position of one chloroethyl group (Fig. (3)). Like CPA, IFO is activated *via* 4-hydroxylation by CYP2B6 and

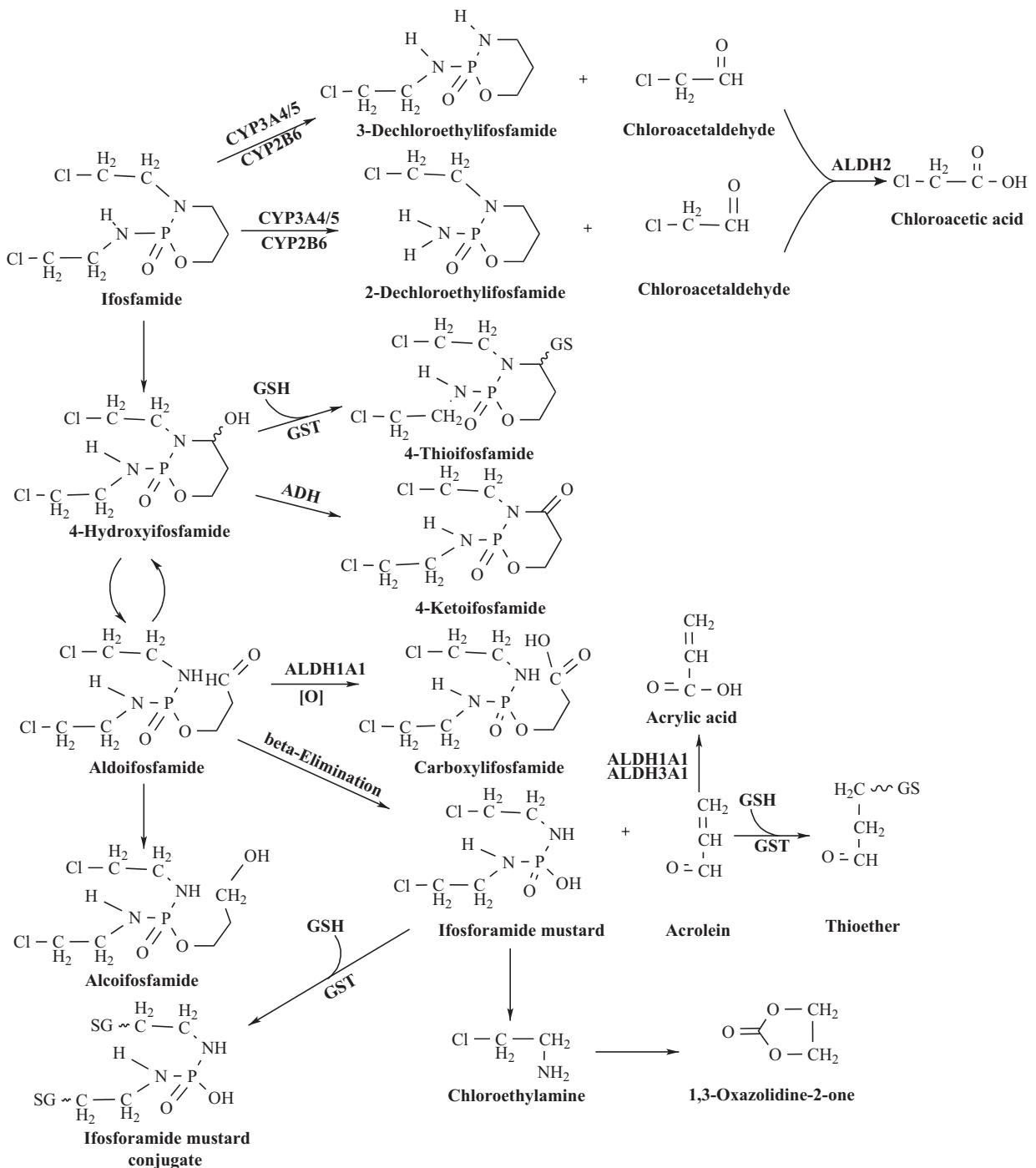
CYP3A4 to form cytotoxic ifosforamide mustard and the byproduct CAA [54, 73, 106-109]. Ifosforamide mustard is the final alkylating metabolite of IFO, which is able to form covalent linkage with electrophilic residues of DNA molecule and cause cross-links between DNA strands [110]. Ifosforamide mustard is also converted to chloroethylamine and 1,3-oxazolidine-2-one, resulting from degradation [111]. Like CPA, 4-OH-IFO is metabolized to its inactive metabolites 4-keto-IFO by ADH and 4-thio-IFO with conjugation of reduced GSH *via* GSTs [112]. Alternatively, the tautomer of 4-OH-IFO aldoifosfamide can be converted into ifosforamide mustard with concurrent formation of acrolein by spontaneous  $\beta$ -elimination and to the inactive metabolite carboxyifosfamide by ALDHs. In addition, aldoifosfamide can be oxidized by ADH and AKR1 to generate non-toxic alcoifosfamide.

Although CPA is almost completely converted to its active 4-hydroxy-metabolite in humans, 25-60% of IFO is metabolized to CAA through *N*-dechloroethylation [71, 113-117]. The inactivation of IFO involves removal of a chloroethyl group from either endo- or exocyclic nitrogen atom to generate non-toxic 3-dechloroethyl-IFO and 2-dechloroethyl-IFO, respectively [71, 109, 118]. Activation and deactivation *via* dechloroethylation of IFO have been proved to be catalyzed by various CYPs [109, 118]. CYP3A4 has been demonstrated to be responsible for both 4-hydroxylation and dechloroethylation [73, 76, 119]. CYP3A5 is involved in the dechloroethylation of IFO and mutations of CYP3A5 affect the metabolism rate [120]. CYP2B6 also significantly contributes to the activation and dechloroethylation of IFO [75].

The liver is the primary organ for the metabolism of both CPA and IFO through which the drugs are activated and eliminated, but metabolism may occur in other sites, including the erythrocytes [80], kidneys [121] and tumor itself [122]. Renal metabolism by CYPs is considered associated with the neurotoxicity of IFO [121]. Various CYPs including CYP1A1, 2A6, 2B6, 2C8/9 and 3A4 are present in a variety of tumors, including those from the central nervous system, breast, colon, lung, ovarian, prostate, and kidney, but their relative levels compared with normal tissue are less [123-125]. Since CYP3A4 and CYP2B6 are the major enzymes for CPA/IFO activation, their intratumoral level may be a useful predictive marker for the efficacy of CPA and IFO treatment.

## 2.5. Auto-Induction

The metabolism of both CPA and IFO is an auto-inducible process [126-136]. Auto-induction results in an increase in the total clearance, increased formation of 4-OH-metabolites, and shortened  $t_{1/2/\beta}$  values, following repeated administration at 12- to 24-hour interval [31, 109, 137]. Auto-induction of IFO and CPA metabolism has been observed within 12 to 24 hours after the start of treatment [134, 136]. Auto-induction is typically observed between day 1 and day 5, but metabolic rates are always reset to initial baseline values at the start of a consecutive course 3 weeks later [138]. Auto-induction of CPA and IFO metabolism exhibits marked and probably unpredictable interpatient variability, both in its incidence and in the



**Fig. (3).** Metabolism of ifosfamide. The metabolism of ifosfamide is similar to that of CPA, but there are some differences in the formation of metabolites as ifosfamide differs structurally from CPA in the position of one chloroethyl group. As a prodrug, ifosfamide is activated *via* 4-hydroxylation to form 4-hydroxyifosfamide which is inactivated to form 4-ketoifosfamide by alcohol dehydrogenase (ADH) and 4-thioifosfamide with conjugation of reduced glutathione. The tautomer of 4-hydroxyifosfamide, aldoifosfamide, can be converted to carboxyifosfamide by ALDH1A1 and alcoifosfamide by aldo-keto reductase (AKR1). Alternatively, aldoifosfamide can decompose to generate cytotoxic ifosforamide mustard with concurrent formation of acrolein by spontaneous  $\beta$ -elimination. Notably, cyclophosphamide is almost completely converted to its active 4-hydroxy-metabolite in humans, whereas 25-60% of ifosfamide is metabolized to chloroacetaldehyde through 2- and 3-dechloroethylation.

magnitude of its effect, in particular when high dosage is used [139]. In the absence of a corresponding increase in the formation of inactive metabolites, time-dependent increase in CPA or IFO metabolism may be of potential clinical benefit.

Prolonged infusions of CPA or IFO would also prevent the development of high peak concentrations, thus reducing possible saturation of metabolizing enzymes when high-dose regimens are used.

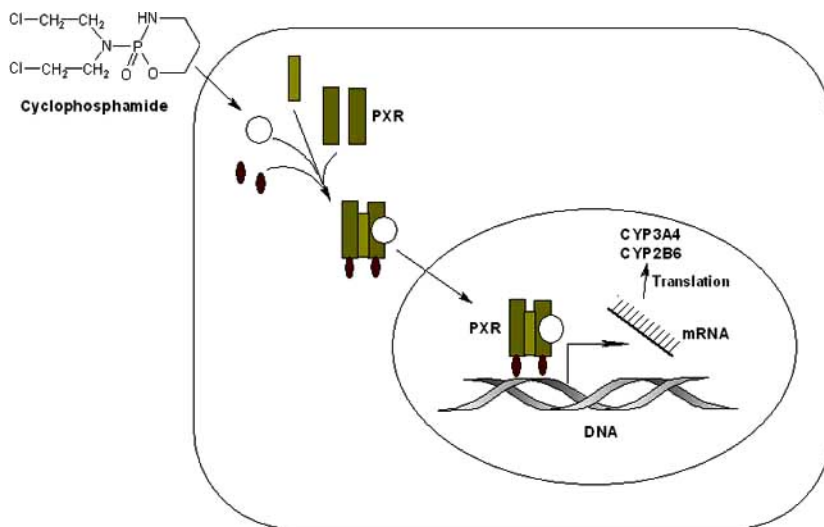
The rate and magnitude of auto-induction of CPA and IFO metabolism are influenced by dose, dosing schedule, comedication and other factors (e.g. genetic factors). A time-dependent increase in CPA metabolism was not observed in patients treated with CPA as a 120-hour infusion [140]. By contrast, auto-induction was found in 87% patients receiving a 96-hour infusion of CPA [127]. Drug-induced enzyme induction is dose-dependent, thus the lack of auto-induction in one study may reflect the smaller administered dose (2 vs 6 g/m<sup>2</sup>) [141]. The metabolic rate of IFO due to auto-induction was 52% lower with long infusion (24-72 hours) compared with short infusion (1-4 hours) [142]. This difference was, however, comparable with its interindividual variability (22%) and was thus considered to be of little clinical importance. Auto-induction caused a less proportional increase in the area under the plasma concentration-time curve (AUC) of IFO and more than proportional increase in metabolite exposure with increasing IFO dose [142]. Following long-infusion treatment, the dose-corrected exposures (AUC/D) of IFO were significantly decreased but 3-dechloroethyl-IFO level was increased compared with short-infusion regimen [142]. Following dose escalation of CPA, dividing the high dose over 2 days instead of one, single infusion may favorably impact the metabolism of CPA in terms of increased activation [143].

