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A REVIEW OF HUMAN CARCINOGENS

This publication represents the views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 14-21 October 2008

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IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

International Agency for Research on Cancer

World Health Organization

CYCLOPHOSPHAMIDE

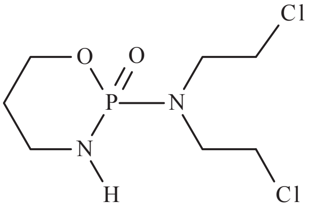
Cyclophosphamide was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 50-18-0
Chem. Abstr. Name: 2*H*-1,3,2-Oxazaphosphorin-2-amine, *N*,*N*-bis(2-chloroethyl)tetrahydro-, 2-oxide
IUPAC Systematic Name: *N*,*N*-Bis(2-chloroethyl)-1-oxo-6-oxa-2-aza-1λ^5-phosphacyclohexan-1-amine
Synonyms: 2-[Bis(2-chloroethyl)amino] tetrahydro-2*H*-1,3,2-oxazaphosphorin 2-oxide; bis(2-chloroethyl)phosphoramide cyclic propanolamide ester; *N*,*N*-bis(*β*-chloroethyl)-*N′*,*O*-trimethylenephosphoric acid ester diamide; *N*,*N*-bis(2-chloroethyl)-*N′*,*O*-propylenephosphoric acid ester diamide; Cytoxan; Endoxan; Neosar
Description: Crystalline solid [anhydrous form] (O'Neil, 2006)

1.1.1 *Structural and molecular formulae, and relative molecular mass*



$C_7H_{15}Cl_2N_2O_2P$
Relative molecular mass: 261.1

1.2 Use of the agent

Cyclophosphamide is an antineoplastic agent metabolized to active alkylating metabolites with properties similar to those of chlormethine. It also possesses marked immunosuppressant properties. It is widely used, often in combination with other agents, in the treatment of several malignant diseases. Information for Section 1.2 is taken from McEvoy, (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Indications

Cyclophosphamide is used in the treatment of chronic lymphocytic leukaemia, lymphomas, soft tissue and osteogenic sarcoma, and solid tumours. It is given orally or intravenously. Cyclophosphamide is inactive until metabolized by the liver.

(a) Hodgkin lymphoma

Cyclophosphamide is used in combination regimens (e.g. bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone [known as BEACOPP]) for the treatment of Hodgkin lymphoma.

(b) Non-Hodgkin lymphoma

Cyclophosphamide is used in combination therapy for the treatment of non-Hodgkin lymphoma, including high-grade lymphomas, such as Burkitt lymphoma and lymphoblastic lymphoma, as well as intermediate- and low-grade lymphomas. Cyclophosphamide is commonly used with doxorubicin (hydroxydaunorubicin), vincristine (oncovin), and prednisone (known as the CHOP regimen), with or without other agents, in the treatment of various types of intermediate-grade non-Hodgkin lymphoma. Cyclophosphamide has also been used as a single agent in the treatment of low-grade lymphomas.

(c) Multiple myeloma

Cyclophosphamide is used in combination with prednisone, or as a component of combination chemotherapy (i.e. vincristine, carmustine, melphalan, cyclophosphamide, and prednisone [VBMCP]) for the treatment of multiple myeloma.

(d) Leukaemia

Cyclophosphamide is used commonly for the treatment of chronic lymphocytic (lymphoblastic) leukaemia. Cyclophosphamide is used in combination with busulfan as a conditioning regimen before allogeneic haematopoietic progenitor cell transplantation in patients with chronic myelogenous leukaemia.

Cyclophosphamide is used in the treatment of acute lymphoblastic leukaemia, especially in children. In the treatment of acute myeloid (myelogenous, non-lymphocytic) leukaemia, cyclophosphamide has been used as an additional drug for induction or post-induction therapy.

(e) Cutaneous T-cell lymphoma

Cyclophosphamide is used alone or in combination regimens for the treatment of advanced mycosis fungoides, a form of cutaneous T-cell lymphoma.

(f) Neuroblastoma

Cyclophosphamide alone is used in the treatment of disseminated neuroblastoma. Combination chemotherapy that includes cyclophosphamide is also used for this neoplasm.

(g) Cancer of the ovary

Cyclophosphamide is used in combination chemotherapy (vincristine, actinomycin D, and cyclophosphamide [VAC]) as an alternative regimen for the treatment of ovarian germ-cell tumours.

Cyclophosphamide has been used in combination with a platinum-containing agent for the treatment of advanced (Stage III or IV) epithelial ovarian cancer.

(h) Retinoblastoma

Cyclophosphamide is used in combination therapy for the treatment of retinoblastoma.

(i) Cancer of the breast

Cyclophosphamide is used alone and also in combination therapy for the treatment of breast cancer.

Combination chemotherapy with cyclophosphamide is used as an adjunct to surgery in premenopausal and postmenopausal women with node-negative or -positive early (TNM Stage I or II) breast cancer. Adjuvant combination chemotherapy that includes cyclophosphamide, methotrexate, and fluorouracil has been used extensively.

Adjuvant combination chemotherapy (e.g. cyclophosphamide, methotrexate, and fluorouracil; cyclophosphamide, adriamycin, and fluorouracil; cyclophosphamide and adriamycin with or without tamoxifen) is used in patients with node-positive early breast cancer (Stage II) in both premenopausal and postmenopausal patients once treatment to control local disease (surgery, with or without radiation therapy) has been undertaken.

In Stage III (locally advanced) breast cancer, combination chemotherapy (with or without hormonal therapy) is used sequentially following surgery and radiation therapy for operable disease or following biopsy and radiation therapy for inoperable disease; commonly employed effective regimens include cyclophosphamide, methotrexate, and fluorouracil; cyclophosphamide, doxorubicin, and fluorouracil; and cyclophosphamide, methotrexate, fluorouracil, and prednisone. These and other regimens also have been used in the treatment of more advanced (Stage IV) and recurrent disease.

(j) Small cell cancer of the lung

Cyclophosphamide is used in combination chemotherapy regimens (e.g. cyclophosphamide, adriamycin, and vincristine [CAV]; cyclophosphamide, adriamycin, and etoposide [CAE]) for the treatment of extensive-stage small cell lung cancer.

(k) Sarcoma

Cyclophosphamide has been used in combination regimens (usually with dactinomycin and vincristine) and as an adjunct to surgery and radiation therapy in the treatment of rhabdomyosarcoma and Ewing sarcoma.

1.2.2 Dosage

Cyclophosphamide is administered orally or by intravenous injection or infusion. Less frequently, the drug has been administered intramuscularly and by intracavitary (e.g. intrapleural, intraperitoneal) injection and direct perfusion.

In patients with no haematological deficiencies receiving cyclophosphamide monotherapy, induction therapy in adults and children is usually initiated with an intravenous cyclophosphamide loading dose of 40–50 mg/kg administered in divided doses over 2–5 days. Other regimens for intravenous administration include 10–15 mg/kg every 7–10 days or 3–5 mg/kg twice weekly.

When cyclophosphamide is administered orally, the usual dose for induction or maintenance therapy is 1–5 mg/kg daily, depending on the tolerance of the patient.

A daily oral dose of 2–3 mg/kg for 60–90 days has been used in children with nephrotic syndrome, and in whom corticosteroids have been unsuccessful. In patients who are to undergo stem-cell transplantation, very high doses of cyclophosphamide such as 60 mg/kg daily for 2 days may be given as part of the conditioning regimen.

