Handbook 1

all-*trans*-Retinoic acid

1. Chemical and Physical Characteristics

1.1 Nomenclature

See General Remarks, section 1.4

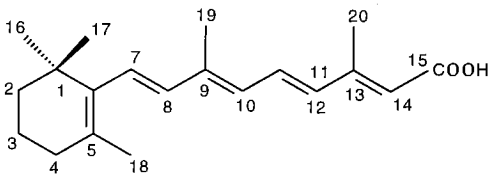
1.2 Name: all-*trans*-Retinoic acid

Chemical Abstracts Services Registry Number
302-79-4

IUPAC Systematic Name
(all-*E*)-9,13-Dimethyl-7(1,1,5-trimethylcyclohex-5-en-6-yl)nona-7,9,11,13-tetraen-15-oic acid (see 1.3), or (all-*E*)-3,7-dimethyl-9-(2,2,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraen-1-oic acid

Synonyms
Vitamin A acid, vitamin A_1 acid, *trans*-retinoic acid, tretinoin; Retin-A, Aberel, Airol, Aknoten, Atra, Cordes Vas, Dermairol, Epi-Aberol, Eudyna, Vesanoid.

1.3 Structural and molecular formulae and relative molecular mass



Composition: $C_{20}H_{28}O_2$
Relative molecular mass: 300.45

1.4 Physical and chemical properties

Description
Yellow crystals from ethanol

Melting-point
180–182°C (Budavari *et al.*, 1996)

Solubility
Soluble in most organic solvents, fats and oils; sparingly soluble in water (0.21 mmol/L) (Szuts & Harosi, 1991).

Spectroscopy
UV and visible: λ_{max} 350 (ethanol), $E\ {}^{1\%}_{1\ cm}$ 1510, E_M 45 300 (Frickel, 1984; Barua & Furr, 1998).

Nuclear magnetic resonance
^{1}H-NMR ($CDCl_3$, 220 MHz): δ 1.02 (1-CH_3), 1.47 (2-CH_2), 1.62 (3-CH_2), 1.72 (5-CH_3), 2.01 (9-CH_3), 2.02 (4-CH_2), 2.37 (13-CH_3), 5.79 (14-H), 6.14 (8-H), 6.15 (10-H), 6.29 (7-H), 6.31 (12-H), 7.03 (11-H); $J_{7,8}$ (16 Hz), $J_{10,11}$ (11.5 Hz), $J_{11,12}$ (15 Hz) (Schweiter *et al.*, 1969; Vetter *et al.*, 1971; Frickel, 1984; Barua & Furr, 1998).

^{13}C-NMR ($CDCl_3$, 68 MHz) δ 12.9 (9-CH_3), 13.9 (13-CH_3), 19.5 (3-C), 21.6 (5-CH_3), 29.0 (1,1-CH_3), 33.3 (4-C), 34.5 (1-C), 40.0 (2-C), 118.5 (14-C), 128.7 (7-C), 129.8 (5-C, 10-C), 131.1 (11-C), 135.5 (12-C), 137.6 (8-C), 138.0 (6-C), 139.3 (9-C), 153.2 (13-C), 168.6 (15-C) (Englert, 1975; Frickel, 1984; Barua & Furr, 1998).

Resonance Raman, infrared and mass spectrometry
(Frickel, 1984; Barua & Furr, 1998).

X-Ray analysis
(Stam & MacGillavry, 1963; Frickel, 1984).

Stability
Unstable to light, oxygen and heat, protected in solution by the presence of antioxidants, such as butylated hydroxytoluene and pyrogallol. A variety of factors influence the stability of all-*trans*-retinoic acid in tissue culture media. Degradation and isomerization are minimized by storing under an inert gas, e.g. argon, at –20 °C or lower temperatures in the dark (Frickel, 1984; Barua & Furr, 1998).

2. Occurrence, Production, Use, Human Exposure and Analysis

2.1 Occurrence

The concentration of all-*trans*-retinoic acid in the plasma of fasting individuals is 4–14 nmol/L (Blaner & Olson, 1994). Most other tissues of the body also contain all-*trans*-retinoic acid, at concentrations of 40–6000 pmol/g wet weight (Napoli, 1994; Zhuang *et al.*, 1995). The concentration of all-*trans*-retinoic acid is < 1% that of all-*trans*-retinol in human plasma and < 5% that of total vitamin A in the tissues of healthy animals and humans, and all-*trans*-retinoic acid is present only in traces in plants, if at all. Thus, all-*trans*-retinoic acid is a very minor constituent of the diet. Some foods, such as dairy products, sugar and comestible oils, have been fortified with vitamin A but not with all-*trans*-retinoic acid, which, unlike vitamin A and carotenoids, is not available as a dietary supplement.

2.2 Production

Attempts to synthesize retinol were initiated soon after its structure was determined by von Euler and Karrer in 1931. all-*trans*-Retinoic acid was synthesized by Arens and van Dorp in 1946. The first successful industrial synthesis of all-*trans*-retinol was devised by Isler in 1947 with the Lindlar catalyst. In the 1960s, Pommer and his colleagues used the Wittig reaction involving phosphonium salts to devise an elegant new industrial method for the synthesis of retinol, retinoic acid and β-carotene in the 1960s. In the early 1970s, Julia and Arnoud devised an effective synthesis of all-*trans*-retinoic acid by using the C-15 sulfone as an intermediate (Frickel, 1984). Other synthetic procedures have since been developed which have been used in the formation of a large number of related compounds (Frickel, 1984; Dawson & Hobbs, 1994).

2.3 Use

all-*trans*-Retinoic acid is used primarily for treating dermatological disorders (Peck & DiGiovanna, 1994; Vahlquist, 1994), but it has also been used to treat several types of human cancer (Hong & Itri, 1994), both in experimental animals and in humans, and to reduce elastase-induced emphysema in rats (Massaro & Massaro, 1997).

The skin disorders that have been treated with all-*trans*-retinoic acid are listed in Table 1. The most efficacious oral doses are 1–2 mg/kg bw per day (Peck & DiGiovanna, 1994; Vahlquist, 1994), although such doses often induce adverse side-effects, as discussed in section 2.4. Daily topical doses of up to 0.1% all-*trans*-retinoic acid in creams or gels are effective in treating acne and photoageing, but adverse side-effects are again common. Both the efficacy of all-*trans*-retinoic acid and the incidence of side-effects are dose-dependent. Thus, retinoids with therapeutic efficacy but little if any toxicity are being sought avidly.

Some of the precancerous conditions and cancers treated with all-*trans*-retinoic acid are summarized in Table 2. Oral doses of 0.5–2 mg/kg bw per day have commonly been used, but oral doses of 5–10 mg/day have also been given. all-*trans*-Retinoic acid has been approved for use in the treatment of acute promyelocytic leukaemia at a dose of 45 mg/m^2 per day, and patients with cervical dysplasia were treated topically with 0.37% all-*trans*-retinoic acid in a sponge or gel (Hong & Itri, 1994).

Table 1. Some skin disorders treated with all-*trans*-retinoic acid [a]

Acne vulgaris
Cystic acne
Keloids
Lichen planus
Photoaged skin
Psoriasis

[a] Modified from Vahlquist (1994) and from Peck & DiGiovanna (1994)

Table 2. Some precancerous conditions and cancers treated with all-*trans*-retinoic acid[a]

Actinic keratosis
Acute promyelocytic leukemia
Basal-cell carcinoma
Cervical dysplasia
Oral leukoplakia

[a] Cited by Hong & Itri (1994)

2.4 Human exposure

As indicated above, the amount of retinoic acids in the diet is very small, probably in the range of 10–100 µg/day. Because all-*trans*-retinoic acid is rapidly metabolized in the body and is not stored in the liver or other organs, it does not accumulate over time (Blaner & Olson, 1994). The amount of all-*trans*-retinoic acid ingested in the diet therefore poses neither a benefit nor a risk. As a consequence, exposure to all-*trans*-retinoic acid is limited, for all practical purposes, to the oral or topical treatment of medical disorders. As indicated in section 2.3, the maximum oral dose is approximately 2 mg/kg bw per day. The many adverse side-effects observed at such doses (Kamm *et al.*, 1984; Armstrong *et al.*, 1994) are described in section 7.1.