Coadministered drugs can also modulate the auto-induction process. Aprepitant (a neurokinin-1 receptor antagonist) abolished the reduced clearance of CPA and reduced the formation of active metabolite [144]. Rifampin was a particularly potent inducer of IFO and CPA 4-hydroxylation *in vitro* [145]. Carbamazepine significantly increased the CL of CPA and IFO in pediatric patients [130]. However, CPA auto-induction is less marked in patients with prior treatment of phenytoin [127, 146]. This may be due to the auto-induction was secondary to maximal prior CYP induction by the anticonvulsant. On the other hand, administration of CPA during conditioning in transplant

recipients reduced the serum cyclosporine concentrations during the 2 weeks following hematopoietic stem cell transplantation, probably resulting from induction effect of CPA or its metabolites on CYP3A4 [147].

The auto-induction is due to an increased expression of CYP3A4 and CYP2B6 that metabolize CPA and IFO [145]. The mechanism for the auto-induction of CPA metabolism has been initially identified based on several *in vitro* studies using cultured primary human hepatocytes [145, 148, 149] or cultured human liver slices [150]. Activation of the orphan nuclear receptor pregnane X receptor (PXR) is considered the predominant mechanism for the inducing effect of CPA on CYP2B6, CYP3A4 and CYP2C9 in human hepatocytes (Fig. (4)) [148]. PXR has been identified as the primary transcriptional regulators of drug-induced CYP3A4 and CYP2B6 expression [151-156]. CPA has been shown to induce CYP2C9 and CYP3A4 expression in a concentration-dependent manner in cultured primary human hepatocytes [145, 148, 149]. Exposure of cultured human hepatocytes to 250 μM CPA resulted in increased CYP3A4-mediated 6β-hydroxylation of testosterone and CYP3A4 protein level in human hepatocytes [145]. However, there was no increase in CYP2B6 protein with this same treatment of CPA. In contrast, treatment of primary human hepatocytes with CPA at 1 mM caused induction of CYP2B6 at both protein and mRNA levels [149]. Additionally, CPA induced the expression of CYP3A4 and CYP2B6, but not CYP2C9, in human liver slices [150]. In contrast, CPA treatment for up to 72 hours did not induce CYP2B6, CYP3A4, CYP2C9 and CYP2C19 at mRNA and protein in HL-60 cells, a human acute promyelocytic leukemia cell line [157], suggesting that the CYP inducibility of CPA is poor in blood cells.

By using transient transfection assay, CPA treatment caused a dose-dependent increase in PXR activation that was maximal at the highest CPA concentration studied (500 μM) [148]. The addition of dexamethasone to CPA resulted in a



**Fig. (4).** Induction of CYP3A4 and CYP2B6 by cyclophosphamide through pregnane X receptor (PXR) activation in human hepatocytes. Cyclophosphamide enters hepatocytes and binds to the cytosolic PXR, producing a complex with recruitment of other functional proteins. The ligand-PXR complex translocates and into the nucleus and binds to pregnane response element in DNA, leading to the initiation of the expression of *CYP3A4* and *CYP2B6*.

minor increase in PXR activation compared with CPA alone [148]. However, the dose response for PXR activation by CPA does not appear to be sufficient to explain entirely on the extent of induction that is observed in primary human hepatocyte cultures. This could be due to a number of factors including the involvement of other nuclear receptors such as constitutive androstane receptor or the formation of active metabolites of CPA that are more efficacious activators of PXR in primary cell culture systems.

Other mechanisms such as increased stability of mRNA, reduced CYP degradation and increased enzyme activity by allosteric binding possibly contribute to auto-induction. IFO has been shown to decrease protein synthesis [158] and as such, may reduce synthesis of proteases responsible for CYP3A4 degradation, resulting in decreased IFO clearance. The ability of IFO to reduce protein synthesis and the rapid rate of auto-induction suggest that this mechanism of auto-induction is the most viable option. In addition, CPA and IFO and their metabolites may bind to a non-catalytic site of a CYP protein, resulting in increased catalytic activity because of an altered spin state of the hemoprotein of the CYP. However, further study is warranted to reveal the evidence for these hypotheses.

## 2.6. Excretion

Both CPA and IFO are primarily (70%) excreted in urine in forms of metabolites and to a less extent, in the feces [109]. However, only ~10-20% is excreted unchanged in the urine [159, 160] and only 4% is excreted in the bile following CPA or IFO administration [161]. CEPM is the dominant inactive metabolite of CPA found in the urine, while 4-keto-CPA is only a minor component in patient urine (<1%) [162]. The renal clearance of CPA in cancer patients was reported to be 15–44 ml/min [127, 163, 164]. The reported renal clearance of CPA metabolites including 4-OH-CPA, dechloroethylifosfamide, keto-CPA, and CEPM in cancer patients is  $60.6 \pm 9.0$ ,  $3.2 \pm 1.0$ ,  $1.3 \pm 0.8$  and  $7.0 \pm 4.5$  ml/min, respectively [164]. The renal clearance of IFO, isophosphoramidate mustard, 2-*N*-dechloroethyl-IFO, and 3-*N*-dechloroethyl-IFO is  $3.5 \pm 0.6$ ,  $24.7 \pm 7.4$ ,  $1.3 \pm 2.6$ , and  $8.7 \pm 6.9$  ml/min, respectively [165]. These values are lower than the glomerular filtration rate (~125 ml/min). During high-dose therapy of CPA (100 mg/kg), the amount of CPA excreted in urine is correlated with the urine flow, whereas this correlation does not exist during conventional dose therapy (500 mg/m<sup>2</sup>) [164]. However, there was no correlation between the renal excretion of any metabolites of CPA and urine flow during either conventional or high dose therapy [164]. These findings indicate that the renal clearance of IFO and its metabolites is lower than creatinine clearance, suggesting a substantial part of tubular reabsorption of IFO and its metabolites when they go through the renal tubules [44, 163]. Urine flow can have a substantial effect of the renal clearance of CPA or IFO that are substantially reabsorbed. Generally, there is no correlation between body clearance, non-renal clearance or renal clearance of IFO and age [137].

The majority of CPA and IFO elimination is by metabolic transformation with the metabolites recovered from the urine and feces. Thus, liver impairment may have

an impact on pharmacokinetics and disposition of CPA and IFO. However, such liver impairment only leads to less production of aldophosphamide; fewer adverse effects were observed in patients with liver dysfunction [166]. Thus, it is not recommended to adjust the dosage of CPA in patients with liver dysfunction.

## 2.7. Pharmacokinetic Parameters and Modelling

A number of pharmacokinetic studies have been conducted with CPA and IFO and the pharmacokinetic parameters reported. The  $t_{1/2\beta}$  of CPA ranges from 3.2–7.6 hours with total body clearance (CL) values of ~2.5 to 4.0 L/h/m<sup>2</sup> (Table 1) [109]. CPA metabolism is probably more rapid in children as a result of increased CYP activity [163]. For IFO, the  $t_{1/2\beta}$  values are 2.1–8.6 hours with clearance of 2.1–5.1 L/h/m<sup>2</sup> [137]. Following intravenous administration, the  $V_d$  of both CPA and IFO approximates the total body water volume (30–50 L), suggesting that distribution of the two agents takes place with minimal tissue binding.

Different modeling methods have been used to describe the pharmacokinetics of CPA and its metabolites. At doses of CPA used in clinical practice, no dose-dependent pharmacokinetics has been found. A model-independent approach or one-compartment model is sufficient to describe the pharmacokinetics of CPA at conventional doses over short infusions in patients with Hodgkins lymphoma [167] or ovarian carcinoma [168]. When CPA was administered at 4.0 g/m<sup>2</sup> over a 90-min infusion in patients with metastatic breast cancer, one-compartment model with Michaelis-Menten saturable elimination in parallel with first-order renal elimination can adequately describe the kinetics of CPA [127]. However, Michaelis-Menten elimination was not apparent in the second course because the plasma concentration of CPA was much lower [127]. More complex models involving time- and concentration-dependent pharmacokinetics have been proposed for prolonged or high dose administration of CPA [127, 143, 164, 169, 170]. This mechanism-based model used a hypothetical enzyme compartment to estimate the effects of auto-induction on the pharmacokinetics of CPA and its metabolites. While these more complex models apply specifically to the concentration of the parent drug in plasma, they also have implications for the systemic formation of active and inactive metabolites. The combination of auto-induction and non-linear metabolism of IFO and CPA needs complex models with incorporation of more parameters to describe its pharmacokinetics. In addition, population pharmacokinetic approach has been used to described the plasma concentration-time profiles of both CPA and 4-OH-CPA [170, 171].