Various cyclophosphamide-containing combination chemotherapy regimens have been used in the treatment of breast cancer. One commonly employed regimen for the treatment of early breast cancer includes a cyclophosphamide dosage of 100 mg/m^2 orally on Days 1 through 14 of each cycle combined with intravenous methotrexate at 40 mg/m^2 on Days 1 and 8 of each cycle, and intravenous fluorouracil at 600 mg/m^2 on Days 1 and 8 of each cycle. In patients older than 60 years of age, the initial intravenous methotrexate dosage is reduced to 30 mg/m^2 and the initial intravenous fluorouracil dosage is reduced to 400 mg/m^2. Dosage is also reduced if myelosuppression develops. Cycles are generally repeated monthly (i.e. allowing a 2-week rest period between cycles) for a total of 6–12 cycles (i.e. 6–12 months of therapy).

Cyclophosphamide is available as 25 and 50 mg tablets for oral administration, and as 200 mg, 500 mg, 1 g, or 2 g vials of powder for reconstitution for parenteral administration.

1.2.3 Trends in use

No information was available to the Working Group.

2. Cancer in Humans

The carcinogenicity of cyclophosphamide in humans was established initially on the basis of a large number of case reports, as well as several epidemiological studies (IARC 1981, 1987a). The interpretation of the epidemiological studies was limited by the small numbers of cases, the difficulty in separating the role of cyclophosphamide from other agents, or both factors.

The most substantial evidence available to previous Working Groups was a Danish study of 602 patients treated "mainly with cyclophosphamide" for non-Hodgkin lymphoma, in which nine cases of acute myeloid leukaemia were observed compared to 0.12 expected (Pedersen-Bjergaard *et al.*, 1985), and a case–control study of leukaemia following ovarian cancer in the former German Democratic Republic where a strong dose–response relationship was observed (Haas *et al.*, 1987). All other studies reported at most three cases of leukaemia or bladder cancer in people who had received cyclophosphamide as the only potentially carcinogenic agent (IARC, 1981; Kinlen, 1985; Greene *et al.*, 1986).

Subsequently, further studies have been published that have provided more detailed information on the carcinogenicity of cyclophosphamide. This review is restricted to epidemiological studies that have used appropriate comparison groups to investigate the role of cyclophosphamide as the cause of specific types of cancer.

There have been several reported cohort studies in which patients treated with cyclophosphamide were followed up, and the occurrence of second cancers investigated. Valagussa *et al.* (1994) followed 2465 women who had received treatment with cyclophosphamide, methotrexate and fluorouracil, a combination in which only cyclophosphamide is considered to have carcinogenic potential in humans. There were three cases of acute myeloid leukaemia observed compared to 1.3 expected, and five cases of bladder cancer compared to 2.1 expected. Statistical significance was not reported but was calculated by the Working Group to be greater than 0.05 for both types of cancer. Smith *et al.* (2003) followed 8563 women who had received cyclophosphamide and doxorubicin as adjuvant therapy for breast cancer and observed 43 cases of acute myeloid leukaemia or myelodysplastic syndromes (AML/MDS). The incidence of AML/MDS overall was seven times higher than expected rates in the general population, and was increased 3-fold in regimens that contained double the cumulative dose of cyclophosphamide.

Several case–control studies have also been reported. For leukaemia, Kaldor *et al.* (1990) investigated 114 cases of a cohort of ovarian cancer patients. The relative risks were, respectively, 2.2 and 4.1 in two increasing dose categories of cyclophosphamide. Neither increase was reported as statistically significant. Travis *et al.* (1994) carried out a study involving 35 cases of leukaemia following non-hodgkin lymphoma, and found that prior treatment with cyclophosphamide was associated with a relative risk of 1.8 that was not statistically significant when comparison was made to treatment with radiotherapy alone. In an investigation by Nandakumar *et al.* (1991) of 97 cases of myeloid leukaemia as second primary cancers, patients receiving cyclophosphamide had a relative risk of 12.6 compared to those treated surgically, and was substantially higher when prednisone was co-administered with cyclophosphamide. Curtis *et al.* (1992) compared 90 women who developed acute myeloid leukaemia following breast cancer to controls, and found that the risk of leukaemia was 2.6 times greater in those who had received cyclophosphamide, compared to women who had been treated by surgery only.

There have also been two case–control studies of bladder cancer in relation to cyclophosphamide. Kaldor *et al.* (1995) investigated 63 cases of bladder cancer following ovarian cancer, and found that in comparison to surgery alone, the relative risk associated with chemotherapy containing cyclophosphamide as the only potential bladder-cancer-causing agent was 4.2 (P = 0.025) in the absence of radiotherapy, and 3.2 (P = 0.08) with radiotherapy. Travis *et al.* (1995) studied 31 cases of bladder cancer and 17 cases of kidney cancer as well as matched controls within a cohort of 2-year survivors of non-Hodgkin lymphoma. The relative risk associated with cyclophosphamide treatment was 4.5 (P < 0.05) for bladder cancer, and 1.3 for kidney cancer.

2.1 Synthesis

The studies summarized above provide a comprehensive epidemiological basis for identifying cyclosphosphamide as an independent cause of acute myeloid leukaemia and bladder cancer, that fully supports the conclusions drawn from earlier case reports, and more limited studies. Several studies have assessed the risk of all second primary cancers following cyclophosphamide treatment, and some have found rates of occurrence that appear to be elevated, but have not provided evidence for risk of other specific cancer types.

3. Cancer in Experimental Animals

Cyclophosphamide has been tested for carcinogenicity by oral administration to mice and rats, by subcutaneous injection to mice, by topical application to mice, by intravenous injection to rats, by intraperitoneal injection to mice and rats, and by perinatal exposure to mice.

Oral administration of cyclophosphamide resulted in skin tumours in transgenic mice (Yamamoto *et al.*, 1996; Eastin *et al.*, 2001), and in urinary bladder carcinoma, leukaemia, and nervous system tumours in rats (Schmähl & Habs, 1979; Habs & Schmähl, 1983). Subcutaneous injection of cyclophosphamide to mice caused a variety of neoplasms, including mammary gland carcinoma and leukaemia (Schmähl & Osswald, 1970; Walker & Bole, 1971, 1973; Walker & Anver, 1979, 1983; Petru *et al.*, 1989).

Intravenous injection of cyclophosphamide to rats caused both benign and malignant neoplasms (Schmähl, 1967, 1974; Schmähl & Osswald, 1970).

Intraperitoneal administration of cyclophosphamide increased the incidences of lung adenoma and adenocarcinoma, bladder papilloma, and leukaemia in mice (Shimkin *et al.*, 1966; Weisburger *et al.*, 1975; Mahgoub *et al.*, 1999), and mammary gland adenoma and carcinoma in rats (Weisburger *et al.*, 1975).

Administration of cyclophosphamide to newborn mice caused lung and liver adenoma and carcinoma, and Harderian gland adenoma (Kelly *et al.*, 1974; McClain *et al.*, 2001).

See Table 3.1.