Topical treatment of acne and photoaged skin with creams or gels containing up to 0.1% all-*trans*-retinoic acid is common (Peck & DiGiovanna, 1994; Vahlquist, 1994). Previously, higher concentrations (0.3–0.4%) were used.

2.5 Analysis

all-*trans*-Retinoic acid in plasma and tissues is commonly measured by high-performance liquid chromatography (HPLC) (Barua & Furr, 1998). The plasma is collected in heparinized tubes, and either plasma or a tissue homogenate is acidified and then extracted several times with a suitable volume of an organic solvent, such as chloroform and methanol, diethyl ether, dichloromethane, acetonitrile, 2-propanol or ethyl acetate. After the combined extract has been dried with anhydrous sodium sulfate, the solvent is evaporated to dryness under yellow light (to avoid isomerization) in nitrogen or argon. The dried powder is immediately dissolved in the HPLC solvent and injected onto the HPLC column. In some cases, a solid-phase extraction or elution step is introduced to remove contaminants.

A reversed-phase C18 column is usually used for the separation, and the compound is usually detected by measuring the absorption at 350 nm and quantified by measuring the area under the absorption peak with an integrator. A known amount of a reference standard, usually all-*trans*-retinyl acetate, is added to the tissue, plasma or serum sample to correct for losses during extraction and analysis. An antioxidant such as butylated hydroxytoluene is also added at the outset to minimize oxidation of the retinoids.

A large number of chromatographic systems have been devised for the separation and quantification of all-*trans*-retinoic acid (Frolik & Olson, 1984; Furr *et al.*, 1992, 1994; Barua & Furr, 1998; Barua *et al.*, 1999).

all-*trans*-Retinoic acid can also be separated as its methyl or pentafluorobenzyl ester by gas–liquid or liquid–liquid chromatography and quantified by mass spectrometry. New ionization methods and tandem mass spectrometry have further enhanced the sensitivity and selectivity with which retinoic acid can be measured (Barua *et al.*, 1999).

3. Metabolism, Kinetics and Genetic Variation

Information on the metabolism, plasma transport and tissue distribution of all-*trans*-retinoic acid in humans and animal models after administration of pharmacological doses is summarized below. More information is given in the General Remarks on the endogeneous (physiological) metabolism of all-*trans*-retinoic acid.

3.1 Humans

3.1.1 Metabolism

Muindi *et al.* (1992) studied the metabolism of all-*trans*-retinoic acid in 13 patients with acute promyelocytic leukaemia who were receiving the drug orally at a dose of 45 mg/m^2. The only metabolite of all-*trans*-retinoic acid measured in plasma before treatment was all-*trans*-4-oxo-retinoic acid, which accounted for < 10% of the circulating all-*trans*-retinoic acid. The urine of these patients was found to contain all-*trans*-4-oxo-retinoyl-β-glucuronide, but urinary excretion of this compound accounted for < 1% of the administered dose. No drug was found in the cerebrospinal fluid.

3.1.2 Kinetics

Muindi *et al.* (1992) also assessed the pharmacokinetics of all-*trans*-retinoic acid. The peak plasma concentration (347 ± 266 ng/ml) was reached 1–2 h after ingestion of the drug, and this decayed in a mono-exponential fashion with a half-life of 0.8 ± 0.1 h. Continued oral administration of all-*trans*-

retinoic acid for an additional 2–6 weeks was associated with a significant decrease in both the peak plasma concentration and the integrated area under the curve of concentration–time (AUC). For a subset of the patients, this decrease occurred within the first 7 days after the start of treatment. The decrease was associated with a 10-fold increase in urinary excretion of all-*trans*-4-oxoretinoyl-β-glucuronide, suggesting that the accelerated clearance of all-*trans*-retinoic acid from plasma was associated with increased drug catabolism.

In patients with either squamous- or large-cell carcinomas of the lung, the mean plasma AUC value calculated after administration of a single oral dose of 45 mg/m² was significantly lower than that of patients with adenocarcinomas ($p = 0.0001$) or control subjects ($p = 0.01$). Individuals with an AUC value < 250 ng-h/mL had a greater likelihood of having squamous- or large-cell carcinoma (odds ratio = 5.9) (Rigas *et al.*, 1996). [It is unclear from these studies whether the phenotype for rapid clearance of all-*trans*-retinoic acid is a cause or an effect of the squamous- or large-cell lung cancer.]

After administration of all-*trans*-retinoic acid at a dose of 30 mg/m² to four children with acute promyelocytic leukaemia, the peak plasma concentration was 20–741 ng/mL and was reached within 60–120 min of administration. The patient with the lowest peak plasma concentration did not achieve complete remission and had a much higher concentration of all-*trans*-4-oxoretinoic acid in plasma than the other three children, who underwent remission. The authors concluded that accelerated metabolism of all-*trans*-retinoic acid to all-*trans*-4-oxoretinoic acid plays an important role in its failure to induce remission in cancer patients (Takitani *et al.*, 1995a,b).

3.1.3 Tissue distribution

No information was available on the tissue distribution of all-*trans*-retinoic acid in humans after its administration as a drug.

3.1.4 Variations within human populations

The studies of Rigas *et al.* (1996) and Takitani *et al.* (1995a,b) suggest that healthy individuals and patients with various types of cancers may have different capacities for the metabolism and plasma clearance of all-*trans*-retinoic acid. No information was available about variations in the metabolism and/or plasma clearance of all-*trans*-retinoic acid in other human populations.

3.2 Experimental models

3.2.1 Metabolism

After female cynomolgus monkeys were given all-*trans*-retinoic acid orally at a dose of 6.7 μmol/kg bw per day for 10 days, the concentration of all-*trans*-retinoyl-β-glucuronide in the plasma rose to a maximum of 231 nmol/L (Creech Kraft *et al.*, 1991a). When 13-*cis*-retinoic acid was similarly administered, the maximal concentration of 13-*cis*-retinoyl-β-glucuronide was 42 nmol/L. The two isomers also partly interconverted, e.g. all-*trans*-retinoic acid to 13-*cis*-retinoic acid and to 13-*cis*-retinoyl-β-glucuronide and 13-*cis*-retinoic acid to all-*trans*-retinoic acid (Creech Kraft *et al.*, 1991b). Creech Kraft *et al.* (1991a) indicated that the extent of retinoyl-β-glucuronide formation from retinoic acid, as assessed pharmacokinetically, is dependent both on the isomer administered and the species studied.

In pregnant females of most but not all species, all-*trans*-retinoyl-β-glucuronide is a major metabolite in the plasma after administration of all-*trans*-retinoic acid (Creech Kraft *et al.*, 1987, 1991b; Eckhoff & Nau, 1990; Eckhoff *et al.*, 1991). In pregnant mice treated with 13-*cis*-retinoic acid, 13-*cis*-retinoyl-β-glucuronide was the most abundant plasma metabolite (Creech Kraft *et al.*, 1991b). In this study, all-*trans*-retinoic acid was transferred to the embryo 10 times more efficiently than 13-*cis*-retinoic acid and 100 times more efficiently than 13-*cis*-retinoyl-β-glucuronide. When retinoids were injected into the amnion of rat embryos on day 10 of gestation, the concentrations of all-*trans*-4-oxoretinoic acid, 13-*cis*-4-oxoretinoic acid and all-*trans*-retinoyl-β-glucuronide required to produce the same dysmorphogenic effects as all-*trans*-retinoic acid (250 ng/mL) were twofold, 10-fold and 16-fold higher, respectively (Creech Kraft & Juchau, 1992). The lack of teratogenicity of all-*trans*-retinoyl-β-glucuronide after oral administration of very high doses to pregnant rats seems to be due to its relatively slow absorption from the intestine, its slow hydrolysis to all-*trans*-retinoic acid, its relatively inefficient transfer across the placenta and its inherently low toxicity (Gunning *et al.*, 1993). all-*trans*-Retinoyl-β-glucuronide was

more teratogenic at equimolar doses than all-*trans*-retinoic acid after subcutaneous application to mice on day 11 of gestation. This effect appears to be due to the extensive hydrolysis of all-*trans*-retinoyl-β-glucuronide after subcutaneous and intravenous administration, suggesting that it is a precursor of all-*trans*-retinoic acid when administered by these routes (Nau et al., 1996).