For IFO, one- and two-compartment models have been used to properly describe plasma concentration-time profiles of IFO following short-duration infusions (1–3 hours) [44, 54, 172]. Model-independent approach was also employed for short infusion regimen [107]. However, these methods did not take into account the effects of auto-induction on the pharmacokinetics of IFO. Prasad *et al.* [173] and Boddy *et al.* [174] reported models that enabled more adequate description of the concentration-time data for IFO infusions of longer duration up to 72 hours. Their models included a lag-time before the development of auto-induction and

**Table 1. Clinical Pharmacokinetic Parameters of Cyclophosphamide and Ifosfamide in Patients**

n of patients	Dose	t <sub>1/2β</sub> (h)	AUC (mmol/L·h)	CL (L/h/m <sup>2</sup> )	V <sub>d</sub> (L/kg)	f <sub>renal</sub> (%)	Ref.
<b>Cyclophosphamide</b>							
23	g/m <sup>2</sup> (i.v)	6.2	1.6 ± 0.3	2.5 ± 0.5	0.57 ± 0.08	13.9 ± 6.2	[483]
16	0.15–0.4 g/m <sup>2</sup> (i.v)	7.6	NR	2.4	0.52	NR	[50]
4	1.6 g/m <sup>2</sup> (i.v)	5.5	2.4 ± 0.2	2.6 ± 0.2	0.52 ± 0.01	12.1 ± 2.8	[483]
38	0.37–2.49 g/m <sup>2</sup> (i.v)	3.2 (1.1–16.8)	NR	2.9 (1.2–10.6)	0.63 (0.26–1.48)	NR	[163]
12	0.8 g (i.v) 5.9 g (i.v)	4.8 ± 1.1 4.8 ± 1.4	0.7 ± 0.1 6.0 ± 1.0	2.7 ± 0.35 2.7 ± 0.43	0.49 ± 0.08 0.45 ± 0.12	14.9 ± 3.7 22.9 ± 5.9	[164]
13	0.6–1.0 g/m <sup>2</sup> (1-h i.v) 0.6–1.0 g/m <sup>2</sup> (24-h i.v)	2.6 (1.2–7.9) 4.5 (1.9–7.1)	NR	3.1 (1.4–6.2) 5.1 (1.7–12.7)	0.48 (0.12–0.78) 0.63 (0.08–1.7)	NR	[134]
19	1.9–2.7 g/m <sup>2</sup>	8.7 ± 4.6 (1 <sup>st</sup> dose) 3.6 ± 0.9 (2 <sup>nd</sup> dose)	3.1 ± 1.1 (1 <sup>st</sup> dose) 1.6 ± 0.7 (2 <sup>nd</sup> dose)	NR	NR	16	[159]
<b>Ifosfamide</b>							
15	5.0 g/m <sup>2</sup> (24-h i.v)	4.7 ± 2.0	5.4 (3.5–8.7)	3.5 ± 0.9	0.56 ± 0.22	14.4 (5.3–19.4)	[108]
7	1.5 g/m <sup>2</sup> (0.5-h i.v)	7.0 ± 2.6	2.4 ± 0.7	3.8 ± 0.9	0.62 ± 0.17	NR	[138]
14	g/m <sup>2</sup> (1-h i.v) 3.0 g/m <sup>2</sup> (24-h i.v)	NR	6.9 ± 2.0 7.0 ± 4.4	NR	NR	21 ± 9 25 ± 14	[484]
9	2.0–3.0 g (i.v.) 2.0–3.0 g (p.o)	NR	2.3 ± 0.3 2.9 ± 0.8	2.1 ± 0.9 1.8 ± 0.8	NR	5.3 ± 1.6 4.9 ± 2.7	[41]
17	9.0 g/m <sup>2</sup> (1-h i.v) 9.0 g/m <sup>2</sup> (72-h i.v)	3.2 ± 1.5 2.1 ± 0.7	6.2 (4.8–12.2) 6.4 (2.3–10.9)	3.8 ± 1.1 5.5 ± 2.7	1.1 ± 0.4 0.7 ± 0.4	19.6 (7.5–42.3) 15.4 (9.8–25.8)	[485]
8	1.8 g/m <sup>2</sup> × 5 d (i.v)	5.2 (4.4–8.8)	2.0 (1.1–2.7)	3.4 (2.0–6.2)	0.66 (0.41–1.41)	NR	[135]
6	0.765–1.1 g/m <sup>2</sup> (10-day i.v)	4.6 ± 0.6	0.50 ± 0.10	2.7 ± 0.8	0.84 ± 0.27	9.0 ± 4.0	[486]
14	1.2–1.355 g/m <sup>2</sup> (10-day i.v)	4.0 ± 1.4	0.53 ± 0.10	2.8 ± 0.7	0.80 ± 0.22	8.0 ± 2.0	[486]
3	1.416–1.562 g/m <sup>2</sup> (10-day i.v)	4.0 ± 0.4	0.53 ± 0.19	2.5 ± 0.4	0.91 ± 0.24	10 ± 2.0	[486]

\*Patients were assumed to have a 70-kg body weight or 1.73-m<sup>2</sup> body surface as appropriate. Data are presented as mean ± SD or median & the range.

AUC = area under the concentration-time curve; CL = total body clearance; f<sub>renal</sub> = fraction of urinary excretion of unchanged drug; NR = Not reported; t<sub>1/2β</sub> = elimination half-life; V<sub>d</sub> = volume of distribution.

properly described the increase of clearance of IFO over time. Population pharmacokinetic approach has also been used to describe the kinetics of IFO in patients with resistant small-cell lung cancer [128]. This modeling approach did not require a lag-time to describe the concentration-time profile of IFO.

## 2.8. Pharmacokinetic Variability

Large interpatient variability in clinical response rate and toxicity has been observed in cancer patients treated with CPA and IFO. This may be explained by differences in the pharmacokinetics of both agents observed in cancer patients [137, 163, 175, 176]. Interpatient variability is often greater

than inpatient variability. Pharmacokinetic parameters of the parent drug vary less (coefficient of variation, 10–30%) than those for the elimination parameters (coefficient of variation, 14–64%) [164]. A 2 to 15-fold variability in the AUC, t<sub>1/2β</sub> and/or CL of CPA, IFO and their metabolites has been reported in cancer patients [137, 163, 170, 175, 176]. Larger variability in the pharmacokinetics of metabolites of CPA was found compared to the parental drug. Such considerable pharmacokinetic variability is considered to arise from differences in the expression of the individual CYPs (in particular CYP3A4, CYP2C9 and CYP2B6) that metabolize CPA [91, 177]. The contribution of CYP2B6 to CPA 4-hydroxylation (activation pathway) has been shown to vary significantly among liver samples due to differences

in the expression of CYP2B6 protein levels in individual livers [75, 178]. A 20- to 250-fold variability in CYP2B6 expression has been reported [179, 180]. In addition, there is a significant variation in the hepatic expression of CYP3A4 based on *in vitro* (35-100-fold) studies using human liver bank [181] and *in vivo* (20-50-fold) using probe drugs such as erythromycin [182] and midazolam [183], and alfentanil [184]. Such a substantial variation is considered to be the result of a number of environmental, physiological and genetic factors [185].

Interindividual variations in the metabolism, transport, distribution and disposition of CPA and IFO can be caused by a number of factors associated with the drugs (e.g. dosage, dosing regimen, route of administration, and drug combination) and patients (e.g. age, gender, renal and hepatic function, and genetic factor).

Dosage and dosing regimen are important factors affecting the pharmacokinetics of CPA and IFO. There is increased interest in the use of high dose of CPA or IFO in cancer chemotherapy. To increase chemotherapy efficacy against human cancer, it is desirable to increase dose intensity to the maximum tolerated dose. High dose oxazaphosphorine chemotherapy is assumed to result in improved antitumor activity due to increased generation of cytotoxic mustards. Moderate and high dose CPA, doxorubicin, and fluorouracil within the standard range result in greater disease-free and overall survival than the low dose regimen [186]. Higher doses ( $>9.0 \text{ g/m}^2$ ) of CPA are usually administered intravenously in either 5% dextrose or 0.9% saline. The drug is often given in a single dose over a period of up to 1 hour, repeated every 3 to 4 weeks.

However, despite the use of myeloid growth factor and sulfhydryl compounds (e.g. mesna and amifostine), myelosuppression continues to be a dose-limiting toxicity of oxazaphosphorines. At higher doses used prior to marrow transplantation, the dose-limited toxicity is cardiac toxicity. Besides cardiac toxicity, hemorrhagic cystitis, water retention and hyponatremia are found in patients receiving high dose CPA. Nephrotoxicity is mostly found in children and is the major dose-limiting factor for IFO administration in children. Importantly, IFO is relatively well tolerated but it can also be associated occasionally with life-threatening complications such as arrhythmias, heart failure, and severe encephalopathy. Mesna administration cannot control these toxicities. Other preventive measures, such as amifostine or methylene blue administration, have not yet been adequately evaluated in a sufficient number of patients. Clinicians should be watchful for early signs of severe toxicity in order to discontinue IFO high dose administration soon enough to avoid occurrence of major or life-threatening toxicity.

High-dose regimens have led to concerns over the existence of dose-dependent pharmacokinetics, with an increase in the production of inactive metabolites as the predominant activation pathway of metabolism is saturated. The degree of renal excretion and inactive metabolite formation was increased at higher dose of CPA, associated with a relative decrease in the formation of the active metabolite [164, 187]. Saturation kinetics at doses of 1 and  $4 \text{ g/m}^2$  of CPA has been observed in cancer patients [127]. As

to IFO, saturation of individual pathways of metabolism may also occur at high doses, although the pharmacokinetics of the parent drug has been shown to be dose-independent up to  $18 \text{ g/m}^2$  [117].

Dosing schedule has significant effect on the pharmacokinetics of CPA and IFO. In pediatric treatment or the highest doses given prior to bone marrow transplantation, the total dose can be fractionated over several days. A single high dose schedule of IFO tends to generate more myelosuppression than fractionated dose schedules. There is no obvious evidence that benefits can be resulted from the prolonged intravenous infusions of CPA [188].

Age is an important factor affecting the pharmacokinetics of CPA and IFO. Elderly patients with non-small cell lung cancer demonstrated a doubled  $t_{1/2\beta}$ , because of an increased  $V_d$  when total body, renal and non-renal clearance remained unaltered [55]. Pediatric patients are a specific group of individuals, because they have distinct physiological features from adults. The  $t_{1/2\beta}$  of CPA has been shown to be shorter than in adults [163]. The differences in body surface area will lead to larger variability of CPA or IFO doses, which makes comparison between pharmacokinetic parameters more difficult. Since  $V_d$  of both CPA and IFO approximates that of body water, the  $V_d$  in children is significantly lower than in adults.

Since CPA is primarily (70%) excreted in urine, renal function may play a role in the pharmacokinetic variability of CPA. Early studies by Bramwell *et al.* [189] and Juma *et al.* [190] found that alterations in renal function did not significantly alter the pharmacokinetics of CPA in patients, and did not result in any clinically-relevant changes in response rate and toxicity. However, a later study by Haubitz *et al.* [191] indicated that the clearance of CPA was decreased in patients with impaired renal function, thereby resulting in an increased systemic drug exposure. It appears that minor to moderate renal function impairment just insignificantly alters the clearance of CPA or its alkylating metabolites and as such there is no necessity to adjust the doses of CPA [189, 190]. However, terminal renal insufficiency may have a major impact on the renal excretion of CPA and its metabolites. In particular, CPA was removed by being taken into the dialysate in hemodialysis-dependent patients [191]. Haubitz *et al.* [191] reported that up to 25% of administered CPA dose ( $1.2 \pm 0.4 \text{ g}$ ) was recovered in the dialysate and thus removed from the body during the dialysis. They concluded that the dialysis should not be initiated earlier than 12 hours after CPA infusion, which can prevent the removal of drug in the early distribution phase [191]. Only when the patients with terminal renal insufficiency are overdosed with CPA, the repetitive dialysis can help remove the drug from the body. Therefore, the severity of renal impairment has to be carefully assessed, dosage of CPA should be accordingly adjusted in patients with renal dysfunction on hemodialysis and therapeutic drug monitoring should always be conducted in these patients.