Table 3.1 Studies of cancer in experimental animals exposed to cyclophosphamide

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Oral administration				
Mouse, Tg ras H2/CB6F1 & B6C6F1 (M), 9 wk 26 wk Yamamoto et al. (1996)	0, 10, 30 mg/kg bw by gavage (in water, volume NR), twice/wk for 25 wk Initial number/group NR	Tg ras H2/CB6F1: Lung (adenomas)– 0/9, 3/16, 3/27 Multiplicity– 0, 0.19, 0.11 tumours/mouse	[NS][a]	Pharmaceutical grade
		CB6F1: Lung (adenomas)– 0/6, 2/18, 2/20 Multiplicity– 0, 0.11, 0.10 tumours/mouse	[NS][a]	
Mouse, Tg.AC (M, F), 8–9 wk 27 wk Eastin et al. (2001)	0, 10, 30, 60 mg/kg bw by gavage (in water 50% ethanol, volume NR); twice/wk for 26 wk 15/sex/group	Skin tumours (at all sites; histologically confirmed): 5/15, 1/2, 5/5, 5/15 (M); 2/15, 5/11, 11/11, 14/15 (F)	[$P < 0.0001$ for 30 and 60 mg/kg bw doses in female mice][a]	Purity NR; Tg.AC mice are transgenic mice that carry a v-Ha-*ras* oncogene
		Skin tumours (squamous cell papillomas of vulva): 2/15, 4/11, 10/11, 12/15 (F)	[$P \leq 0.0003$ for 30 and 60 mg/kg bw doses in female mice][a]	
		Leukaemia (erythrocytic): 0/15, 0/15, 4/15, 1/15 (F)	$P < 0.05$, for 30 mg/kg bw group	
Rat, Sprague-Dawley (M, F) Lifetime Schmähl & Habs (1979)	0, 0.31, 0.63, 1.25, 2.5 mg/kg bw in drinking-water, 5 ×/wk for life 40/sex/group	Malignant tumours: 4/38, 11/34, 14/36, 15/35, 13/31 (M); 5/34, 11/37, 13/37, 11/33, 9/27 (F)	[$P < 0.05$, for 3 highest doses]	Purity NR
		Urinary bladder (carcinomas): 0/38, 2/34, 2/36, 5/35, 7/31 (M); 0/34, 0/37, 0/37, 0/33, 1/27 (F)	[$P \leq 0.02$ for 2 highest doses in males][a]	
		Lymphoid and haematopoietic tissue (leukaemia): 0/72, 3/71, 6/73, 6/68, 4/58 (M, F)	[$P \leq 0.04$ for 3 highest doses for combined males and females][a]	
		Nervous system (sarcomas): 1/72, 7/71, 5/73, 6/68, 1/58 (M, F)	[$P \leq 0.05$ for 0.31 and 1.25 mg/kg doses for combined males and females][a]	

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (M), 100 d 20 mo Habs & Schmähl (1983)	0, 2.5 mg/kg bw in drinking-water, 5 ×/wk for 20 mo 100/group	Urinary bladder (papillomas or transitional-cell carcinomas): 0/63, 24/80 Nervous system tumours: 1/63, 11/80	[$P < 0.0001$][a] [$P < 0.0076$][a]	Reported as "chemically pure"
Rat, Sprague-Dawley (M), 100[a] Lifetime Schmähl & Habs (1983)	0, 2.5 mg/kg bw in drinking-water, 5 times/wk for life 100/group	Urinary bladder (papillomas): 0/100, 15/100 Urinary bladder (transitional-cell carcinomas): 0/100, 17/100	[$P < 0.0001$][a] [$P < 0.0001$][a]	Purity NR; only data on bladder tumours reported
Subcutaneous injection				
Mouse, NMRI (F) 52 wk Schmähl & Osswald (1970)	0, 26 mg/kg bw/wk (in solvent NR), for 5 wk 50/group	Malignant tumours (primarily mammary carcinomas): 3/46, 28/46	[$P < 0.001$][b]	Purity > 98%
Mouse, New Zealand Black/New Zealand White (F) 64 wk Walker & Bole (1971)	0, 8 mg/kg bw (in saline; volume NR), daily for 64 wk 16, 10	Neoplasms (mainly lymphomas): 0/16, 6/10	$P = 0.00002$	Purity NR
Mouse, New Zealand Black/New Zealand White (M, F) 93 wk Walker & Bole (1973)	0, 1, 8 mg/kg bw (in 100 µL saline), daily for 93 wk 20, 10, 10 per sex	Neoplasms (mainly lymphomas): 2/16, 3/9, 8/9 (M); 1/20, 1/10, 9/9 (F)	$P = 0.003$ for 8 mg/kg bw males; $P < 0.0001$ for 8 mg/kg bw females	Purity NR
Mouse, New Zealand Black/New Zealand White (F) Lifetime Walker & Anver (1979)	0, 5.7, 16 mg/kg bw (in 100 µL saline), daily for life 15, 17, 21	Neoplasms (mainly mammary carcinomas): 0/13, 15/15, 17/19 Mammary carcinomas: 0/13, 5/15, 16/19	[$P < 0.0001$ for 5.7 and 16 mg/kg bw groups][a] [$P \leq 0.03$ for 5.7 and 16 mg/kg bw groups][a]	Purity NR; treatment groups not started simultaneously
Mouse, New Zealand Black/New Zealand White (F), 6 wk Lifetime Walker & Anver (1983)	0, 56 mg/kg bw (in 100 µL saline), weekly for life 15, 22	Neoplasms: 0/13, 17/19	[$P < 0.0001$][a]	Purity NR; groups not started simultaneously; Neoplasms were mainly mammary gland carcinomas, lung adenomas and lymphomas

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, NMRI & AKR (F), 7 wk, Lifetime Petru et al. (1989)	0, 13, 26 mg/kg bw (in saline, volume NR), weekly for life 30/group	Leukaemia (NMRI mice): 2/30, 16/30, 10/30 Leukaemia (AKR mice): 30/30, 25/30, 19/30	$P \leq 0.027$ for 13 & 26 mg/kg bw groups $P \leq 0.006$ for 13 & 26 mg/kg bw groups	Purity NR [negative trend in AKR mice]
Skin application				
Mouse, Tg.AC (M, F), 8–9 wk 27 wk Eastin et al. (2001)	0, 10, 30, 90 mg/kg bw (in 50% ethanol, 3.3 mL/kg bw), 2 ×/wk for 26 wk 15/sex/group	Skin tumours (at site of application): 1/15, 0/15, 2/15, 3/15 (M); 1/15, 0/15, 0/15, 2/15 (F) Skin tumours (at all skin sites): 1/15, 2/15, 3/15, 3/15 (M); 4/15, 3/15, 9/15, 14/15 (F)	[NS][a] [$P = 0.0002$ for 90 mg/kg females][a]	Purity NR; Tg.AC mice are transgenic mice that carry a v-Ha-*ras* oncogene
Intravenous administration				
Rat, BR 46 (M) 23 mo Schmähl (1967)	0, 15 mg/kg bw (vehicle and volume NR), weekly (750 mg/kg bw total dose) 50, 40	Neoplasms (benign and malignant combined): 1/50, 14/26	[$P < 0.001$][b]	Purity > 98%
Rat, BR 46 (M) 23 mo Schmähl & Osswald (1970)	0, 13 mg/kg bw (vehicle and volume NR), weekly for 52 wk 89, 48	Neoplasms: 3/65, 4/36 (benign); 4/65, 6/36 (malignant)	[NS][b]	Purity > 98%
Rat, BR 46 (M) 23 mo Schmähl & Osswald (1970)	0, 33 mg/kg bw (vehicle and volume NR), 5 times every 2 wk 89, 96	Neoplasms: 3/65, 5/66 (benign); 4/65, 16/66 (malignant)	[$P < 0.01$, malignant tumours][b]	Purity > 98%
Rat, Sprague-Dawley (M) 700 d Schmähl, (1974)	0, 13 mg/kg bw (vehicle and volume NR), weekly (670 mg/kg bw total dose) 52, 32	Neoplasms (malignant): 6/52, 14/32	[$P < 0.001$][b]	Purity > 98%
Intraperitoneal administration				
Mouse, dd (M, F) 48 wk Tokuoka (1965)	0 or 5 mg/kg bw (in saline 5 mL/kg), 2 injections/wk for 15 wk 20, 29	Lung (adenomas or carcinomas): 1/20, 3/29 Liver (adenomas): 0/20, 2/29 Testis (interstitial cell tumours): 0/20, 4/29 Mammary gland (carcinomas): 1/20, 3/29	NS NS NS NS	Purity NR