3.2.2 Kinetics

After intravenous administration of all-*trans*-retinoic acid to male DBA mice at a dose of 10 mg/kg bw, the serum concentrations showed a distribution phase that decreased rapidly over 30 min and was followed by a non-exponential phase. The mean serum concentration of all-*trans*-retinoic acid was 17 ± 1.1 µg/mL 5 min after treatment and < 0.1 µg/mL 6 h after injection. The concentrations of all-*trans*-retinoic acid in liver, kidney, lung, brain and small intestine were generally higher than those in serum throughout the study. By 8 h after injection, the brain still contained relatively high concentrations of all-*trans*-retinoic acid (1.8 ± 0.2 µg/g tissue), even though the dose had been effectively cleared from the circulation (Wang *et al.*, 1980).

Fasted female B6D2F_1 mice received all-*trans*-retinoic acid intragastrically at a dose of 10 mg/kg bw, and clearance was followed for up to 12 h. all-*trans*-Retinoic acid was detected in the plasma by a more sensitive HPLC procedure within 30 min of administration; the plasma concentration was essentially constant for the first 4 h but decreased exponentially from 5–9 h after dosing. Subsequently, the compound was not detected at concentrations higher than those present endogenously in mouse plasma. The authors estimated that the half-life of all-*trans*-retinoic acid was 0.5 h (McPhillips *et al.*, 1987).

In a study of the effects of pretreatment of male BDF_1 mice with phenobarbital (80 mg/kg bw intraperitoneally for 3 days), 3-methylcholanthrene (40 mg/kg intraperitoneally for 3 days) or all-*trans*-retinoic acid (10 mg/kg intragastrically for 3 days) on the disposition of an orally administered dose of 10 mg/kg bw of all-*trans*-retinoic acid, the AUC values for serum and for liver, lung, small intestine, kidney, spleen, large intestine, fat, heart, brain, muscle, testis and bladder were reduced relative to those of controls by an average of 54% after pretreatment with all-*trans*-retinoic acid and by 37% after phenobarbital. Pretreatment with 3-methylcholanthrene did not affect the disposition of all-*trans*-retinoic acid (Kalin *et al.*, 1984) .

A dose of 25 mg of all-*trans*-retinoic acid was administered by intravenous infusion over 30 s into the cephalic vein of four anaesthetized male dogs with bile-duct cannulae. The concentration of all-*trans*-retinoic acid in blood over time was biphasic, with an estimated elimination half-life of 4.5 h, an apparent volume of distribution of 1 L/kg bw and a blood clearance rate of 23 mL/min. The mean AUC was 1190 ± 330 µg-min/mL. At 4 h after administration, only 0.63% of the dose was recovered in the bile, about 25% of which was unconjugated, the remainder occurring as a conjugated derivative. Only 0.04% of the dose was recovered in the bile as 13-*cis*-retinoic acid in the 4 h after infusion of all-*trans*-retinoic acid (Patel *et al.*, 1982).

Cynomolgus monkeys were given all-*trans*-retinoic acid at doses of 2 or 10 mg/kg bw for 10 days. The maximum mean plasma concentration of animals receiving 2 mg/kg bw was 420 ± 100 ng/ml on day 1 and 210 ± 10 ng/ml on day 10, while that of animals receiving 10 mg/kg bw was 1200 ± 345 ng/ml on day 1 and 460 ± 125 ng/ml on day 10. The mean plasma AUC value was 928 ± 233 ng-h/mL on day 1 and 432 ± 196 ng-h/mL by day 10 in animals at 2 mg/kg bw and 4607 ± 1194 ng-h/mL on day 1 and 1557 ± 484 ng-h/mlL on day 10 for animals given 10 mg/kg bw. Thus, the AUC values were proportional to the dose administered (Creech Kraft *et al.*, 1991a).

3.2.3 Tissue distribution

In mice, significant quantities of all-*trans*-retinoic acid were observed in all tissues examined (liver, kidney, lung, brain, testis and small intestine) after administration of 10 mg/kg bw intravenously. The highest concentrations were found in liver at each time studied between 5 and 480 min, followed by kidney, in which the concentrations were about 70% of those in liver. The testis took up the least, accounting for 5–15% of the concentration observed in liver (Wang *et al.*, 1980).

The AUC values in mice after an oral dose of all-*trans*-retinoic acid were reported for liver, lung, small intestine, kidney, spleen, large intestine, fat, heart, brain, muscle, testis and urinary bladder. All tissues took up the retinoid (Kalin *et al.*, 1984).

3.2.4 Intra- and inter-species variation

As discussed in section 3.2.1, different species have very different capacities for the metabolism and clearance of pharmacological doses of all-*trans*-retinoic acid. No one species appears to reflect the situation in humans.

4. Cancer-preventive Effects

4.1 Humans

4.1.1 Epidemiological studies

No data were available to the Working Group.

4.1.2 Intervention trials

No data were available to the Working Group.

4.1.3 Intermediate end-points

4.1.3.1 Skin

The use of all-*trans*-retinoic acid and etretinate was studied in the treatment of patients who had received renal transplants and who had more than 50 skin lesions, consisting of actinic keratosis, squamous-call carcinomas of the skin and warts. Seven patients received all-*trans*-retinoic acid topically plus etretinate systemically (10 mg/day), and four patients received it alone. After three months of therapy, six of seven patients receiving all-*trans*-retinoic acid plus etretinate and three of four of those receiving all-*trans*-retinoic acid alone showed clinical improvement, on the basis of at least a 25% decrease in the number of apparent actinic keratoses and a reduction in the size of warts. After six months of therapy, three of four evaluable patients receiving the two retinoids and two of three receiving all-*trans*-retinoic acid alone showed at least a 50% decrease in the number of lesions or in the number of new actinic keratoses or squamous-call carcinomas of the skin (Rook *et al.*, 1995). [The Working Group noted that no control group was included and that actinic keratoses may regress spontaneously.]

Two randomized trials were conducted of the use of topical all-*trans*-retinoic acid to reverse actinic keratoses. In both studies, patients were assigned randomly to the retinoid or to the vehicle, and treatment was continued for six months. In one study, 266 patients were given 0.05% all-*trans*-retinoic acid and compared with 261 patients given the vehicle; in the second study, 226 patients were given 0.1% all-*trans*-retinoic acid and 229 received the vehicle. Treatment with 0.05% retinoid resulted in a rate of regression of 42%, whereas the rate in the controls was 34% (not significant). At the higher dose, a statistically significantly higher rate of regression of lesions was seen among patients receiving all-*trans*-retinoic acid (55%) than among those given the vehicle (41%; $p < 0.001$) (Kligman & Thorne, 1991). [The Working Group noted the inadequate reporting of the study, that the rate of spontaneous regression in the control group was high and that actinic keratoses may regress spontaneously.]