As to IFO, renal dysfunction has few implications for its elimination, since renal clearance is only minor [116]. However, renal insufficiency might increase the risk of neurotoxicity because of more extensive deactivation of IFO

as a result of decreased renal excretion. Impaired renal function may reduce the renal clearance of IFO metabolites, leading to their accumulation in the body. Dose reduction in patients with renal failure has been suggested [192].

Both CPA and IFO are extensively metabolized by hepatic CYP2B6, 2C9 and 3A4, indicating that liver impairment has a major impact on pharmacokinetics of CPA and IFO. However, such liver impairment only leads to less formation of aldophosphamide; fewer adverse effects were observed in patients with liver dysfunction [166]. Therefore, it is not recommended to adjust the dosage of CPA in patients with liver dysfunction. Although biliary concentrations of CPA are comparable to plasma concentrations, only a low fraction of CPA (1.8%) is found in stool [161]. Liver impairment is known to slow the metabolism of IFO without shifting the metabolism towards more activation or deactivation [137]. Complete hepatic failure resulted in a total blockage of metabolism and a first-order pharmacokinetics of IFO with renal elimination being the only clearance pathway [137].

Disease status is also an important factor for pharmacokinetic variability. The activation of CPA was inhibited in tumor-bearing rats compared to healthy control [193]. The clearance of CPA was found to reduce in children with Fanconi's anemia, probably as a result of altered CYP oxidase-reductase cycling [194]. Recently, the risk of recurrence of non-Hodgkin's lymphoma in children is related to inadequate clearance of CPA to active metabolites [88].

Importantly, genetic factors may affect the pharmacokinetics of CPA and IFO. There are increased studies on the role of genetic polymorphisms of genes encoding various proteins involved in the distribution, metabolism, and transport of CPA and IFO in the pharmacokinetic variability and therapeutic outcomes. Theoretically, polymorphisms of CYP3A4, CYP2B6, CYP2C9, ALDH1A1, ALDH3A1, GSTT1, GSTM1, GSTP1, and MRP2 may play a role in the disposition of CPA and IFO, thus resulting in wide interpatient variability in exposure to CPA and its active metabolites, with important clinical consequences in cancer chemotherapy.

There is some evidence for polymorphisms in CYP2B6, CYP2C9 and CYP3A [195-198]. Altered activity of these CYPs responsible for activation and deactivation of CPA and IFO due to genetic polymorphism may lead to an altered formation of active metabolites and organ toxicity of CPA and IFO. For example, site-specific mutations of *CYP2B6* altered the formation of active metabolite of CPA [29]. Lang *et al.* [199] found nine polymorphisms in the *CYP2B6* gene, five of which resulted in amino acid substitutions. The authors showed that a polymorphism in exon 9 was associated with significantly reduced *CYP2B6* protein expression and *S*-mephenytoin *N*-demethylase activity in human liver specimens. Ariyoshi *et al.* [200] reported a G to T nucleotide change at position 516 in *CYP2B6* gene, with an allelic frequency of 20% in Japanese. The variant allele exhibits increased catalytic activity for *O*-deethylation of 7-ethoxycoumarin *in vitro*, whereas its *in vivo* effect has yet to be determined. Recently, more allelic variants of *CYP2B6*

have been reported [201-204]. Genetic polymorphism of CYPs resulting in interindividual variation in CPA and IFO metabolism is considered to contribute to certain extent to the differences in the pharmacokinetics, activity and susceptibility to organ toxicity [205]. Therefore, further research is needed to correlate these *CYP2B6* mutations with CPA and IFO disposition, activity and toxicity.

Multiple GSTs are involved in the conjugation of CPA and IFO and their metabolites. These Phase II enzymes represent an important detoxification mechanism for cytotoxic drugs such as CPA and IFO. A number of genetic polymorphisms of GSTs have been reported in humans [206, 207]. These mutations are highly likely to affect the disposition of CPA and consequently modulate the efficacy and toxicity. In pediatric patients with steroid-sensitive nephrotic syndrome, *GSTM1* null polymorphism gave a significantly better rate of sustained remission than in patients with the heterozygous or homozygous *GSTM1* wildtype [208]. In contrast, children with *GSTP1* heterozygous or homozygous polymorphism had a significantly lower rate of sustained remission compared to homozygous wildtype [208]. The *GSTT1* genotype did not alter the outcome of CPA treatment. Patients with the combination of *GSTM1* null and *GSTP1* wildtype did not relapse in 50%, compared to 6% in other children. These findings indicate that the polymorphic expression of *GSTM1* and *GSTP1* has a significant impact on the long-term remission rate of CPA treatment in children with steroid-sensitive nephrotic syndrome. *GSTM1* null mutation increases the efficacy of CPA, whereas *GSTP1* polymorphism seems to be related to enhanced susceptibility to further relapses.

ALDH superfamily has at least 17 members in human genome [209-215]. These genes encode NADP-dependent enzymes that oxidize a wide range of aliphatic and aromatic aldehydes to their corresponding carboxylic acids. Based on primary sequence analysis, three major classes of mammalian ALDH1, 2, and 3 have been identified. Mutations in several ALDH genes including ALDH1 and ALDH3 have been reported [216]. Polymorphism in ALDH2 is associated with altered acetaldehyde metabolism, decreased risk of alcoholism and increased risk of ethanol-induced cancers [216-218]. Polymorphisms in ALDH3A2, ALDH4A1, ALDH5A1 and ALDH6A1 are associated with metabolic diseases generally characterized by neurological complications [210, 211, 216]. Mutations in ALDH3A2 cause loss of enzymatic activity and are the molecular basis of Sjogren-Larsson syndrome [216, 219, 220]. Since ALDH1A1 is the major enzyme oxidizing cytotoxic 4-OH-CPA and 4-OH-IFO by forming CEPM, 4-keto-CPA and 4-keto-IFO, respectively [221], and human ALDH3 can also oxidize aldophosphamide to a less extent [222]. Mutations in genes encoding these enzymes may have an impact on CPA and IFO detoxification and thus alter the efficacy and toxicity profiles.

In addition, genetic polymorphisms of MRP2 have been reported in humans [223, 224]. MRP2 is a member of the ATP-binding cassette transporters associated with multidrug resistance. Mutations of MRP2 have been associated to

Dubin-Johnson syndrome characterized by conjugated hyperbilirubinemia [225]. Together with Pgp, breast cancer resistance protein (BCRP), and other MRPs, MRP2 are increasingly recognized for their ability to modulate the absorption, distribution, metabolism, excretion, and toxicity of a number of xenobiotics including anticancer agents [226, 227]. Since GSCY, a conjugate of CPA metabolite, is excreted into the bile by MRP2 [63], mutations of MRP2 may affect the excretion of CPA.

## 2.9. Pharmacokinetic Drug Interactions

Since both CPA and IFO undergo extensive CYP-catalyzed metabolism through which they are activated, deactivated and eliminated, drug interactions may arise due to modulation of the pharmacokinetics, in particular, when inhibition or induction of the relevant CYPs is implicated.

A number of drug interactions with CPA have been reported in humans. It seems that the underlying mechanism is inhibition of CYP enzymes for the drug interactions with allopurinol [163], chloramphenicol [228], sulphaphenazole [228], chlorpromazine [163, 229], fluconazole [230], ranitidine [231], and thiotepa (triethylenethiophosphoramide) [232]. Drug interactions have also been reported with dexamethasone [163], prednisolone [233], phenobarbitone [234], and phenytoin [169] due to induction of CPA metabolism. Phenytoin induces the *N*-dechloroethylation of the *S*-enantiomer of CPA to a greater extent than that of the *R*-enantiomer [235]. The clinical significance of these drug interactions is unclear. In addition, an altered toxicity profile of CPA was observed when combined with paclitaxel in a schedule-dependent manner [236], but pharmacokinetic modulation cannot provide an explanation.

However, IFO has lesser interactions with various CYP inducers and inhibitors compared with CPA. In a study by Lokiec *et al.* [237], modulation was not detected when IFO was coadministered with the CYP inducer phenobarbital. This failure could, however, be explained by the fact that enzyme induction by barbiturates typically requires several days to develop. Therefore, the simultaneous administration of phenobarbital was not likely to result in heteroinduction of IFO metabolism. Pretreatment of phenobarbital for a few days was shown to induce the metabolism of IFO in rats [238]. Rifampin, a potent CYP3A4 and CYP2B6 inducer, increased the clearance of IFO at the start of therapy at 102% in patients [239]. The fraction of IFO metabolized to the dechloroethylated metabolites was increased, whereas exposure to the metabolites was decreased due to increased elimination. The fraction metabolized and the exposure to 4-OH-IFO was insignificantly affected. Ketoconazole (a CYP3A4 inhibitor) did not alter the fraction metabolized or the exposure to the dechloroethylated metabolites, whereas both parameters were reduced with 4-OH-IFO [239]. However, coadministration of IFO with ketoconazole or rifampin did not alter the pharmacokinetics of the parent or cytotoxic metabolites [239].

On the other hand, CPA and IFO may alter the pharmacokinetics and pharmacodynamics of coadministered drugs. CPA reduced digoxin absorption has been reported [240]. Thiotepa inhibited the activation of CPA and

decreased the efficacy and toxicity of CPA [232, 241], because of the mechanism-based inhibition of CYP2B6 [242, 243]. Conversely, CPA induced the conversion of thiotepa to its metabolite TEPA [244], thioTEPA is frequently given in conjunction with CPA in high-dose chemotherapy regimens in preparative regimens before autologous bone-marrow and peripheral stem-cell transplantation. Therefore, the sequence and schedule of these two drugs should be critically concerned and it has been recommended that these two agents should not be combined [133, 241]. Furthermore, the clearance of doxorubicin was significantly reduced (50%) when in combination with CPA [245]. The combination of CPA and doxorubicin also caused a 10% decrease in clearance of etoposide, but this is of little clinical significance [246, 247]. CPA is often combined with doxorubicin and etoposide in the treatment of breast cancer. Therefore, proper therapeutic drug monitoring is needed when CPA is used in combination with doxorubicin and etoposide.