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, A (M, F) 48 wk Tokuoka (1965)	0 or 5 mg/kg bw (in saline 10 mL/kg), 2 injections/wk for 15 wk 16, 25	Lung (adenomas or carcinomas): 2/16, 6/25 Testis (interstitial cell tumours): 0/16, 3/25	NS NS	Purity NR
Mouse, A/J (M, F, equally split) 39 wk Shimkin et al. (1966)	0, 32.2, 129, 516, 1609 µmol/kg bw (total dose; in 200 µL water), 3 injections/wk for 4 wk 360, 30, 30, 30, 30	Lung (adenomas or adenocarcinomas): 107/339, 12/30, 11/26, 20/27, 2/4 (incidence); 0.38, 0.4, 0.6, 1.3, 2.5 (tumours per mouse)	[$p < 0.001$ (for 516 µmol/kg bw dose, incidence)][b]	Purity NR
Mouse, Swiss-Webster-derived (M, F) 18 mo Weisburger et al. (1975)	0, 12, 25 mg/kg bw (vehicle and volume NR), 3 injections/wk for 6 mo 101, 25, 25 (M) 153, 25, 25 (F)	Lung (adenomas or adenocarcinomas): 10/101, 7/30 (M); 21/153, 10/35 (F) Bladder (papillomas or carcinomas): 3/101 & 4/30 (M)	$P = 0.031$ (M) and $P = 0.027$ (F) (combined 12 & 25 mg/kg bw vs control) $P = 0.048$ (combined 12 & 25 mg/kg bw vs control)	Purity NR; not all control mice were treated with the vehicle
Mouse, 129/Sv & 129/Sv X C57BL/6 *Nf1*$^{+/+}$ & *Nf1*$^{+/-}$(sex NR), 6–10 wk 15 mo Mahgoub et al. (1999)	0 or 100 mg/kg bw/wk (solvent and volume NR) for 6 wk 129/Sv *Nf1*$^{+/+}$: 31 & 5 mice 129/Sv *Nf1*$^{+/-}$: 46 & 12 mice 129/Sv X C57BL/6 *Nf1*$^{+/+}$: 14 & 15 mice 129/Sv X C57Bl/6 *Nf1*$^{+/-}$: 412 & 25 mice	Leukaemia (129/Sv *Nf1*$^{+/+}$): 2/31, 0/5 Leukaemia (129/Sv *Nf1*$^{+/-}$): 8/46, 7/12 Leukaemia (129/Sv X C57BL/6 *Nf1*$^{+/+}$): 0/14, 2/25 Leukaemia (129/Sv X C57BL/6 *Nf1*$^{+/-}$): 0/12, 7/25	 $P = 0.004$	Purity NR
Rat, Sprague-Dawley (M, F) 18 mo Weisburger et al. (1975)	0, 5, 10 mg/kg bw (vehicle and volume NR), 3 injections/wk for 6 mo 179, 25, 25 (M) 181, 25, 28 (F)	Mammary gland (adenomas): 2/105 & 24/53 (F; combined 5 & 10 mg/kg bw) Mammary gland (carcinomas): 13/105 & 9/53 (F; combined 5 & 10 mg/kg bw)	$P = 0.028$ $P = 0.035$	Purity NR; not all control rats were treated with the vehicle

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Perinatal exposure				
Mouse, CD-1 (M, F) 79 wk Kelly et al. (1974)	i.p. injection 0, 0.8, 4.0, 20.0 mg/kg bw (in 10 µL/kg saline), on postnatal Days 1, 3, 6 30/sex/group	Lung (adenomas): 0/28, 2/29, 4/27, 0/21 (M); 1/25, 2/27, 2/28, 3/21 (F)	$P < 0.05$ for 4 mg/kg bw males (life-table analysis)	Purity NR; the 20 mg/kg dose caused marked bw changes and nearly 100% mortality
Mouse, CD-1 (M, F) 1 yr McClain et al. (2001)	Oral 0, 10, 20, 40, 60 mg/kg bw by gavage (100 µL and 200 µL) on postnatal Days 8 & 15 [solvent NR] 48 (control), 24/sex	Liver (adenomas): 2/48, 2/24, 4/24, 6/24, 5/24 (M)	[$P < 0.04$ for 40 & 60 mg/kg bw][a]	Purity NR
		Liver (carcinomas): 0/48, 0/24, 1/24, 6/24, 1/24 (M)	[$P = 0.0009$ for 40 mg/kg bw][a]	
		Lung (adenomas): 3/48, 0/24, 8/24, 12/24, 13/24 (M); 7/48, 3/24, 6/24, 16/24, 13/24 (F)	[$P < 0.005$ for 20, 40, & 60 mg/kg bw (M); 40 & 60 mg/kg bw (F)][a]	
		Lung (carcinomas): 0/48, 1/24, 0/24, 6/24, 3/24 (M); 0/48, 1/24, 3/24, 3/24, 0/24 (F)	[$P < 0.03$ for 40 & 60 mg/kg bw (M); 20 & 40 mg/kg bw (F)][a]	
		Harderian gland (adenomas): 2/48, 1/24, 1/24, 1/24, 5/24 (F)	[$P < 0.04$ for 60 mg/kg bw][a]	
Pre and postnatal exposure				
Mouse, BR 46 (M, F) 24 mo Roschlau & Justus (1971)	i.p injection 25 mg/kg bw on gestation Day 14 [solvent and volume NR]. Male and female offspring treated every 2 wk for a total of 30 times Initial number NR	Lung (adenomas): male offspring 4/16, 2/16; female offspring 5/12 & 1/18	NS	Purity NR
		Lung (carcinomas): male offspring 0/16, 3/16; female offspring 0/12, 4/18	NS	

[a] Current Working Group analysis (Fisher Exact test)
[b] Previous Working Group analysis
bw, body weight; d, day or days; F, female; i.p., intraperitoneal; M, male; mo, month or months; NR, not reported; NS, not significant; vs, versus; wk, week or weeks, yr, year or years

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In most species, cyclophosphamide is rapidly absorbed, metabolized, and excreted. Its metabolic pathway has been studied in several species including mice, rats, hamsters, rabbit, dogs, sheep, and monkeys. Cyclophosphamide is not cytotoxic *per se,* because it requires metabolic activation before it can act as an alkylating agent. Activation takes place predominantly in the liver, although this may occur in other tissues (IARC, 1981).

Cyclophosphamide undergoes metabolism to several intermediates with alkylating activity. The principal metabolites identified are phosphoramide mustard, and acrolein. Phosphoramide mustard can undergo dephosphoramidation to yield nornitrogen mustard, which also has alkylating activity. Metabolites of cyclophosphamide can interact with DNA and proteins, resulting in the formation of adducts. The metabolism of cyclophosphamide and DNA adducts formation are summarized in Fig. 4.1.

A minor pathway results in dechloroethylation and the formation of 2-dechloroethylcyclophosphamide and another alkylating agent, chloroacetaldehyde (Balu *et al.*, 2002).

The other compounds such as 4-ketocyclophosphamide and propionic acid derivative are relatively non-toxic, and are the major urinary metabolites of cyclophosphamide in several species (IARC, 1981).