The effects of all-*trans*-retinoic acid in patients with dysplastic naevi have been evaluated in two studies. In one trial (Edwards & Jaffe, 1990), eight patients were randomly assigned to topical treatment with 0.05% all-*trans*-retinoic acid and 13 received placebo. Five treated patients and 11 given placebo completed the four-month intervention, and their lesions were biopsied. The authors reported marked changes in the clinical and histological appearance of naevi in three of the five evaluable treated patients and in none of the 11 controls ($p < 0.001$). [The Working Group noted that losses from the treatment group and the unspecified methods of statistical analyses complicate interpretation of this report].

A second trial (Halpern *et al.*, 1994) involved five male patients with multiple dysplastic naevi who received applications of a 0.005% solution of all-*trans*-retinoic acid on the right or left half of the back, the side being chosen at random. Treatment was continued for six months, at which time all naevi were assessed clinically and four naevi were excised from each side and examined for histological appearance. A statistically significant ($p < 0.0001$) improvement in the clinical appearance of naevi on the treated side of the back was reported. Four of the 16 excised treated and 13 of the 16 untreated naevi met histological criteria for dysplasia. The histological results for naevi from one patient who did not finish the course of treatment were not reported.

4.1.3.2 Uterine cervix

In a randomized trial, all-*trans*-retinoic acid or placebo was given topically to 301 patients with moderate cervical intra-epithelial neoplasia ($n = 151$), or severe cervical intra-epithelial neoplasia or dysplasia ($n = 150$) as a 0.372% solution on a collagen sponge in a cervical cap daily for four days and

then daily for two days after three and six months. The patients were evaluated by serial colposcopy, cytology and cervical biopsy. The dose, schedule and delivery system were determined in prior single-arm phase I and II trials (Meyskens *et al.*, 1983; Graham *et al.*, 1986). A total of 52 patients were lost to follow-up. Among the 141 patients with moderate dysplasia, a higher rate of complete response was observed in those receiving all-*trans*-retinoic acid (43%) than in the group given placebo (27%; $p = 0.041$). No significant difference in the rates of regression of dysplasia were seen among treated and untreated patients with severe dysplasia. Signs of acute toxicity were infrequent, mild and reversible, consisting primarily of local (vaginal and vulvar) irritation and occurring in less than 5% of treated subjects (Meyskens *et al.*, 1994). [The Working Group noted the uncertain compliance of the patients lost to follow-up, which limits the interpretation of the results of this trial.]

4.2 Experimental models

4.2.1 Cancer and preneoplastic lesions

These studies are summarized in Table 3.

4.2.1.1 Skin

Female Swiss albino mice weighing 20–22 g were treated with 150 µg of 7,12-dimethylbenz[*a*]-anthracene (DMBA) by local application for initiation of skin papillomas. After three weeks, croton oil (0.5 mg in acetone) was applied twice weekly for three to eight months as a promoter. This treatment induced four to eight papillomas per mouse. all-*trans*-Retinoic acid was given at a dose of 200 or 400 mg/kg bw by intraperitoneal injection or gavage once a week for two weeks. The sum of the diameters of the papilloma was determined for each mouse and the average value calculated for each group. Treatment with all-*trans*-retinoic acid reduced the average of the papilloma diameters per animal from 25 to 16 mm and from 22 to 11 mm at the two doses, respectively ($p < 0.05$, Student's *t* test) (Bollag, 1974).

Groups of 25 female Sencar mice, seven to eight weeks of age, were treated topically with 5 µg of DMBA for initiation and two weeks later received either basal diet (controls) or a diet supplemented with 40 mg/kg all-*trans*-retinoic acid. The tumour incidence 30 weeks after initiation was 4% in controls and 68% with all-*trans*-retinoic acid ($p < 0.01$, log rank analysis). In the same study, 25 mice received all-*trans*-retinoic acid topically at a concentration of 30 nmol twice weekly for 28 weeks. The incidence of papillomas was enhanced from 4% in controls to 58% ($p < 0.01$, log rank analysis) (McCormick *et al.*, 1987).

Groups of 30 male and 30 female hairless albino mutant mice received daily topical treatment with 0.001% or 0.01% all-*trans*-retinoic acid from 7 to 30 weeks of age. From day 15 of treatment, the mice were exposed daily for 2 h to simulated sunlight from a 6000-W Xenon-arc lamp for 28 weeks. At the end of the experiment at 55 weeks, the tumour multiplicity was 1.2 carcinomas per mouse in controls and 6.1 and 10 in the two groups given all-*trans*-retinoic acid. The cumulative tumour incidence (% tumour-bearing animals) was 45% in controls, 100% at the low dose and 94% at the high dose (Forbes *et al.*, 1979). [The Working Group noted the lack of statistical analysis.]

Groups of 30 female Sencar mice, six weeks of age, received a single topical application of 10 nmol/L DMBA in acetone, a dose that does not induce skin papillomas. Subsequently, the mice received twice weekly applications of 2 µg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and all-*trans*-retinoic acid at doses of 1 and 10 µg for the remainder of the experiment of 18 weeks. The incidence of papillomas was 100% in controls and 76% and 50% at the low and high doses of all-*trans*-retinoic acid, respectively. The multiplicity of papillomas was reduced from 7.4 per mouse in controls to 3.5 and 1.4 per mouse, respectively. In a second part of the study, all-*trans*-retinoic acid had no effect on two-stage tumour promotion, tested by treating initiated skin with 2 µg of TPA twice a week for two weeks and subsequently with 2 µg of mezerein twice a week for 18 weeks. The mice receiving both TPA and mezerein had a papilloma incidence of 92%, with 4.2 papillomas per mouse. Treatment with 10 µg of all-*trans*-retinoic acid during TPA application (stage I promotion) had little or no effect (88% papilloma incidence and 4 papillomas per mouse), whereas treatment with all-*trans*-retinoic acid during mezerein application (stage II promotion) inhibited papilloma development (34% papilloma incidence and 0.8 papillomas per mouse) (Slaga *et al.*, 1980). [The Working Group noted that no statistical analysis was given.]

Table 3. Effects of all-*trans*-retinoic acid on carcinogenesis in animals

Cancer site	Species, sex, age at carcinogen treatment	No. of animals per group	Carcinogen, dose, route	all-*trans*-Retinoic acid dose, route	Duration in relation to carcinogen	Incidence		Multiplicity		Efficacy	Reference
						Control	Treated	Control	Treated		
Skin	Swiss albino mice, female	11	150 µg DMBA + 0.5 µg croton oil 2 x wk for 3–8 months	200 mg/kg bw, i.p.	For 2 wks after papillomas developed	100	100	NA	NA	Reduced tumour size	Bollag (1974)
				400 mg/kg bw, orally		100	100	NA	NA		
Skin	Hairless albino mice, male and female	60	2 h/day UVR exposure for 28 wks	0.001%,	– 2 to + 30 wks	45	100	1.2	6.1	Tumour enhancing effect	Forbes *et al.* (1979)
				0.01% topically		45	94	1.2	10.0		
Skin	SENCAR mice, female, 6 wks	30	10 nmol DMBA + TPA for 18 wks	1 µg	+ 1 wk to end	100	76	7.4	3.5	Effective	Slaga *et al.* (1980)
				10 µg		100	50	7.4	1.4	Effective	
			TPA (2 wks) + mezerein 18 wks	10 µg	+ 1 wk to +3	92	90	4.2	4.5	Ineffective	
			TPA (2 wks) + mezerein 16 wks	10 µg topically	+ 3 wks to end	92	38	4.2	1.0	Effective	
Skin	SENCAR mice, female, 7 wks	30	10 nmol DMBA + 2 µg TPA 3 x/wk/13 wks	5 µg	+ 1 wk to end	100	90	10.0	6*	Effective	Fischer *et al.* (1985)
				20 µg topically		100	90	10.0	6	Effective	
		30	10 nmol DMBA	5 µg	+ 2 to 24 wks	0	25	0	0.5	Tumour enhancing effect	
				20 µg topically		0	50	0	0.9		
		30	10 nmol DMBA	5 µg + 2 µg mezerein	+ 1 to 3 wks	17	37	0.3	1.3	Tumour enhancing effect	
				10 µg + 2 µg mezerein	+ 1 to 3 wks	17	43	0.3	1.6		
Skin	CD-1, female, 5–7 wks	30	200 nmol DMBA + 444 nmol anthralene daily/32 wks	1.7 nmol	+ 2 wks to end	64	41*	1.5	NA	Effective	Dawson *et al.* (1987)
				17 nmol		64	34*	1.5	NA	Effective	
				170 nmol for 32 wks		64	28*	1.5	NA	Effective	