IFO can potentiate the anticancer activity of a variety of cytotoxic agents including cisplatin and paclitaxel [248]. This may be not only related to the alkylating property of IFO, but also to its ability to deplete intracellular GSH. IFO significantly reduces intracellular GSH levels *in vitro* in malignant cell lines and *in vivo* in peripheral blood lymphocytes from cancer patients [249]. The intracellular GSH level was decreased maximally at 2 hours after drug was administered and was returned to normal within 24 hours [249]. Recently, the topoisomerase I inhibitor camptothecins such as irinotecan (CPT-11) and topotecan combined with IFO have been studied. In the study on the combination of IFO and irinotecan for the treatment of osteosarcoma in children, IFO reduced the AUC of SN-38, which is the active metabolite of irinotecan [250]. However, IFO did not affect the pharmacokinetics of topotecan in patients with advanced malignancies refractory to standard therapy [251]. These findings suggest that concerns may arise when IFO is combined with irinotecan.

## 2.10. Stereochemistry

Both CPA and IFO are chiral and are administered as racemic (50:50) mixtures of the two isomers. Studies of the clinical pharmacology, metabolism and disposition of these agents indicate that stereochemistry plays a minor role in the efficacy and toxicity of CPA [252]. There are some differences in the elimination of the enantiomers of CPA in cancer patients, with the *S*-enantiomer being eliminated more rapidly [253].

Chirality is an influencing factor in IFO-induced neurotoxicity [252]. A rat study has demonstrated that the administration of *R*-IFO retained the antitumor efficacy while limiting the generation of neurotoxic metabolites [254]. In contrast to CPA, *S*-IFO and *R*-IFO differ in their disposition and metabolism and thus show differential cytotoxicity. *In vitro* studies indicated that tumor cells expressing CYP3A4 were the most sensitive to *R*-IFO, whereas tumor cells expressing CYP2B1 or CYP2B6 were most sensitive to CPA [255]. Correspondingly, CYP3A4-expressing cells and cDNA-expressed CYP3A4 metabolized *R*-IFO to generate the active 4-hydroxylated metabolite at a 2

to 3-fold higher rate than *S*-IFO or CPA. CYP2B6-expressing cells and cDNA-expressed CYP2B6 metabolized CPA almost exclusively by 4-hydroxylation, whereas *R*-IFO and *S*-IFO were substantially converted to the inactive *N*-dechloroethylated metabolites [255]. Therefore, *R*-IFO is expected to exert greater anti-cancer activity than *S*-IFO or CPA against tumors that express CYP3A enzymes, whereas tumors expressing CYP2B6 may be more sensitive to CPA treatment.

From studies in patients with i.v administration of the racemic IFO, the urine and plasma samples are enriched with *R*-IFO [173, 256], indicating that *S*-IFO is preferentially metabolized by CYP3A4 and CYP2B6 due to higher affinity. *N*-dechloroethylation of *S*-IFO is primarily catalyzed by CYP2B6, whereas CYP3A4 and CYP3A5 are major contributors for the *N*-dechloroethylation of *R*-IFO [257]. Theoretically, *R*-IFO is less *N*-dechloroethylated and more activated than *S*-IFO, leading to more development of neurotoxicity with *R*-IFO [252].

### 3. PHARMACODYNAMICS OF CYCLOPHOSPHAMIDE AND IFOSFAMIDE

#### 3.1. Mechanism of Action

Like other alkylating agents, the alkylating nitrogen mustards of CPA and IFO work through the covalent bonding of highly reactive alkyl groups with nucleophilic groups of nucleic acids. Covalent binding to cellular proteins is also possible. Following metabolic activation, bifunctional alkylating nitrogen mustards of CPA and IFO are generated, which are capable of reacting with the nitrogen-7 atom of purine bases in DNA, especially when they are flanked by adjacent guanines (Fig. (4)) [258]. At alkaline or neutral pH, the nitrogen mustard is converted to chemically reactive carbonium ion through imonium ion. Carbonium ion reacts with the N<sup>7</sup> of the guanine residue in DNA to form a covalent linkage. The second arm in phosphoramidate mustard can react with a second guanine moiety in an opposite DNA strand or in the same strand to form crosslinks [259]. The O<sup>6</sup> atom of guanine may also be a target for oxazaphosphorines [260]. The different intramolecular distance between the chloroethyl groups in CPA or IFO mustards results in a different range of cross-linked DNA.

Despite a good understanding of nature of the chemical reactions between alkylating species and DNA molecules [261], the mechanisms linking adduct and crosslink formation with tumor cell death are not fully identified. CPA and IFO, as with all other alkylating agents, destroy tumor cells through apoptosis (programmed cell death) initiated by DNA damage, modulation of cell cycle and other anti-proliferative effects [262-264]. It is generally accepted that the main mechanism that results in cell death is inhibition of DNA replication, as the interlinked strands do not allow separation of the two strands [265]. Apoptosis is characterized by a cascade-like activation of intracellular cysteine-proteases (i.e. caspases). Caspases pre-exist as zymogens that are activated by proteolytic cleavage by other caspases or by autocatalysis. Distinct caspase cascades are involved in receptor-mediated or chemical-induced apoptosis [266]. Drug-induced apoptosis is always mediated by the mitochondrial pathway leading to activation of the initiator

caspase-9, which in turn activates the effector caspases-3 and caspase-7. Because CPA and IFO can damage DNA during any phase of the cell cycle and their cytotoxicity is independent of the cell-cycle [267, 268], they are not cell-cycle phase specific.

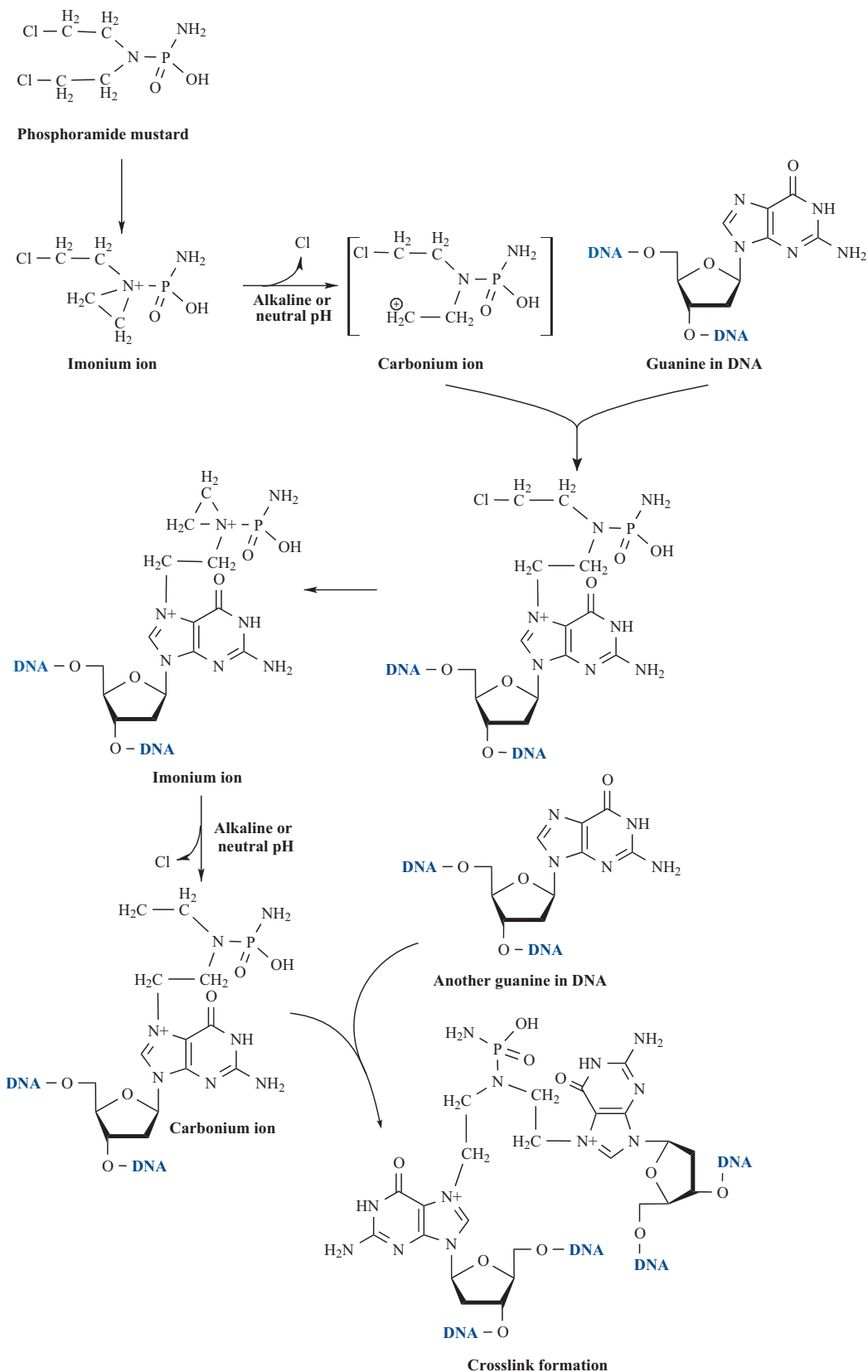
In addition to the final alkylating metabolites, CAA and acrolein are also related to the cytotoxicity of IFO and CPA. CAA induces depletion of intracellular sulfhydryl agents including GSH, resulting in cytotoxicity [115]. Therefore, stimulation of cellular GSH synthesis may protect against cytotoxicity of CPA and IFO. Generation of CAA may explain in part the clinically observed lack of complete cross-resistance between CPA and IFO and the higher remission rates achievable by IFO in certain tumor types [114, 269]. Moreover, other factors like the greater cross-linking arm length of IFO and its longer half-life may contribute to this difference [270]. Acrolein is able to induce single strand DNA break that is possibly associated to the cytotoxicity of CPA and IFO. Such cytotoxicity can be prevented by coadministration of mesna [264].