4.2 Genetic and related effects

4.2.1 Interaction with DNA

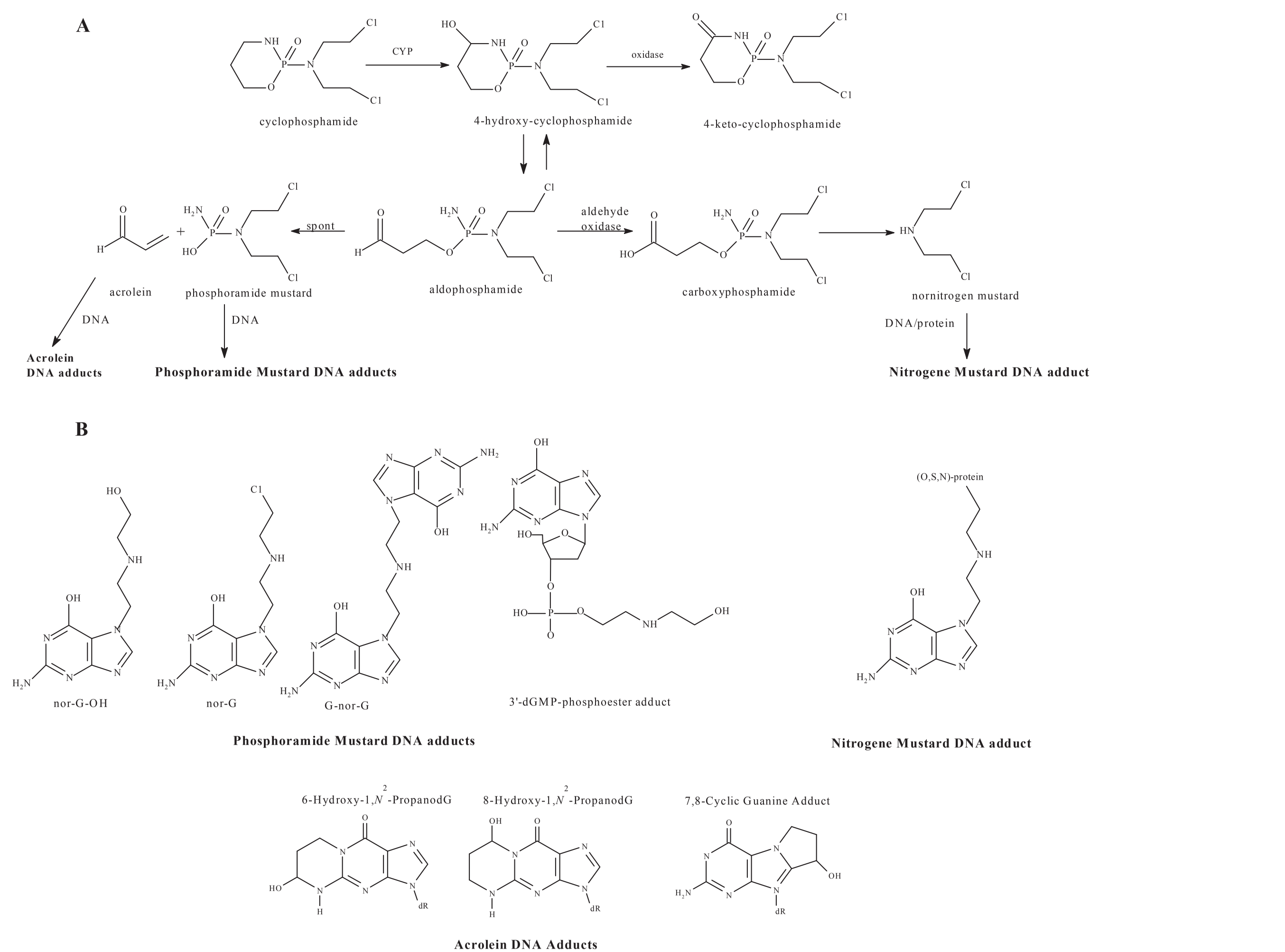
Using 4-hydroperoxycyclophosphamide as an activated form of cyclophosphamide, Mirkes *et al.* (1992) identified by mass spectrometric analysis the formation of the monofunctional adduct *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl]amine (nor-G) and the bifunctional adduct *N,N*-bis[2-(7-guaninyl)ethyl]amine (G-nor-G) in rat embryos in in-vitro culture. The monofunctional adduct *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine (nor-G-OH) was detected in bladder tissue of rats injected with [^{3}H] cyclophosphamide (Benson *et al.*, 1988). Using ^{32}P-postlabelling analysis, a phosphotriester was shown to be formed: (1) when phosphoramide mustard was reacted with deoxyguanosine 5′-monophosphate, (2) when cyclophosphamide was incubated with calf thymus DNA in the presence of reconstituted cytochrome P450 (CYP) metabolizing system, and (3) in liver DNA from mice injected intraperitoneally with cyclophosphamide (Maccubbin *et al.*, 1991).

Nornitrogen mustard reacts with guanosine and with guanine bases in DNA to form nor-G initially, but this is converted to a hydroxylated derivative (nor-G-OH), and to a cross-linked (between guanines) adduct (G-nor-G) (Hemminki, 1987). Both monofunctional adducts, but not the cross-linked adduct, were also detected when phosphoramide mustard was reacted with DNA (Cushnir *et al.*, 1990). Acrolein reacts with DNA to form O^6-(*n*-propanalyl)guanine, and the product of chloroacetaldehyde reaction with DNA is O^6-(ethanalyl)guanine (Balu *et al.*, 2002). Acrolein can produce exocyclic adducts in DNA, including 1,N^2-hydroxypropanodeoxyguanosine and 1,N^6-hydroxypropanodeoxyadenosine (Chung *et al.*, 1984; Foiles *et al.*, 1990; Smith *et al.*, 1990). The former was detected in acrolein-treated human fibroblasts and in peripheral blood lymphocytes of a dog treated with cyclophosphamide (Wilson *et al.*, 1991).

Nornitrogen mustard also reacts covalently with proteins, and a method for the detection of cysteine-34 residue adducts in human serum albumin has been described (Noort *et al.*, 2002).

The single-cell gel comet assay is used to detect single-strand breaks and other alkali-labile lesions in DNA exposed to cyclophosphamide.

Fig. 4.1 Metabolic pathway of cyclophosphamide



A. Metabolism of cyclophosphamide to phosphoramide mustard, acrolein, and nornitrogen mustard. Cyclophosphamide is metabolized by CYP enzymes to 4-hydroxycyclophosphamide, wich equilibrates with aldophosphamide to spontaneously yield phosphoramide mustard and acrolein. Aldophosphamide is also metabolized by aldehyde oxidase to carboxyphosphamide, which produces nornitrogen mustard. 4-Hydroxy-cyclophosphamide can be oxidized to the inactive 4-keto-cyclophosphamide.

B. Phosphoramide mustard produces multiple monofunctional and bifunctional adducts with guanine, and acrolein forms exocyclic adducts. Nornitrogen mustard forms mono- and bifunctional adducts with guanine.

From Povirk & Shuker (1994), Anderson et al. (1995), Khan et al. (1998)

CYP, cytochrome P450; nor-G, *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl]amine; G-nor-G, *N,N*-bis[2-(7-guaninyl)ethyl]amine; nor-G-OH, *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl] amine; dR, deoxyribose

In vitro studies have demonstrated the comet-forming activity of cyclophosphamide in human hepatoma (Hep G2) cells (Uhl *et al.*, 2000; Yusuf *et al.*, 2000), in primary cultures of rat and human urinary bladder cells (Robbiano *et al.*, 2002), in primary cultures of human leukocytes in the presence of metabolic activation system S9 mix (Hartmann *et al.*, 1995; Hartmann & Speit, 1995; Frenzilli *et al.*, 2000), and in extended-term cultures of human T-lymphocytes, also in the presence of S9 (Andersson *et al.*, 2003). Comet formation was also detected *in vivo* in the urinary bladder mucosa of rats given cyclophosphamide orally (Robbiano *et al.*, 2002), and in peripheral blood cells of patients administered the drug (Hartmann *et al.*, 1995).