Table 3 (contd)

Cancer site	Species, sex, age at carcinogen treatment	No. of animals per group	Carcinogen, dose, route	all-*trans*-Retinoic acid dose, route	Duration in relation to carcinogen	Incidence Control	Incidence Treated	Multiplicity Control	Multiplicity Treated	Efficacy	Reference
Skin	SENCAR mice, female, 7–8 wks	25	5 µg DMBA	40 mg/kg diet 30 nmol topically	+ 2 wks to end	4	68*	NA	NA	Tumour enhancing effect	McCormick *et al.* (1987)
				2 x wk	+ 2 wks to end	4	58*	NA	NA	Tumour enhancing effect	
Skin	SENCAR mice, female, 6 wks	24	20 nmol DMBA + 3.3 nmol TPA 2 wks + 2 mmol diacyl-glycerol for 17 wks	17 nmol topical	+ 2 wks to end	67	38*	5.3	0.7*	Effective as inhibitor of 2nd-stage promotion	Verma (1988)
Skin	SENCAR mice, 3 wks, male and female	30–40	20 µg DMBA + TPA 2 µg once a wk for 20 wks	3 µg/kg diet 30 µg/kg diet 3/30 µg/kg diet 30/3 µg/kg diet	0 to 45 wks		50 18.5 23.1 23.1		0.7 0.2* 0.2* 0.2*	Effective**	Chen *et al.* (1994)
Liver	B6D2F$_1$/Hsd mice, female, 4 wks	35	50 mg NDEA ip 100 mg NDEA ip	30 mg/kg diet	+ 1 week to end at 6 mths	37 44	86 90	NA NA	NA NA	Tumour enhancing effect	McCormick *et al.* (1990)
Mammary gland	Sragué-Dawley rats, female, 50 days	12–24	MNU 50 mg/kg bw	60 mg/kg diet 120 mg/kg diet	+ 1 to 4.5 mths	100 100	91 83	3.6 3.6	3.3 2.8	Ineffective	Anzano *et al.* (1994)

wk, week; DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; NDEA, *N*-nitrosodiethylamine; ip, intraperitoneal; NA, not available; MNU, *N*-methyl-*N*-nitrosourea

* Statistically significant (see text)

** Conversion of papilloma to carcinoma was inhibited.

In similar studies, Sencar mice received topical applications of a single dose of 10 nmol/L DMBA and then 5 or 20 µg of all-*trans*-retinoic acid simultaneously with 2 µg of TPA thrice weekly for 13 weeks. Both doses of all-*trans*-retinoic acid reduced the tumour yield, from approximately 10 per mouse in controls to 6 per mouse, and the papilloma incidence was 100% and 90%, respectively. The authors reported that papilloma development was delayed in the animals given all-*trans*-retinoic acid. In the same series of experiments, all-*trans*-retinoic acid was applied instead of TPA to the skin of Sencar mice initiated with 10 nmol/L DMBA. After 24 weeks, the papilloma yield was about 0.5 papillomas per mouse at 5 µg of all-*trans*-retinoic acid and 0.9 papillomas per mouse at 20 µg, with tumour incidences of about 50% and 25%, respectively. In another experiment, 2 µg of mezerein were applied to the skin of mice initiated with 10 nmol/L DMBA both as a first-stage promoter three times per week for two weeks and as a second-stage promoter three times per week for the subsequent 15–18 weeks. The papilloma yield per mouse was 0.3, and the papilloma incidence was 17%. When 10 µg of all-*trans*-retinoic acid were applied instead of mezerein during the first stage of promotion, the papilloma yield was 1.6 per mouse and the incidence was 43% per mouse (Fischer *et al.*, 1985). [The Working Group noted that no statistics were given, and the papilloma yields and incidences were gleaned from graphs.]

In another two-stage tumour promotion study in female SENCAR mice initiated with DMBA, TPA was applied as a stage-I promoter and L-α-dioctanoylglycerol (a protein kinase C inducer) as a stage-II promoter. all-*trans*-Retinoic acid (17 nmol/L) was topically applied 1 h before L-α-dioctanoylglycerol. The papilloma incidence at 17 weeks was 67% in controls and 38% in mice given all-*trans*-retinoic acid [no statistics given], and the tumour multiplicity was 5.3 and 0.3, respectively ($p < 0.01$ [method not given]) (Verma, 1988).

Groups of 30 female CD-1 mice, five to seven weeks of age, were treated topically with 200 nmol/L DMBA in acetone; two weeks later, the mice were treated with 444 nmol/L anthralene alone or with 1.7, 17 or 170 nmol/L all-*trans*-retinoic acid for 32 weeks. The incidences of skin papillomas were 64% in the control group and 41%, 34% and 28% at the low, intermediate and high doses of all-*trans*-retinoic acid ($p < 0.01$, Student's *t* test) (Dawson *et al.*, 1987).

Pregnant Sencar mice were placed on diets containing all-*trans*-retinoic acid at 3 or 30 µg/g of diet. Their pups were raised on the same diets, were initiated with a single dose of 20 µg DMBA at three weeks of age and promoted with 2 µg of TPA once a week for 20 weeks. At that time, half of the animals given the diet containing all-*trans*-retinoic acid at 3 µg/g were switched to the diet containing 30 µg/g, and half of those given 30 µg/g were switched to the diet containing 3 µg/g. The papilloma incidences in the four groups were not significantly different, but the carcinoma incidence and yield were significantly lower in the three groups that were maintained either continuously or for some period on the diet containing all-*trans*-retinoic acid at 30 µg/g ($p < 0.05$, Fisher's exact test). When the animals were 26 weeks of age, 27–40 in each group were still alive; the experiment was terminated when they were 45 weeks old. The cumulative carcinoma incidence was 50% in animals maintained continuously on the diet with the low concentration of all-*trans*-retinoic acid and 18–23% in animals kept continuously or for some period on the diet with the high concentration. The carcinoma yield was 0.68 in the mice maintained continuously at the low dose and 0.19–0.23 in animals maintained continuously or intermittently at the high dose (Chen *et al.*, 1994).

4.2.1.2 Liver

Groups of 35 female B6D2F$_1$/hsd, mice, four weeks of age, received intraperitoneal injections of 50 or 100 mg *N*-nitrosodiethylamine (NDEA) and dietary supplements of 0.1 mmol of all-*trans*-retinoic acid per kg of diet beginning one week after carcinogen treatment until the end of the study at six months. The combined incidence of benign and malignant liver tumours in animals given the low dose of NDEA was 37% in controls and 86% in those given all-*trans*-retinoic acid ($p < 0.05$; χ^2 test). The incidence of hepatocellular carcinoma in animals at the high dose of NDEA was 44% in controls and 90% with all-*trans*-retinoic acid ($p < 0.01$; χ^2 test). Since all-*trans*-retinoic acid alone did not induce any liver tumours, these results suggest that it enhanced the hepatocarcinogenicity of NDEA (McCormick *et al.*, 1990).