Recently, there is accumulating evidence indicating the action of CPA and IFO on the immune system. CPA has modulating effects on both humoral and cell-mediated immunity [79, 271-276] and thus beneficial effects are obtained when used as an immunosuppressive drug. CPA also augmented the efficacy of antitumor immune responses in animals and humans by depleting CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells and increasing T lymphocyte proliferation and T memory cells [277-282]. The immunostimulatory effect of CPA is associated with the marked inhibition of inducible nitric oxide synthase [10]. Furthermore, CPA kills circulating endothelial progenitors that are a marker of tumor angiogenesis [283], whereas 4-OH-CPA readily destroys various hematopoietic progenitors cells including marrow stromal progenitors [284]. These findings may provide a solid rationale for the use of CPA or IFO as an immunosuppressive agent in the treatment of autoimmune diseases or an integral component in combination with other immunotherapy in cancer treatment.

#### 3.2. Antitumor Activity

CPA is the most widely used alkylating agent in the treatment for hematological malignancies and a variety of solid tumors, including leukemia [12, 13, 285], breast cancer [286-291], lung cancer [292-295], lymphomas [296-298], prostate cancer [299, 300], ovarian cancer [301-306], and multiple myeloma [307, 308]. Although its role in the treatment for ovarian cancer and small-cell lung cancer is declining, CPA continues to be used in treatment of breast cancer as a critical component of the CMF, CEF (CPA, epirubicin, and 5-fluorouracil), MVC (mitoxantrone, vinblastine, and CPA) and TAC (docetaxel, doxorubicin and CPA) regimen [289, 290, 309-312]. Higher doses of CPA are used in the treatment prior to bone marrow transplantation for aplastic anemia, leukemia and other malignancies [11-16].

In comparison with CPA, IFO is more effective in a wide range of malignant diseases. IFO has shown antitumor activity against a variety of tumors, including small-cell and



**Fig. (5).** Alkylation of 7-nitrogen atom of guanine in DNA molecules by phosphoramidate mustard resulting from cyclophosphamide activation. At alkaline or neutral pH, the nitrogen mustard is converted to chemically reactive carbonium ion through imonium ion. Carbonium ion reacts with the N<sup>7</sup> of the guanine residue in DNA to form a covalent linkage. The second arm in phosphoramidate mustard can react with a second guanine moiety in an opposite DNA strand or in the same strand to form crosslinks.

non-small-cell lung carcinoma (NSCLC) [313-316], breast cancer [317-319], ovarian cancer [320, 321], bladder cancer [322-324], cervical cancer [325], osteosarcoma [326], neuroblastoma [327, 328], leukemia [329, 330], multiple myeloma [331], and lymphomas [332, 333]. IFO could be used in both single agent and combination therapy with many other cytotoxic agents in clinical practice, such as etoposide, doxorubicin and mitomycin. For example, VIM [IFO, etoposide (VP-16) and methotrexate] and MINE [mitoguanzone (an inhibitor of S-adenosylmethionine decarboxylase, i.e. a key enzyme in the polyamine biosynthetic pathway), IEV (IFO, epirubicin and etoposide), and IVE [IFO, vinorelbine (navelbine) and etoposide] regimens have been often used in the treatment of relapsed non-Hodgkin's lymphoma and Hodgkin's lymphoma, respectively [333-335]. ICE (IFO, carboplatin, and etoposide) is commonly used for the treatment of refractory small-cell lung cancer [315], while MICE (mitomycin-C, IFO, cisplatin, and vindesine) is effective in NSCLC [336].

The difference in the efficacy between CPA and IFO is due to the different pharmacokinetic profiles of their final alkylating mustard metabolites. Ifosforamide mustard, the final alkylating mustard of IFO, has a higher affinity to alkylate DNA in comparison to the final alkylating mustard of CPA, phosphoramidate mustard [337]. Ifosforamide mustard has one 2-chloroethyl group on the exo and one on the endocyclic oxazaphosphorine nitrogen atom, whereas phosphoramidate mustard has 2 exocyclic 2-chloroethyl groups (shown in Fig. (3)). The different intramolecular distance between the chloroethyl groups in these two mustards results in the different orientation of its alkylating moieties and the different range of cross-linked DNA [259].

### 3.3. Toxicity

In cancer patients, CPA and IFO are primarily activated by CYP3A4, CYP2C9 and CYP2B6 in the liver (see Section 2.4), followed by erythrocyte-mediated transport of the activated metabolites to the tumor tissue *via* blood circulation. However, these activated metabolites also gain entry into normal tissues, where they may induce host toxicity. For CPA, the usual dose-limited toxicity is myelosuppression [269]. At higher doses used prior to marrow transplantation, the dose-limited toxicity is cardiac toxicity [338]. Besides cardiac toxicity, hemorrhagic cystitis, water retention and hyponatremia are found in patients receiving high dose CPA.

Acrolein is the causal agent to hemorrhagic cystitis. Using mesna (sodium-2-mercaptoethanesulfonate) can reduce the incidence of hemorrhagic cystitis [339]. A direct effect of CPA on the renal tubules leads to excess water retention. This can be managed by hydration with isotonic fluids [340]. In women receiving CPA, methotrexate and fluorouracil for treatment of breast cancer, the severe toxicity thromboembolic events have been reported [341]. Elevation of serum level of aminotransferases in patients treated with CPA has also been reported. This CPA-induced liver injury results from metabolites of CPA, especially acrolein and is mainly dose-dependent [342]. Metabolites of CPA have been demonstrated to be teratogens and carcinogens in animals.

Malformations have been associated to first trimester exposure to CPA [343].

Several studies suggested CPA as a single chemotherapeutic agent induced leukemia in human [344]. The mechanisms are unknown, but this may be associated with the cytogenetic toxicity of CPA [345-349]. Patients with breast cancer and inheritance of a combined gene deletion of *GSTM1* and *GSTT1* might bear an increased risk to develop a secondary CPA-induced hematological neoplasia [350].

IFO has a wide variety of toxicities including hematological toxicity, urotoxicity, nephrotoxicity, and neurotoxicity [351]. The principal dose-limited hematological toxicity is myelosuppression, especially leucocytopenia. The incidence of myelosuppression is dose-dependent. A single high dose schedule of IFO tends to generate more myelosuppression than fractionated dose schedules [352]. Hematological recovery will be enhanced by treatment with granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, or interleukin-3 [353, 354]. Urotoxicity is another dose-limiting toxicity of IFO and is usually manifested as hemorrhagic cystitis [351]. Hemorrhagic cystitis is more often seen in patients receiving IFO compared with CPA. Acrolein and CAA have been regarded as the causative factor of urotoxicity of IFO. Mesna reacts specifically with acrolein in the urinary tracts and prevents urotoxicity of IFO [100, 351].

In addition, nephrotoxicity is another non-hematological toxicity of IFO which is not associated with CPA [355]. Nephrotoxicity is mostly found in children and is the major dose-limiting factor for IFO administration in children [356]. Nephrotoxicity is characterized by Fanconi syndrome, which is predominant in pediatric populations and glomerular toxicity [357]. IFO-induced nephrotoxicity is age-dependent, with younger children (<3 years) substantially more vulnerable [355]. Because CPA does not show nephrotoxicity and extensive dechloroethylation as with IFO, CAA has been considered as the possible causative agent of nephrotoxicity [358-360]. It has been reported that renal cells are able to metabolize IFO to dechloroethyl-IFO, indicating that CAA is locally generated by renal cells [165, 358]. From the *in vitro* studies and clinical experience, mesna does not eliminate the nephrotoxicity of IFO [361]. Interestingly, melatonin has been demonstrated to prevent the Fanconi syndrome caused by IFO in rats [362].

IFO is relatively well tolerated but it can also be associated occasionally with life-threatening complications such as arrhythmias, heart failure, and severe encephalopathy. Mesna administration cannot control these toxicities. Other preventive measures, such as amifostine or methylene blue administration, have not yet been adequately evaluated in a sufficient number of patients. Clinicians prescribing IFO, especially using high doses, should be watchful for early signs of toxicity in order to discontinue IFO administration soon enough to avoid development of major or life-threatening toxicity.

Both CPA and IFO have serious organ toxicities, but there are significant differences in their toxicity profiles between these two agents [363]. CPA exhibits relatively

little non-hematopoietic toxicity and has been used in both adults and children with high-dose therapy regimens [109]. In contrast to CPA, IFO shows increased hematological toxicity, neurotoxicity, urotoxicity and nephrotoxicity. Hence, IFO is dose-limited when used in pediatric patients and has to be used with at least an equimolar dose of mesna, which is an uroprotective agent and can prevent hemorrhagic cystitis.

#### 4. PHARMACOKINETIC-PHARMACODYNAMIC RELATIONSHIP

As CPA and IFO are prodrugs, no correlation between the efficacy and/or toxicity and the plasma concentration of the parent drugs has been expected. Attempts to assess the relationship between pharmacokinetics and pharmacodynamics of CPA would require frequent blood sampling, but a limited sampling strategy to relate CPA pharmacokinetics with pharmacodynamics was developed [364].

AUC is a better indicator of pharmacodynamic responses than administered dosage. Significant low AUC of CPA in patients who developed cardiac toxicity has been reported. It supports that increased CPA activation may lead to less relapse and increased cardiac toxicity [365]. The high inpatient variability in pharmacokinetic parameters such as AUC,  $t_{1/2\beta}$  and CL was observed in patients with chemotherapy of CPA. The pharmacokinetic information of CPA obtained from one treatment course would not be useful in the decision of dosage of next treatment [139, 175]. It is clear that the active metabolites of CPA, not the parent compound, are responsible for the activity. The pharmacokinetics of the metabolites are not predictable by those of CPA [49, 146]. The relationship between the pharmacology and treatment responses of CPA is not completely understood and further studies are required.

The pharmacokinetic-pharmacodynamic relationships of IFO and its metabolites have been studied with a focus on the role of metabolites in its efficacy and toxicity. It has been found that the plasma AUC of the final active alkylating metabolite ifosforamide mustard has a substantial negative correlation with progression-free interval and survival, whereas the dechloroethylated metabolites of IFO have a positive correlation with survival [108].

The exposure to IFO and its metabolite 3-dechloroethyl-IFO is influenced by the duration of IFO infusion. The AUC of 3-dechloroethyl-IFO was increased with short infusion, while AUC of IFO was significantly decreased [142]. Acrolein is associated with urotoxicity of IFO, whereas CAA and dechloroethyl metabolites are associated with neurotoxicity and nephrotoxicity of IFO. No pharmacokinetic-pharmacodynamic relationship was found between hematological toxicity and 4-hydroxy-IFO in patients with soft tissue sarcoma [131].