4.2.2 Genotoxic effects in humans

There are few reports of DNA-adduct formation by cyclophosphamide in humans. Acrolein-derived DNA adducts, detected by immunochemical methods, were found in blood leukocytes of cancer patients receiving cyclophosphamide (McDiarmid *et al.*, 1991). In another study, mono-adducts and inter-strand cross-links derived from phosphoramide mustard were detected in a single patient administered 1 g/m^2 cyclophosphamide (Souliotis *et al.*, 2003). Increased DNA damage (comet formation) was also observed in the lymphocytes of patients administered cyclophosphamide (Hartmann *et al.*, 1995).

Increased frequencies of several biomarkers of genotoxicity have been observed in the lymphocytes of patients treated with cyclophosphamide, relative to control subjects. These include mutations at the hypoxanthine-(guanine) phosphoribosyl transferase (*HPRT*) locus (Palmer *et al.*, 1986, 1988; Tates *et al.*, 1994; Sanderson *et al.*, 2001), and sister chromatide exchange (Raposa & Várkonyi, 1987; McDiarmid *et al.*, 1990; Sardaş *et al.*, 1994; Mertens *et al.*, 1995; Hartmann *et al.*, 1995).

Other studies reported positive findings for elevated chromosomal aberrations frequencies (Sessink *et al.*, 1994; Rubes *et al.*, 1998; Burgaz *et al.*, 2002), and micronuclei (Yager *et al.*, 1988; Tates *et al.*, 1994; Zúñiga *et al.*, 1996; Burgaz *et al.*, 1999; Rekhadevi *et al.*, 2007) in medical personnel exposed to cyclophosphamide. Increases in frequencies of micronuclei were also detected in buccal cells in some studies (Cavallo *et al.*, 2005; Rekhadevi *et al.*, 2007), but not in another (Burgaz *et al.*, 1999).

4.2.3 Genotoxic effects in experimental systems

(a) Mutagenic effects in vitro

The previous *IARC Monograph* (IARC, 1987b) states that cyclophosphamide induced chromosomal aberrations, sister chromatid exchange, and DNA damage in human cells *in vitro*. It also induced morphological transformation, chromosomal aberrations, sister chromatid exchange, mutation, and unscheduled DNA synthesis (UDS) in rodent cells *in vitro*. It further induced aneuploidy, mutation, recombination, gene conversion, and DNA damage in fungi. It was also reported to act as a mutagen and DNA-damaging agent in bacteria.

The mutagenicity of cyclophosphamide in *Salmonella typhimurium* was enhanced by increased induction of CYPs in S9 liver fractions by a combination of β-naphthoflavone and sodium phenobarbital (Paolini *et al.*, 1991a). Comparison of S9 from liver and kidney of pregnant mice revealed that liver S9 was more effective in activating cyclophosphamide to mutagenic metabolites in *S. typhimurium*, and also in inducing sister chromatid exchange in human peripheral lymphocytes, and Chinese hamster ovary (CHO) cells (Winckler *et al.*, 1987).

In *Saccharomyces cerevisiae*, higher rates of mitotic gene conversion and point mutation by cyclophosphamide were associated with induction of class 2B CYPs in co-cultured epithelial cell lines from fetal mouse liver (Paolini *et al.*, 1991b). A recombinant plasmid containing a full-length cDNA encoding the rat cytochrome CYP2B1 introduced into *S. cerevisiae* also increased the mutation frequency induced by cyclophosphamide (Black *et al.*, 1989).

CYP2B1 expressed in Chinese hamster V79-derived SD1 cell lines also potentiated cyclophosphamide mutagenesis (6-thioguanine resistance), whereas CYP1A1 expressed in V79-derived XEM_2 cell lines did not (Doehmer *et al.*, 1990, 1992).

Cyclophosphamide was weakly mutagenic (detected by induction of resistance to 6-thioguanine) in differentiated Reuber hepatoma cells H4IIEC3/G^-, but markedly cytotoxic and clastogenic (micronucleus formation) (Roscher & Wiebel, 1988), and also mutagenic in a Chinese hamster epithelial liver cell line (6-thioguanine resistance) (Turchi *et al.*, 1992), and in Chinese hamster lung (CHL) cells in the presence of S9, as measured at microsatellite loci (Kikuno *et al.*, 1995).

Using 4-hydroperoxycyclophosphamide and phosphoradiamidic mustard, the role of different repair enzymes in defining sensitivity was investigated by Andersson *et al.* (1996) in CHO cells. Mutations in excision repair cross-complementing *ERCC1* and *ERCC4* genes caused hypersensitivity to the cyclophosphamide analogues.

Cyclophosphamide induced sister chromatid exchange in mouse primary bone-marrow and spleen cells (Soler-Niedziela *et al.*, 1989), and micronuclei in mouse lymphoma in L5178Y $tk^{+/-}$ cells (Kirsch-Volders *et al.*, 2003), and in parental V79 cells (Kalweit *et al.*, 1999) in the presence of rat liver S9. Of several V79 cell lines engineered to express rat CYPs, increases in micronuclei (Ellard *et al.*, 1991) and sister chromatid exchange (Kulka *et al.*, 1993) were seen in the cells expressing CYP2B1. The rat hepatoma cells lines H4IIEC3/G^- and 2sFou were also susceptible to micronuclei induction by cyclophosphamide (Tafazoli *et al.*, 1995).

Human T-lymphocytes were more susceptible than B-lymphocytes to both chromosomal aberrations and sister chromatid exchange induction by cyclophosphamide in the presence of rat liver S9 (Miller 1991a, b). This difference between T- and B-lymphocytes was not found with mouse cells treated with 4-hydroxycyclophosphamide or phosphoramide mustard (Kwanyuen *et al.*, 1990). In another study (Kugler *et al.*, 1987), rat liver microsomal mix was more effective than rat liver S9 in activating cyclophosphamide to induce chromosomal aberrations. Human lymphocytes from women carrying mutations in the breast cancer susceptibility gene *BRCA1* were more susceptible to micronuclei induction than cells from non-carriers (Trenz *et al.*, 2003). Hep G2 human hepatoma cells were susceptible to sister chromatid exchange and micronuclei induction by cyclophosphamide (Natarajan & Darroudi, 1991) and, in analogous studies, the S9 microsomal fraction of these cells were shown to be capable in activating cyclophosphamide to induce sister chromatid exchange and micronuclei in CHO cells (Darroudi & Natarajan, 1993). Human dental pulp cells formed chromosomal aberrations when exposed to cyclophosphamide in the presence of rat liver S9 (Tsutsui *et al.*, 2006).

In the presence of rat liver S9, cyclophosphamide induced morphological transformation of BALB/3T3 mouse embryonic fibroblast cells (McCarvill *et al.*, 1990).

(b) *Mutagenic effects in vivo*

The previous *IARC Monograph* (IARC, 1987b) states that cyclophosphamide was found to bind to kidney, liver and lung DNA in mice. It also induced dominant lethality, chromosomal aberrations, micronuclei, sister chromatid exchange, mutations, and DNA damage in rodents *in vivo*. In *Drosophila*, it induced aneuploidy, heritable translocations, and somatic and sex-linked recessive lethal mutations. In patients administered cyclophosphamide, increased incidences of chromosomal aberrations and sister chromatid exchange in peripheral lymphocytes and bone marrow were observed.

In *Drosophila melanogaster*, cyclophosphamide tested positive for the somatic white-ivory mutation (Batiste-Alentorn *et al.*, 1994), and produced chromosome breaks in spermatocytes (Zijlstra & Vogel, 1989).

Several studies have examined the mutagenic effects of cyclophosphamide in transgenic mice. In MutaMouse, mutation induction was observed in bone marrow (other tissues not studied) (Hoorn *et al.*, 1993). In Big Blue mice, mutation frequencies were elevated in the liver, but not in the testis or spleen in one study (Hoyes *et al.*, 1998), and in another study, in the lung and urinary bladder, but not in the kidney, bone-marrow or splenic T-cells (Gorelick *et al.*, 1999). Another study compared the *lacI* locus in Big Blue mice with the *Hprt* locus in conventional B6C3F1 mice, and cyclophosphamide induced mutations in the endogenous gene in splenic lymphocytes, but not in the transgene (Walker *et al.*, 1999). In rats, cyclophosphamide produced the 'common deletion' mutation in liver mitochondrial DNA, and folic acid supplementation was found to be protective against this damage (Branda *et al.*, 2002).