4.2.1.3 Mammary gland

Groups of 24 control and 12 treated female Sprague-Dawley rats, 50 days of age, received intravenous injections of 50 mg/kg bw *N*-methyl-*N*-nitrosourea and, one week later, all-*trans*-retinoic acid at 60 or 120 mg/kg of diet for 4.5 months. The incidences of mammary adenocarcinoma were 100% in controls and 83% and 91% at the high and low concentrations of all-*trans*-retinoic acid, respectively. The tumour multiplicity was 3.6 in controls and 3.3 and 2.8 with all-*trans*-retinoic acid, respectively. The differences were not statistically significant (Anzano *et al.*, 1994).

4.2.2 Intermediate biomarkers

Ornithine decarboxylase is considered to be a useful biomarker in experimental studies of skin carcinogenesis. Mice were given 0.2 mmol of DMBA in 0.2 mL of acetone as an initiator followed by twice weekly applications of 17 nmol/L TPA topically 1 h after application of 1.7 or 17 nmol of all-*trans*-retinoic acid in 0.2 ml of acetone. The mice were killed 4.5 h after the last of seven treatments with TPA, and ornithine decarboxylase was measured. all-*trans*-Retinoic acid suppressed the TPA-induced ornithine decarboxylase activity almost completely (Verma *et al.*, 1979).

4.2.3 In-vitro models

4.2.3.1 Models of carcinogenesis

Studies with cells in culture have provided important information on the pleiotropic effects of all-*trans*-retinoic acid that may be relevant for understanding the mechanisms of its chemopreventive effects. The types of cell that have been used to study the effects of retinoids include normal cells in short-term culture, cells immortalized spontaneously or by viral genes such as HPV 16 E6 and SV40 large T antigen or oncogenes such as H-ras, and cells derived from solid tumours or haematological malignancies and used in primary cultures or established as cell lines. Although some important information was gained from each of these cell systems, that obtained with non-malignant cells is more relevant to chemoprevention of cancer. A wide range of concentrations (10^{-11}–10^{-4} mol/L) was used in these studies.

Normal epithelial cells can be maintained in culture for a limited number of cell divisions, as they usually senesce and die. Specific media, which are often serum-free, have been developed to culture epithelial cells and exclude mesenchymal (stromal) cells. Investigations of the effects of retinoids on normal cells included studies on changes in cell growth and differentiation and on the prevention of malignant transformation. Because many of the cells in epithelial tissues *in vivo* are quiescent, adaptation of culture conditions to maximize cell proliferation may select for cells of higher proliferative capacity or allow the cells to exhibit an acquired proliferative potential. Proliferating normal cells in culture can be considered to be hyperplastic cells.

(a) Inhibition of carcinogen-induced neoplastic transformation

In studies with immortalized murine C3H/10 T1/2 murine fibroblasts, all-*trans*-retinoic acid inhibited 3-methylcholanthrene-induced neoplastic transformation when it was added to the medium seven days after removal of the carcinogen and weekly treatments were given for the four-week duration of the experiment. Activity was thus expressed in the promotion phase of transformation and required a concentration of about 10^{-6} mol/L (Bertram, 1980). The low activity was explained by the finding that these cells rapidly catabolized all-*trans*-retinoic acid. When this was blocked by liarazole, an inhibitor of a cytochrome P450 4-hydroxylase, all-*trans*-retinoic acid inhibited transformation at concentrations as low as 10^{-10} mol/L, in the absence of cytoxicity (Acevedo & Bertram, 1995).

(b) Rat tracheal epithelial cells

Primary rat tracheal epithelial cells grown in an air–liquid interface in the presence of all-*trans*-retinoic acid differentiate into normal mucociliary epithelium and produce large amounts of mucin glycoproteins (Kaartinen *et al.*, 1993). The differentiated cultures were shown to express the mucin genes *MUC1* and *MUC5* (Guzman *et al.*, 1996). After removal of all-*trans*-retinoic acid from the medium, the cells assumed a stratified squamous morphology and developed a cornified apical layer. Biochemical analysis revealed loss of expression of transglutaminase type II, keratin 18 and both *MUC1* and *MUC5* and aberrant expression of the squamous markers transglutaminase type I and keratin 13 (Kaartinen *et al.*, 1993; Guzman *et al.*,

1996). Addition of all-*trans*-retinoic acid to squamous differentiated rat tracheal epithelial cultures resulted in a rapid (24 h) down-regulation of prostaglandin H synthase-1 (*PGHS-1*) mRNA expression and a slower (three days) up-regulation of the expression of cytosolic phospholipase A2 and *PGHS-2* genes coincident with re-differentiation of the culture to a mucociliary phenotype (Hill *et al.*, 1996).

The rat tracheal epithelial cell system is useful for identifying potential chemopreventive agents because the cells can be transformed by exposure to chemical carcinogens such as the directly acting *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Exposure of primary rat tracheal epithelial cells *in vitro* to MNNG led to the appearance of initiated stem cells that grew under selective conditions in culture and differed from normal stem cells in that their probability of self-renewal was increased (Nettesheim *et al.*, 1987). When all-*trans*-retinoic acid was included in the medium for only three days at concentrations that did not affect cell survival (3–33 nmol/L), it inhibited transformation by 65–75% in a dose-dependent manner. Longer treatment at higher concentrations caused more than 90% inhibition, with no cytotoxicity. When treatment was delayed for three weeks after exposure to MNNG, 60% inhibition of transformation frequency was still achieved. all-*trans*-Retinoic acid inhibited the growth of normal rat tracheal epithelial cells. It was suggested that the mechanism of the preventive effect of all-*trans*-retinoic acid on tracheal cell transformation was inhibition of cell proliferation. Exposure of the rat tracheal epithelial cells to MNNG for more than five weeks resulted in loss of sensitivity to the growth inhibitory effect of all-*trans*-retinoic acid. This was deduced from the finding that the concentration of all-*trans*-retinoic acid required to cause 50% inhibition of colony formation increased from 0.1–0.3 nmol/L for cells isolated from 3–5-week-old transformed colonies to over 100-times higher concentrations for cells isolated from 12-week-old cultures. Rat tracheal epithelial cell lines established from cells in advanced stages of transformation also showed increased resistance to all-*trans*-retinoic acid, and two of five cell lines even formed more colonies in its presence (Fitzgerald *et al.*, 1986). In this model, cells in early stages of transformation retain responsiveness to factors that constrain proliferation, and most of their descendants differentiate and do not express transformed characteristics. These are the cells that respond to all-*trans*-retinoic acid. Progression of the MNNG-initiated cells to the second stage of transformation, when the cells are immortalized, is accompanied by loss of responsiveness to the growth inhibitory effects of all-*trans*-retinoic acid. In this model, early stages of transformation are likely to respond better than later stages (Fitzgerald *et al.*, 1986; Nettesheim *et al.*, 1987).

Rat tracheal epithelial cells can also be transformed by benzo[*a*]pyrene. Inhibition of transformation by this carcinogen was developed as an assay for screening chemopreventive agents that act by altering metabolism or by inhibiting early stages of carcinogenesis. all-*trans*-Retinoic acid was active in this assay when added simultaneously with benzo[*a*]pyrene, due either to increased cytochrome P450 activity or restoration of differentiation (Steele *et al.*, 1990).

Immortalized rat tracheal epithelial 2C5 cells cultured in serum-free medium undergo squamous differentiation after the addition of serum. Concentrations of 0.1–1 nmol/L all-*trans*-retinoic acid inhibited this differentiation, as evidenced by suppression of several markers, including cross-linked envelope formation and keratin K13 expression (Denning & Verma, 1994).