#### 5. RESISTANCE TO CYCLOPHOSPHAMIDE AND IFOSFAMIDE

##### 5.1. Resistance Phenomena

Resistance to oxazaphosphorine chemotherapy due to lack of sensitivity to the drugs is common in many types of tumors, in particular, those solid tumors resulting in

therapeutic failure in cancer patients [366]. Like other alkylating agents, resistance to CPA and IFO has been demonstrated by *in vitro* studies [367] and murine models [368]. It is considered that tumor resistance to CPA and IFO also occur in patients based on disappointing response rates in some tumor types. Moreover, resistance to CPA in patients with autoimmune diseases has been observed. For example, up to 40% of patients with lupus nephritis failed to achieve renal remission, and up to 20% of patients developed end-stage renal disease after a long course (30 months or longer) of pulse CPA therapy [369-371].

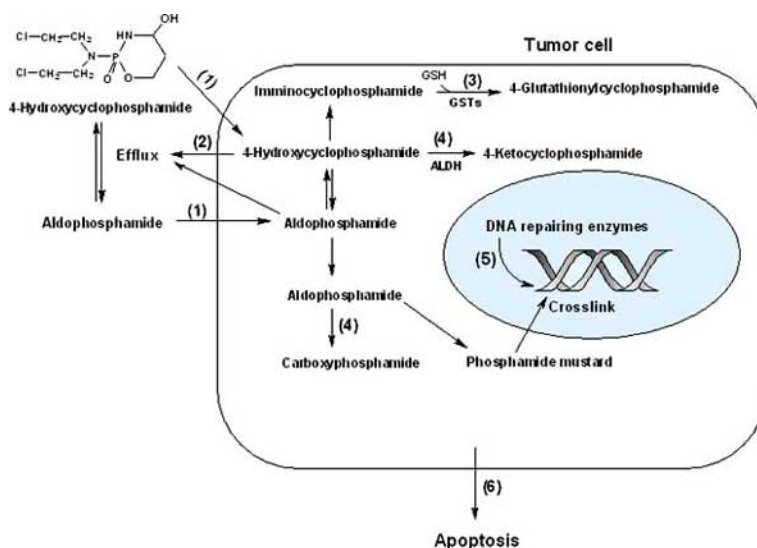
Unlike natural product anticancer drugs, drug resistance to CPA and IFO is not associated with the multidrug resistance phenotype or elevated cellular drug transporters such as P-glycoprotein and MRPs [69, 70]. For those natural product anticancer drugs, resistance to multiple anticancer drugs will appear after a period of treatment. This so-called multidrug resistance phenomenon is not seen with CPA and IFO, indicating that the two agents are not typical substrates for P-gp. Cross-resistance with cisplatin and other alkylating agents is probably due to that fact that all of these agents act on DNA molecules and share some detoxification mechanisms such as conjugation by GSTs, followed by excretion mediated by MRP1 and MRP2 [68].

However, CPA and IFO and their metabolites may modulate multidrug resistance related proteins and thus may alter the pharmacokinetic and pharmacodynamics of the substrate drugs for multidrug resistance related proteins. Acrolein and CAA were found to reverse the multidrug resistance to daunorubicin and vinblastine mediated by MRP1 *in vitro* [372]. The cellular pharmacokinetics of CPA and IFO and the effects on MRP1 and other transporters and the clinical significance should be explored.

##### 5.2. Mechanism Consideration

The mechanisms of resistance are unclear, but several factors associated with drugs and tumor cells have been suggested [366] (see Fig. (6)). These include decreased activation by CYP3A4, CYP2C9 and CYP2B6, decreased entry into or increased exit from cells, increased deactivation of the agents inside cells, increased cellular thiol level [373], increased activity of glutathione *S*-transferases [374, 375], increased ALDH1A and ALDH3 activity [376-379], increased DNA repair capacity [110, 260, 367, 376, 380-382], and deficient apoptosis [383] due to lack of cellular mechanisms to result in cell death following DNA damage.

CYP and IFO require activation through 4-hydroxylation by CYP2B6, CYP3A4 and CYP2C9, resulting in cytotoxic species that cause their cytotoxicity to tumor cells [75, 178]. Thus, altered activity or expression of these CYP enzymes by inducers, inhibitors or genetic polymorphisms will change the formation of active metabolites and consequently, the response and toxicity to CPA and IFO. For example, the metabolism of IFO could be increased by concomitant therapy with inducers of CYP3A4 such as phenytoin [384] and phenobarbital [385], or be interfered by the inhibitors of CYP3A4. These inducers or inhibitors also shift the metabolic pathways of IFO and thus affect the formation of cytotoxic metabolites. Pretreatment of Caco-2 cells and



**Fig. (6).** Proposed mechanisms for resistance to cyclophosphamide. Resistance of tumor cells to cyclophosphamide occurs due to decreased intracellular accumulation of active metabolite (1), increased efflux of active metabolite (2), increased formation of inactive 4-ketocyclophosphamide and carboxyphosphamide (3), increased conjugation by glutathione due to increased cellular glutathione (GSH) level and increased activity of glutathione *S*-transferases (GSTs) (4), increased DNA repair capacity (5), and deficient apoptosis due to lack of cellular mechanisms to result in cell death following DNA damage (6).

primary hepatocytes with antisense phosphorodiamidate morpholino oligomers caused reduced CYP3A4 expression and rendered cells significant resistance to CPA [386]. More importantly, there is a report where CYP2B6 and CYP2C19 mutations are associated with altered response or survival in patients with proliferative lupus nephritis when treated with pulse CPA [387]. Patients who were either heterozygous or homozygous for *CYP2C19\*2* had a significantly lower risk of developing premature ovarian failure [387]. Patients homozygous for *CYP2B6\*5* or *CYP2C19\*2* had a higher probability of reaching end-stage renal disease with a doubled serum creatinine level [387]. In a mouse study, at least 26 polymorphisms in *CYP* genes that may influence response to CPA have been reported [388]. The role of many of these variants in oxazaphosphorine treatment is still unknown and more evidence of these variants is needed to better assess clinical outcomes.

To overcome the problem that CYP expression is generally high in liver, resulting in a first-pass effect, and low in tumor cells, CYP-based cancer gene therapy has been demonstrated with CPA and IFO [389-391]. Preclinical studies have shown that the chemosensitivity of tumors to prodrugs such as CPA can be dramatically increased by CYP gene transfer, which confers the capability to activate the prodrug directly within the target tissue [389-391]. Gene transfer of activating enzymes (e.g. CYP3A4) into tumor cells has been suggested to improve selectivity of CPA and IFO without increasing host toxicity [30, 389, 391-394].

Cancer cells resistant to the cytotoxic effects of alkylating agents have higher levels of intracellular GSH. Multiple metabolites of CPA and IFO can react with GSH to result in various conjugates at different sites (see Fig. (2)) and Fig. (3)). Some of these reactions with GSH may be reversible, while others are irreversible. Intracellular GSH level is related to cytotoxicity of CPA and other oxa-

zaphosphorines [94, 373, 395, 396]. Increased intracellular thiols conferred on tumor cells resistance to CPA [396, 397]. GSH appears to prevent the crosslink formation by 4-OH-CPA and thus desensitize the tumor cells to the drug [398].

GST activity is another important factor determining sensitivity to oxazaphosphorines [374, 375]. Up-regulated GST activity increased resistance of tumor cells to CPA *in vitro* [396]. *GSTP1* gene-transduced hematopoietic progenitor cell transplantation overcome the bone marrow toxicity of CPA in mice [399]. Transfection of the *GSTP1-1* antisense expression vector into tumor cell lines decreased intracellular GSTP1-1 concentration and significantly increased the sensitivity of transfectants to 4-OH-CPA and other alkylating agents [400, 401]. However, a study in ovarian cancer tissue specimens from patients indicated that GSTA or GSTP was an independent factor of resistance to CPA and carboplatin [402]. Coordinated expression of GSTP1-1 with MRP1 failed to enhance resistance to 4-OH-CPA [403]. These findings indicate that the activity of GSTs is not the only factor affecting the sensitivity of tumor cell to CPA and IFO. Other proteins associated with the disposition, attack of targets and toxicity of CPA and IFO may interplay with GSTs.

ALDH1A1, ALDH3A1 and ALDH5A1 are involved in the detoxification of cytotoxic 4-OH-CPA and 4-OH-IFO by forming respective CEPM, 4-keto-CPA and 4-keto-IFO [80-89, 404]. Thus, an altered activity of ALDH1A1 may affect the cytotoxicity of CPA and IFO. Increased ALDH activity by induction, intrinsic mechanism or gene transfer has been shown to cause CPA resistance [377-379, 405-425]. The formation of CEPM *via* ALDH1A1 rendered relative resistance of early hematopoietic precursors (P388 cells) and L1210 leukemia cells to CPA, 4-OH-CPA and other oxazaphosphorines [426-428]. Inhibition of ALDH1A expression by antisense RNA sensitizes tumor cells to 4-OH-

CPA *in vitro* [429]. The known inhibitors of ALDH, disulfiram, diethylthiocarbamate, and cyanamide partially restored the sensitivity of these resistance cells to CPA, 4-OH-CPA and other oxazaphosphorines [427, 430, 431]. Retinoic acid [a substrate for ALDH [432]] down-regulated ALDH1A1 and 3A1 enzyme activity and increased cytotoxicity of 4-OH-CPA and acetaldehyde in A549 and other lung cancer cell lines [89].

The expression of ALDH3A1 is also an influencing factor for resistance to the oxazaphosphorines. Overexpression of ALDH3A1 by induction or intrinsic mechanism caused significant resistance to oxazaphosphorines including CPA and mafosfamide in various tumor cell lines [433-435]. In addition, inhibition of ALDH3A1 by compounds resulted in sensitization of tumor cells that express significant amounts of this enzyme to oxazaphosphorines [436, 437].

A retrospective study indicated that ALDH1A1 level was significantly higher in metastatic tumors that did not respond to subsequent treatment with CPA-based chemotherapeutic regimens than in those that did respond to such regimens [438]. The therapeutic outcome of CPA-based chemotherapy corresponded to cellular ALDH1A1 levels in 77% of cases. This finding suggests that cellular ALDH1A1 level may serve as a useful predictor of therapeutic responses to CPA-based chemotherapy of breast cancer. ALDH1 and ALDH3 have been detected in human tumor tissues [439, 440]. Thus, they may play a role in resistance to CPA and IFO. However, another study in patients with ovarian cancer indicated that neither ALDH nor GSTA and GSTP were independent factors of resistance to CPA and carboplatin [402]. Further studies are needed to explore the role of ALDH1A1 in resistance to CPA and IFO.