In two related studies investigating oncogene and tumour-suppressor gene expression in mice, cyclophosphamide was found to induce expression of several genes, including *c-Myc* and *Tp53*, in the spleen and thymus, but not in other tissues (Ember *et al.*, 1995; Ember & Kiss, 1997).

Many studies have investigated the cytogenicity of cyclophosphamide in newts, rodents, dogs, and non-human primates. Results are invariably positive for this compound, and are summarized in Table 4.1.

(c) Mutagenic effects in germ cells

Anderson *et al.* (1995) reviewed the activity of cyclophosphamide in germ cells, and in summary, the germ cell stages that are most sensitive to cyclophosphamide are the postmeiotic stages. Tests for germ-cell damage that examine effects in F_1 progeny in which cyclophosphamide gave positive results include dominant lethality, heritable translocations, specific locus mutations, and malformations. Although cyclophosphamide is not an effective aneugen, it causes structural and numerical chromosomal damage in second meiotic metaphases and first cleavage metaphases, and in F_1 embryos. It is also positive for inducing sister chromatid exchange in germ cells and causes abnormal sperm-head morphology. Most studies have been carried out in mice, but positive results have also been observed in rats and rabbits, e.g. induction of unscheduled DNA synthesis in the testes (reviewed in Anderson *et al.*, 1995), and also in hamsters (Waters & Nolan, 1995).

More recent studies in mice have demonstrated the dominant lethal effects of cyclosphosphamide (Dobrzyńska *et al.*, 1998) as well as intrachromosomal gene conversion and mutation events primarily in meiotic stage cells (Schimenti *et al.*, 1997). In female rats, administration of cyclophosphamide at 16 days of gestation significantly increased nucleolar and synaptonemal complex fragmentation (Cusidó *et al.*, 1995), and in male rats chronic exposure to cyclophosphamide disrupted meiotic events before pachynema during spermatogenesis (Barton *et al.*, 2003).

(d) Modulation of mutagenicity by other agents

A large number of studies have investigated the effects of agents in modulating the genotoxicity of cyclophosphamide, and are summarized in Table 4.2.

4.3 Mechanisms of carcinogenesis

All of the available evidence indicates that cyclophosphamide exerts its carcinogenic activity via a genotoxic mechanism (McCarroll *et al.*, 2008). The metabolite widely thought to be responsible for the antitumour activity

Table 4.1 Positive cytogenicity studies of cyclophosphamide in newts, rodents, dogs, and non-human primates

Species	Cytogenetic end-point investigated	Additional considerations	Reference
Mouse	SCE	Bone-marrow cells. Reduction in frequency with increasing numbers of cell division	Morales-Ramírez et al. (1990)
Mouse	SCE	Bone-marrow cells. A comparison of wild and laboratory mice	Huang et al. (1990)
Mouse	MN	Bladder epithelial cells	Konopacka (1994)
Mouse	CA	Bone-marrow cells. Effects of malnutrition and alcohol	Terreros et al. (1995)
Mouse	MN	Peripheral blood reticulocytes and PCE in bone marrow	Hatanaka et al. (1992)
Mouse	MN	Splenocytes	Benning et al. (1992)
Mouse	MN	Bone-marrow PCE. Comparison of i.p. and p.o. administration	Wakata et al. (1989)
Mouse	MN	7 organs compared (bone marrow, forestomach, stomach, small intestine, large intestine, urinary bladder, lung)	Sycheva (2001)
Mouse	Intrachromosomal recombination	Spleen cells Transgenic mouse model with *lacZ* transgenic expression depending on somatic interchromosomal inversion	Sykes et al. (1998)
Mouse	MN	PCE in adult bone-marrow cells and fetal liver cells. Male, female, pregnant female, and fetal mice compared	Harper et al. (1989)
Mouse	MN SCE	Transplacental exposure; fetal liver cells	Porter & Singh (1988)
Mouse	MN CA	Bone-marrow and peripheral blood cells (CA) and peripheral blood erythrocytes (MN). Chronic ingestion of cyclosphosphamide; results positive for MN, negative for CA	Director et al. (1998)
Mouse	MN	Bone-marrow cells. In-vivo/in-vitro assay	Odagiri et al. (1994)
Mouse	CA SCE	Bone-marrow and spleen cells. In-vivo/in-vitro assay vs in-vivo assay	Krishna et al. (1987)
Mouse	SCE	Bone-marrow and spleen cells. In-vivo/in-vitro assay vs in-vivo assay	Krishna et al. (1988)
Rat	CA	Liver cells of neonates exposed *in utero*	Saxena & Singh (1998)
Rat	CA	Bone-marrow cells. Comparison in liver cells before and after partial hepatectomy of treated rats	Rossi et al. (1987)
Rat	CA SCE	Bone-marrow cells. Regenerating hepatocytes (SCE)	Masuda et al. (1990)
Rat	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Hayashi et al. (1992)
Mouse	MN	Bone-marrow PCE (positive), hepatocytes (negative)	Parton & Garriott (1997)
Rat	MN	Bone-marrow cells and peripheral blood reticulocytes. 14 rat strains compared	Hamada et al., (2001)
Rat	MN	Bone-marrow cells and peripheral blood reticulocytes. Effect of ageing studied	Hamada et al. (2003)
Rat	MN	Pre-estrous vaginal cells	Zúñiga-González et al. (2003)

Table 4.1 (continued)

Species	Cytogenetic end-point investigated	Additional considerations	Reference
Rat	CA MN	Bone-marrow cells. Simultaneous evaluation of two end-points in the same animal	Krishna et al. (1991)
Rat	MN	Bone-marrow, spleen, peripheral blood cells	Abramsson-Zetterberg et al. (1999)
Rat	MN	Embryos, treatment during pre-implantation period	Giavini et al. (1990)
Rat	MN CA	Bone-marrow and spleen cells. In-vivo/in-vitro assay	Moore et al. (1995)
Newt	MN	Larvae exposed to agent. Red blood cells	Fernandez et al. (1989)
Mouse, Chinese hamster	CA SCE	Bone-marrow cells Comparison of different routes of administration	Jenderny et al. (1988)
Rat, mouse	MN SCE Sperm morphology	Bone-marrow cells (MN). Splenocytes (SCE). Rats more susceptible than mice	Simula & Priestly (1992)
Rat, mouse, Chinese hamster	MN SCE	Bone-marrow cells. Species comparison Susceptibility ranked into the order rat > mouse > Chinese hamster	Madle et al. (1986)
Mouse, rat, Chinese hamster, Armenian hamster, guinea-pig	CA	Bone-marrow cells. Interspecies comparison Susceptibility ranked into the order guinea-pig > rat > mouse > Chinese hamster > Armenian hamster	Nersessian et al. (1992)
Dog (beagle)	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Harper et al. (2007)
Monkey	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Hotchkiss et al. (2008)
Marmoset	MN	Peripheral blood erythrocytes	Zúñiga-González et al. (2005)

CA, chromosomal aberrations; i.p., intraperitoneal; MN, micronuclei; PCE, polychromatic erythrocytes; p.o., per oral; SCE, sister chromatid exchange; vs, versus

Table 4.2 Studies of modulation of cyclophosphamide genotoxicity *in vivo* and *in vitro*