(c) Immortalized and transformed human bronchial epithelial cells

Normal bronchial epithelial cells were immortalized by SV40 large T antigen by Reddel *et al.* (1988) and designated BEAS-2B cells. The cells were then used to develop transformed and tumorigenic derivatives by culturing them on de-epithelialized rat tracheas, transplanting them into rats and exposing the rats to cigarette smoke condensate. Cell lines were derived from tumours which developed in the transplanted tissue in some animals. Certain cell lines were considered to be premalignant, while others, such as 1170-I cells, were tumorigenic and considered to be malignant (Klein-Szanto *et al.*, 1992). The effects of retinoids were compared in primary cultures of normal bronchial epithelial cells, the immortalized BEAS-2B cell line and premalignant and malignant cell lines in a model of multistage tracheobronchial carcinogenesis. The sensitivity to the

growth inhibitory effects of all-*trans*-retinoic acid diminished with progression in this cell system: the most advanced cell line 1170-I was resistant, whereas the growth of normal and immortalized cells was inhibited (Kim *et al.*, 1995; Lee *et al.*, 1997).

In another model for carcinogen-induced transformation *in vitro* (Langenfeld *et al.*, 1996), SV40-T-immortalized human bronchial epithelial BEAS-2B cells are exposed to the carcinogenic agents present in cigarette smoke condensate or to the purified tobacco carcinogen *N*-nitrosamino-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and transformation is scored as increased anchorage-independent growth or acquired tumorigenicity in immune-compromised mice. When the BEAS-2B cells were treated with all-*trans*-retinoic acid during exposure to the transforming agents, the ability of the cells to form colonies in semi-solid media and to form tumours was inhibited. all-*trans*-Retinoic acid inhibited DNA synthesis in immortalized BEAS-2B cells and in their carcinogen-transformed derivative BEAS-2BNNK (Boyle *et al.*, 1999).

In human tracheal gland epithelial cells immortalized by adenovirus 12-simian virus 40 (Ad12-SV40) hybrid, all-*trans*-retinoic acid inhibited both cell proliferation and anchorage-independent growth in a dose-dependent manner when applied at concentrations of 1 nmol/L to 1 μmol/L. all-*trans*-Retinoic acid up-regulated *p53* but had no effect on the expression of *TGF-α* or *TGF-β 1* genes. These results suggest that all-*trans*-retinoic acid regulates the growth of human tracheal gland epithelial cells by up-regulating the expression of *p53* (Joiakim & Chopra, 1993).

[The Working Group noted that the relevance of these cell culture models is uncertain, since the original immortalizing agent, SV40 large T antigen, is not an etiological agent for human lung cancer. Since one of the main effects of the large T antigen is to decrease *p53* levels, the findings may be relevant only to carcinogenesis that involves defects in the *p53* pathway. The immortalized cells expressed only low levels of wild-type *p53* and not mutated *p53* as many human lung cancers do.]

(d) Mouse epidermal keratinocytes

A cell culture model system analogous to initiated mouse epidermis was established by exposing cells of the keratinocyte cell line 308, derived from adult mouse skin, to DMBA. These cells behaved like initiated cells in that they formed papillomas when grafted onto the backs of athymic mice. Normal keratinocytes can normally inhibit the colony-forming ability of these cells in a medium containing a high concentration of Ca^{++}, but exposure to TPA for several weeks allowed initiated colonies to form. This action of TPA could be blocked by all-*trans*-retinoic acid at 10^{-6} mol/L (Hennings *et al.*, 1990).

In another model, treatment of the murine epidermal cell line JB6 with the tumour promoter TPA resulted in transformation into anchorage-independent tumorigenic cells, which was blocked by all-*trans*-retinoic acid at doses of 10^{-10}–10^{-6} mol/L (De Benedetti *et al.*, 1991).

(e) Human papillomavirus type 16-immortalized human epidermal keratinocytes

The transforming ability of human papillomavirus (HPV) type 16 (HPV16), which has been implicated in the development of cervical cancer, resides in the oncogenes *E6* and *E7*. HPV-16 DNA was used to transfect human foreskin epidermal keratinocytes and thus obtain several immortalized cell lines. Treatment of normal keratinocytes with all-*trans*-retinoic acid at 1 nmol/L, during or immediately after transfection with HPV-16 DNA, inhibited immortalization by about 95% (Khan *et al.*, 1993). If the cells were first immortalized, all-*trans*-retinoic acid inhibited their growth by reducing the expression of the HPV-16 early genes (*E2*, *E5*, *E6* and *E7*) at the level of mRNA and protein (Pirisi *et al.*, 1992; Khan *et al.*, 1993). The HPV-16-immortalized cells were about 100 times more sensitive than their normal counterparts to growth inhibition by all-*trans*-retinoic acid in both clonal and mass culture growth assays. They were also more sensitive to modulation of keratin expression than normal cells.

This model was developed further to include more advanced stages of transformation by continuous culture, which resulted in the selection of variants that acquired independence from epidermal growth factor and growth factors present in bovine pituitary extract, which are required at early stages of transformation. The advanced stage cells could be transformed into tumorigenic cells by transfection with viral Harvey *ras* or herpes

simplex virus type II DNA. all-*trans*-Retinoic acid inhibited the early stages of this progression, but the cells lost their sensitivity as they progressed in culture (Creek *et al.*, 1995). all-*trans*-Retinoic acid induced TGFβ1 and β2 expression in these cells. TGFβ was a potent inhibitor of the growth of early stages of progression in this model but the later-stage cells were resistant to this negative growth factor, which the authors concluded is the basis for the accompanying loss of response to all-*trans*-retinoic acid.

(f) Spontaneously immortalized human keratinocytes and their ras*-transformed derivatives*

Although spontaneous immortalization is a rare event, it occurred in a human keratinocyte culture which gave rise to a cell line designated HaCaT. These cells have been also transformed with *c-Ha-ras* oncogene, and benign and malignant clones have been isolated. The various cell types have maintained their ability to differentiate into stratified epithelium and in their response to regulation of keratins by all-*trans*-retinoic acid. The immortalized and *ras*-transformed cells expressed keratins K1 and K10 in medium depleted of retinoids, but the expression of these keratins was fully suppressed when the concentration of all-*trans*-retinoic acid was increased (Breitkreutz *et al.*, 1993). Lotan (1993) suggested that all-*trans*-retinoic acid can regulate differentiation of normal, premalignant and malignant human keratinocytes and can suppress the expression of squamous differentiation markers in malignant squamous-cell carcinomas.

(g) Human papillomavirus-immortalized human cervical cells

Because 90% of human cervical tumours contain HPV DNA, it is assumed that the virus plays a role in the development of this cancer, especially since the DNA can immortalize epithelial cells *in vitro* through the *E6* and *E7* oncogenes. In several immortalized ectocervical epithelial cell lines derived with HPV-16 DNA, all-*trans*-retinoic acid and other retinoids suppressed the expression of squamous differentiation markers like keratins (Agarwal *et al.*, 1991). all-*trans*-Retinoic acid inhibited the growth of the immortalized cells, although it had no effect on the growth of normal ectocervical cells (Sizemore & Rorke, 1993). It suppressed the differentiation markers keratins K5 and K16 and transglutaminase type 1 more effectively in HPV-immortalized cells than in normal ectocervical cells (Choo *et al.*, 1995).

HPV-immortalized keratinocytes can grow in organotypic cultures on a collagen gel substratum that contains fibroblasts, and a three-dimensional tissue-like growth is obtained, which can be viewed as a cervical carcinoma *in situ*. In such cultures, the expression of squamous markers can be suppressed by all-*trans*-retinoic acid, although higher concentrations of all-*trans*-retinoic acid were required to block terminal differentiation in these cultures than in control organotypic cultures of normal cells in which 30 times higher concentrations were required to suppress *K1* mRNA (Merrick *et al.*, 1993). The reason for the difference between the normal and immortalized cells is unknown, nor is it known why in another laboratory (Agarwal *et al.*, 1991, 1996) the immortalized cells were more sensitive than normal cells to all-*trans*-retinoic acid. [The Working Group noted that the two groups of investigators used different culture methods.]