Clinical studies indicate that both BCRP and MRP1 play a role in resistance to CPA-based chemotherapy. MRP1 expression was associated with increased risk for failure in breast cancer patients with small tumors, in node-negative patients and in node-positive patients who received adjuvant systemic chemotherapy of CMF [441]. A clinical study in 516 premenopausal, hormone receptor-positive breast cancer patients with stage I or II disease demonstrated that MRP1 expression level was associated with the clinical resistance to adjuvant CMF chemotherapy [288]. Another clinical study also found that there was a correlation between clinical response and the expression of BCRP and MRP1 in the subgroup of breast cancer patients treated with anthracycline-based chemo-therapy (5-fluorouracil, adriamycin/epirubicin, and CPA), whereas such an association was lacking in the CMF-treated subgroup of patients [442]. All these findings are suggestive of a role of MRP1 and BCRP in clinical tumor resistance to CPA-based chemotherapy.

The ability of the cell to repair alkylating agent-induced DNA lesions, possibly through nucleotide excision repair or other processes, may be a key contributor to drug resistance. Resistance to CPA and IFO has been linked to increased DNA repair capacity [110, 260, 367, 376, 380-382]. Inhibition of DNA repairing enzymes (UBE2N and APEX) by (E)-5-(2-bromovinyl)-2'-deoxyuridine (RP101) reversed the resistance to IFO [443]. UCN-01, a cell cycle checkpoint abrogator, inhibited DNA repair in chronic lymphocytic

leukemia lymphocytes and increased cytotoxicity to 4-OH-CPA [444]. Similar results were observed with fludarabine and clofarabine (both nucleotide analogs) that inhibited DNA repair [445]. Additionally, increased O<sup>6</sup>-methylguanine-DNA methyl transferase (MGMT), a DNA repair protein, has been associated with tumor resistance to CPA [260, 446, 447]. O<sup>6</sup>-Benzylguanine, a potent inactivator of MGMT, increased the sensitivity of Chinese hamster ovary cells to the toxic effects of 4-OH-CPA and other alkylating agents [448]. Pentoxifylline, O<sup>6</sup>-benzylguanine and ethacrynic acid increased mafosfamide-induced cytotoxicity in lymphocytes from patients with chronic lymphatic leukemia [449]. MGMT activity correlated with sensitivity to CPA and cisplatin in human lung tumor xenografts [446]. However, tumor MGMT expression was not predictive of response to CPA in breast cancer patients and a low MGMT expression was significantly related to poor survival [450].

The mismatch repair gene plays a key role in the correction of DNA damage, and the loss of MMR has been implicated in resistance to a variety of chemotherapeutic drugs. DNA mismatch repair proteins function as a detector for adducts produced by 6-thioguanine, N-methyl-N'-nitro-N-nitrosoguanidine, cisplatin, and carboplatin but not for melphalan and perfosfamide [451]. RCC1 and ERCC4 gene products are crucial for the repair of 4-OH-CPA-induced DNA damage [452]. Breast cancer patients on CMF regimen with a lack of hMLH1 expression had a poor prognosis [453].

Moreover, signaling proteins associated with cellular proliferation and apoptosis play a role in resistance to anticancer agents including CPA and IFO. Transfection of Bcl-xL (an anti-apoptotic protein) into neuroblastoma cells decreases CPA-induced apoptosis *in vitro* [383]. Hematopoietic stem cells and other hematopoietic cells showed broad resistance to chemotherapeutic agents including CPA *in vivo* when overexpressing Bcl-2 [454]. Expression of Bcl-2, but not Bax or P53, decreased the resistance to anticancer drugs including CPA, mitomycin C, 5-fluorouracil, and adriamycin in breast carcinoma [455]. Furthermore, Bcl-2/Bcl-xL bispecific antisense treatment sensitized breast carcinoma cells to CPA, doxorubicin and paclitaxel [456]. Bax expression correlated with cellular drug sensitivity to CPA, doxorubicin, and chlorambucil, but not fludarabine or cladribine in B cell chronic lymphocytic leukemia [457]. These findings indicate the important role of signalling proteins in regulating downstream events in apoptosis and sensitivity to chemotherapy.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Much information on pharmacokinetics, pharmacodynamics and toxicology of CPA and IFO has been gained from a large number of preclinical and clinical studies. Such information constitutes the basis of the wide clinical use of these two agents in the management of a variety of malignancies. However, the relationships between pharmacokinetics and pharmacodynamics of CPA and IFO, in particular, the dose-response or dose-toxicity relationships are not fully established. The lack of such important information makes it difficult in optimizing the dosage and regimen when CPA or IFO is used in cancer chemotherapy.

Dose-limiting toxicity and resistance are important reasons for the failure of CPA and IFO chemotherapy. Resistance to oxazaphosphorines is poorly understood, although several possible mechanisms have been implicated including increased aldehyde dehydrogenase activity and DNA repair. Although CPA and IFO share a common metabolic pathway, 4-hydroxylation of IFO occurs at a slower rate and to a lesser extent than that of CPA. This difference significantly alters the toxicity profile of the two drugs. Leukopenia is the dose-limiting toxicity of CPA, whereas neurotoxicity is the dose-limiting toxicity of IFO when preventive measures are taken to reduce urotoxicity. With recent findings concerning their basic and clinical pharmacology, the therapeutic index of CPA and IFO can be improved.

Although CPA and IFO have been used in clinical practice for more than 30-40 years and have contributed to the effective treatment of many thousands of patients with a variety of tumors, the enigma of the mechanism of action, toxicity and resistance persists. In particular, there is little information on the molecular targets associated with the efficacy, toxicity and resistance of CPA and IFO at genome-wide level. A full identification of these targets by using proteomic and genomic approaches can provide deep insights into the molecular events of CPA/IFO activity and toxicity and may allow the identification of useful and novel strategies to overcome the resistance and toxicity and of reliable biomarkers for monitoring clinical responses during chemotherapy. Recently, cDNA microarray gene expression analysis has been used to investigate the genes associated with activity, toxicity and resistance of CPA or IFO [458-462]. For example, by using this technique, it was found that Id-1, MIF and GSTP1 gene overexpression increased the resistance to CPA in hormone independent prostate cancer cells [458]. In addition, expression profile analysis using cDNA microarray consisting of 23,040 genes was conducted in osteosarcoma samples from patients treated with IFO in combination with doxorubicin and cisplatin [461]. This study identified 60 genes whose expression levels were likely to be correlated with the response to chemotherapy and thus they might be useful to predict the response to chemotherapy [461].

Suitable biomarkers are needed in the clinical evaluation of CPA and IFO with regard to efficacy and toxicity. Such biomarkers should enable the characterization of patient populations and quantitation of the extent to which CPA and IFO reach intended targets (DNA and other potential targets), alter proposed pathophysiological mechanisms and achieve clinical outcomes [463-466]. However, the challenge is to identify unique biomarkers in complex biological mixtures that can be unambiguously correlated to biological events in order to validate drug targets and predict drug response. For example, leucocyte nadir that correlates with distant disease free survival and toxicity has been used as a biomarker to evaluate efficacy of anticancer drugs [467, 468]. MRP1 expression independently predicted shorter relapse-free survival and overall survival in patients treated with CPA, methotrexate, and fluorouracil hormone receptor-positive breast cancer patients [288]. Furthermore, serum level of tumor necrosis factor- $\alpha$  correlates with response to

neoadjuvant chemotherapy composed of CPA and doxorubicin in patients with locally advanced breast cancer [469]. More studies are needed to identify sensitive, reliable and efficient biomarkers for clinical assessment of CPA and IFO.

Pharmacogenetics is the study of how the genetic variations affect drug response in individual patients [470-472]. Because cancer chemotherapy is relatively non-specific and has narrow therapeutic indices, there is great potential for pharmacogenetics to improve treatment outcomes by either reducing toxicity or increasing efficacy [473]. Detailed pharmacogenetic studies are needed to explore the role of mutation in genes encoding CYP3A4, CYP2B6, CYP2C9, GSTs, MMR, ALDH1A1, ALDH3 and MRP2 in the pharmacokinetic and pharmacodynamic variability in patients treated with CPA or IFO. The findings would provide insights into genetic factors influencing CPA and IFO pharmacokinetics and pharmacodynamics and make individualized medication possible in cancer chemotherapy.

Therapeutic drug monitoring plays a critical role in the optimal use of some anticancer drugs (e.g. methotrexate, teniposide, etoposide, carboplatin, and mercaptopurine) [474-478]. These drugs are always used at maximum dose despite narrow therapeutic index and show substantial pharmacokinetic variability [479, 480]. This approach is achieved by assessing the pharmacokinetics of drugs or specific covariates correlating with pharmacokinetics and/or pharmacodynamics, with the construction of extensive databases of population pharmacokinetic and pharmacodynamic parameters [476, 477, 481]. However, concentration-effect relationships of most antineoplastic agents have not been well defined, thus limiting the widespread clinical application of therapeutic drug monitoring for cancer chemotherapy. Recently, pharmacogenetics-oriented therapeutic drug monitoring has been developed and both CPA and IFO have been proposed as potential candidates for this novel and efficient approach [476, 481, 482]. Based on the knowledge in pharmacokinetics, pharmacodynamics, toxicology and pharmacogenetics of CPA and IFO and effective use of therapeutic drug monitoring, optimal use of CPA and IFO will become possible.

#### ACKNOWLEDGEMENTS

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#### ABBREVIATIONS

ADH	=	Alcohol dehydrogenase
AKR	=	Aldo-keto reductase
ALDH	=	Aldehyde dehydrogenases
AUC	=	Area under concentration-time curve
BCRP	=	Breast cancer resistance protein
CAA	=	Chloroacetaldehyde
CEPM	=	<i>O</i> -carboxyethylcyclophosphoramide mustard
CL	=	Total body clearance
CPA	=	Cyclophosphamide

CYP	=	Cytochrome P450
$f_{\text{renal}}$	=	Fraction of urinary excretion of unchanged drug
GSCY	=	4-glutathionylcyclophosphamide
GSH	=	Glutathione
GST	=	Glutathione S-transferase
IFO	=	Ifosfamide
MGMT	=	O <sup>6</sup> -methylguanine-DNA methyl transferase
MRP	=	Multidrug resistance associated protein
NAD	=	Nicotinamide adenine dinucleotide
PXR	=	Pregnane X receptor
$t_{1/2\beta}$	=	Elimination half-life
$V_d$	=	Volume of distribution

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