Agent	Experimental system	End-point measured	Effect	Reference
Retinol Retinoic acid	CHEL cells *in vitro*	SCE	Inhibitory	Cozzi et al. (1990)
Apigenin	Human lymphocytes + S9 *in vitro*	SCE CA	Inhibitory	Siddique et al. (2008)
β-carotene Retinal α-tocopherol Riboflavin	Human lymphocytes + S9 *in vitro*	SCE	Inhibitory	Edenharder et al. (1998)
Vitamin C	Human lymphocytes *in vitro*	SCE	Enhancing	Edenharder et al. (1998)
Vitamin K_1	Human lymphocytes *in vitro*	SCE	Inhibitory or enhancing (dependent on timing)	Edenharder et al. (1998)
Melatonin	CHO cells + S9 *in vitro*	SCE CA	Inhibitory	De Salvia et al. (1999)
Melatonin	CHO cells + S9 *in vitro*	Comet formation (DNA damage)	Inhibitory	Musatov et al. (1998)
O^6-alkylguanine-DNA alkyltransferase (AGT)	CHO cells *in vitro*	*Hprt* mutation	Inhibitory	Cai et al. (1999)
Buthionine sulfoximine	V79 cells and CHO +S9 *in vitro*	SCE	Enhancing	Köberle & Speit (1990)
Prostaglandin E_2	Mouse lymphoid L1210 leukaemia cells *in vivo*	SCE	Enhancing	Mourelatos et al. (1995)
Garlic extract	Swiss albino mice *in vivo*	CA (bone-marrow cells)	Inhibitory	Shukla & Taneja (2002)
Indole-3-carbinol	Swiss albino mice *in vivo*	CA (bone-marrow cells)	Inhibitory	Shukla et al. (2004)
Ascorbic acid	Pregnant CBA/CaH mice *in vivo*	CA SCE (pre-implantation embryos)	Inhibitory (SCE no effect)	Kola et al. (1989)
Ascorbic acid	Pregnant NMRI Kisslegg mice *in vivo*	CA SCE (pre-implantation embryos)	Inhibitory (SCE no effect)	Vogel & Spielmann (1989)
β-glucan	Male CD-1 mice *in vivo*	CA (bone-marrow and spermatogonial cells)	Inhibitory	Tohamy et al. (2003)

Table 4.2 (continued)

Agent	Experimental system	End-point measured	Effect	Reference
Nafenopin	Male Wistar rats *in vivo*	CA MN	Enhancing CA in bone marrow and MN in hepatocytes. Inhibitory on MN in bone marrow	Voskoboinik et al. (1997)
Prostaglandin E_2	BALB/c mice inoculated with Ehrlich ascites tumour cells *in vivo*	SCE (Ehrlich ascites tumour cells)	Inhibitory	Mourelatos et al. (1993)
Ginsenoside Rh_2	Male C57BL/6 mice *in vivo*	MN (bone-marrow cells) Comet formation (DNA damage) (white blood cells)	Inhibitory	Wang et al. (2006)
Verapamil	Male BALB/c and C57BL/6 mice *in vivo*	CA (bone-marrow cells)	Enhancing	Nesterova et al. (1999)
Citrus extract	Male BALB/c mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Hosseinimehr & Karami (2005a)
Captopril	Male NMRI mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Hosseinimehr & Karami (2005b)
Spirulina fusiformis	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Premkumar et al. (2001a)
Saffron (*Crocus sativus* L.)	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Premkumar et al. (2001b)
Melatonin and its derivatives	Male albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Elmegeed et al. (2008)
Vitamin C	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Ghaskadbi et al. (1992)
Malaria infection	Female C57BL/6 mice	MN (bone-marrow cells)	Inhibitory	Poça et al. (2008)
Lipoic acid	Male Wistar rats *in vivo*	MN (bone-marrow cells and peripheral blood cells)	Inhibitory	Selvakumar et al. (2006)
Folic acid	Newborn Wistar rats (fetal exposure) *in vivo*	MN (peripheral blood erythrocytes)	Inhibitory	Gómez-Meda et al. (2004)
Taenia taeniformis infection	Sprague-Dawley rats	MN (peripheral blood erythrocytes)	Enhancing	Montero et al. (2003)
O^6-methylguanine-DNA methyltransferase	C57BL/6 wild type and *Mgmt*$^{-/-}$ mice	*Hprt* mutation (splenic lymphocytes)	Inhibitory (non-significant)	Hansen et al. (2007)

CA, chromosomal aberrations; CHEL, Chinese hamster epithelial liver; CHO, Chinese hamster ovary; Hprt, hypoxanthine(guanine)phosphoribosyl transferase; MN, micronuclei; SCE, sister chromatid exchange of cyclophosphamide is the phosphoramide mustard (Povirk & Shuker, 1994). This metabolite is also generally considered to be the most genotoxic, but the contribution of acrolein, which is highly toxic, to the genotoxic activity of cyclophosphamide is less clear.

It is well established that the treatment of cancer patients with cyclophosphamide results in inflammation of the urinary bladder (haemorrhagic cystitis), which is not seen with other alkylating agents (Forni *et al.*, 1964; Liedberg *et al.*, 1970). In rats, cyclophosphamide treatment resulted in cystitis as well (Crocitto *et al.*, 1996), and in mice, mutagenic activity has been detected in urine following cyclophosphamide treatment (Te *et al.*, 1997). The ultimate alkylating metabolite of cyclophosphamide, phosphoramide mustard, is metabolized but was not shown to cause cytotoxicity and had minimal morphological effects on the mouse bladder, but an intermediate in the formation of the acrolein metabolite, diethylcyclophosphamide administered by intraperitoneal injection, caused severe cystitis in male rats, and less extensive inflammation in female rats (Cox, 1979). Acrolein administered to rats by intraperitoneal injections increased urothelial cell proliferation (Sakata *et al.*, 1989). Acrolein is the only metabolite of cyclophosphamide that is known to be both reactive and cytotoxic (IARC, 1995). Collectively, these data indicate that acrolein is the likely causative agent in cyclophosphamide-induced cystitis. Cystitis is an established condition associated with the development of both squamous cell and urothelial bladder cancers (Michaud, 2007). However, intraperitoneal injections of acrolein by itself only induced bladder hyperplasia, not cancer (Cohen *et al.*, 1992), and oral administration studies in mice and rats did not result in carcinogenic effects (IARC, 1995). Thus it is plausible that acrolein-induced cystitis plays a promoting role in cyclophosphamide bladder tumorigenesis that is initiated by other cyclophosphamide metabolites.

The protective effect of O^6-alkylguanine-DNA alkyltransferase (AGT) against cyclophosphamide mutagenicity (*Hprt* mutations) (Cai *et al.*, 1999), and cytotoxicity (Friedman *et al.*, 1999) in CHO cells implies some involvement of acrolein-derived DNA damage. However, mice deficient in this protein (called O^6-methylguanine-DNA methyl transferase [MGMT] in this study) were less susceptible to cyclophosphamide tumorigenesis, not more (Nagasubramanian *et al.*, 2008). Studies of sister chromatid exchange induced in human lymphocytes by acrolein and phosphoramide mustard suggest that phosphoramide mustard is the more potent genotoxic agent (Wilmer *et al.*, 1990). Furthermore, analysis of *TP53* mutations in cyclophosphamide-associated human bladder cancers suggests that the mutations detected are characteristic of DNA damage caused by phosphoramide mustard, rather than by acrolein (Khan *et al.*, 1998).

4.4 Synthesis

Cyclophosphamide, after its bioactivation to alkylating metabolites, is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of cyclophosphamide. Cyclophosphamide causes cancer of the bladder, and acute myeloid leukaemia.

There is *sufficient evidence* in experimental animals for the carcinogenicity of cyclophosphamide.

Cyclophosphamide is *carcinogenic to humans (Group 1).*

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