In HPV-16-immortalized endocervical cell lines grown in organotypic culture, all-*trans*-retinoic acid prevented the dysplastic morphology and cytokeratin differentiation markers of carcinoma *in situ* (Shindoh *et al.*, 1995). Tumorigenic variants of HPV-immortalized cervical cells derived by treatment with cigarette smoke condensate were less sensitive to all-*trans*-retinoic acid than normal and immortalized non-tumorigenic cells. They formed organotypic epithelium resembling severe dysplasia which was persistent even in the presence of all-*trans*-retinoic acid, whereas the immortalized cells formed only a thin epithelium. The investigators predicted that similar resistance to all-*trans*-retinoic acid may occur clinically (Sarma *et al.*, 1996).

In a comparison of the sensitivity of cell lines derived from cervical intraepithelial neoplasia (CIN), HPV DNA-transfected cell lines and cervical carcinoma cell lines to all-*trans*-retinoic acid, the retinoid had comparable effects on growth, detected as a decrease in DNA synthesis, in all but two carcinoma cell lines, which were resistant. all-*trans*-Retinoic acid inhibited growth in cervical neoplastic cell lines, including cervical carcinoma cells (Behbakht *et al.*, 1996).

[See the comment of the Working Group in section *(c)*, above.]

4.2.3.2 Effects on differentiation of normal cells

(a) Normal human tracheobronchial epithelial cells

Normal human tracheobronchial epithelial cells cultured on collagen gels in medium containing all-*trans*-retinoic acid and triiodothyronine expressed a mucociliary phenotype. Removal of the retinoid from the medium caused the cultures to differentiate into a squamous epithelium, accompanied by decreased mucin secretion and reduced expression of the mucin genes *MUC2* and *MUC5AC*, indicating that all-*trans*-retinoic acid plays a major role in differentiation of the mucociliary epithelium (Yoon *et al.*, 1997). In normal human tracheobronchial epithelial cell cultures, all-*trans*-retinoic acid down-regulated the squamous marker cornifin α and upregulated *MUC2*, *MUC5AC* and *MUC5B* mRNAs sequentially at 24, 48 and 72 h, respectively (Koo *et al.*, 1999a). It has been suggested that nuclear RARα and, to a lesser extent, RARγ play a role in the control of mucin gene expression, since RARα- and RARγ-selective agonists strongly induced mucin mRNAs in a dose-dependent manner, whereas an RARβ-selective retinoid only weakly induced mucin gene expression at the high concentration of 1 μmol/L. Furthermore, an RARα antagonist inhibited mucin gene induction and mucous cell differentiation caused by all-*trans*-retinoic acid and by RARα- and, surprisingly, by RARγ–selective retinoids (Koo *et al.*, 1999b).

Treatment of primary cultures of human bronchial epithelial cells with all-*trans*-retinoic acid caused accumulation of cells in the G1 phase of the cell cycle and inhibition of DNA synthesis (Boyle *et al.*, 1999). all-*trans*-Retinoic acid also suppressed epidermal growth factor signalling in normal human tracheobronchial epithelial cells grown on collagen gels (Moghal & Neel, 1998).

(b) Hamster trachea explants

Explants of hamster trachea prepared from vitamin A-deficient animals have been used to measure the ability of retinoids to prevent the squamous metaplasia which normally results when this tissue is cultured in the absence of vitamin A. all-*trans*-Retinoic acid dissolved in dimethyl sulfoxide inhibited metaplasia, with a median effective dose (ED_{50}) of 3 x 10^{-11} mol/L when applied over a 10-day period. Control cultures showed over 90% metaplasia (Newton *et al.*, 1980).

4.2.3.3 Cell lines established from tumours

The effects of all-*trans*-retinoic acid have been studied in numerous cell lines established from various malignancies. Many of these cancer cell lines maintained responsiveness to some of the pleiotropic effects of retinoids, including suppression of proliferation in monolayer culture, inhibition of colony formation in semi-solid media and induction of differentiation, sometimes including complete suppression of tumorigenic potential. The results of many of these studies have been reviewed (Gudas *et al.*, 1994; Lotan, 1995; and General Remarks to this volume). They are not included here because their relevance to cancer prevention is indirect.

4.2.3.4 Antimutagenicity in short-term tests for mutagenicity

Although most studies have focused on the role of all-*trans*-retinoic acid in the promotion and progression of cancer, the results of a few studies have indicated that it may sometimes act as an anti-initiator. It has been shown to modulate chemically-induced genotoxicity in a number of short-term assays, in both bacteria (Table 4) and mammalian cells (Table 5). [The Working Group noted that many of the reports do not give the isomer designation for the retinoic acid used. When the source of the retinoic acid was shown, the company was contacted and asked about the availability of different isomers at different times; for example, retinoic acid obtained from Sigma before 1988 was all-*trans*-retinoic acid, since it was the only form available at that time. In other cases, the author was contacted.]

(a) Salmonella typhimurium

Of the studies in which the ability of all-*trans*-retinoic acid to inhibit the action of standard mutagens was tested in *Salmonella typhimurium* (Table 4), only one examined indirect DNA damage by assaying *umu* C gene expression in *S. typhimurium* TA1535/pSK1002; the others followed the standard assay of Ames. all-*trans*-Retinoic acid

Table 4. Inhibition by all-*trans*-retinoic acid of standard mutagens in the *Salmonella*/microsome test

Retinoid (tested dose)[a]	Mutagen (tested dose)[a]	*S. typhimurium* strain	S9 mix	Result[b]	LED/HID[c]	Reference
all-*trans*-RA (0.0003–300 µmol/plate)	3-Amino-3,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) (0.2 µg/ml)	TA1535/pSK1002	+	+	0.54 µmol/plate (ID_{50})	Okai *et al.* (1996)
all-*trans*-RA (0.003–30 µmol/plate)	Adriamycin (3 µg/ml)	TA1535/pSK1002	–	–	30 µmol/plate	Okai *et al.* (1996)
all-*trans*-RA (0.003–30 (µmol/plate)	Mitomycin C (0.3 µg/ml)	TA1535/pSK1002	–	–	30 µmol/plate	Okai *et al.* (1996)
RA (Sigma)[d] (0.1–10 µmol/plate)	Hydrogen peroxide (5 µmol/plate)	TA104	–	–	10 µmol/plate	Han (1992)
all-*trans*-RA (25–500 nmol/plate)	Cigarette smoke condensate (100–400 µg/plate)	TA98	+	–	500 nmol/plate	Wilmer & Spit (1986)
RA (Sigma)[e] (2.5–40 µg/plate)	Benzo[*a*]pyrene (5 µg/plate)	TA98	+	–	40 µg/plate	Qin & Huang (1985)
RA (Sigma)[e] (2.5–40 µg/plate)	Aflatoxin B_1 (0.5 µg/plate)	TA98	+	+	2.5 µg/plate	Qin & Huang (1985)
all-*trans*-RA (0.26–2600 nmol/plate)	Aflatoxin B_1 (50 ng/plate)	TA98	+	+	26 nmol/plate LED; 860 nmol/plate; 55% decrease in revertants	Whong *et al.* (1988)
RA (Sigma)[e] (0.2–2000 nmol/plate)	Aflatoxin B_1 (200 ng/plate)	TA98	+	+	0.2 nmol/plate; 70% decrease in revertants	Raina & Gurtoo (1985)
RA (Sigma)[e] (0.2–2000 nmol/plate)	Aflatoxin B_1 (200 ng/plate)	TA100	+	–	2000 nmol/plate	Raina & Gurtoo (1985)
RA (Sigma)[e] (0.1, 0.5 µmol/plate)	Aflatoxin B_1 (400 ng/plate)	TA100	+	+	0.1 µmol/plate; 50% decrease in revertants	Bhattacharya *et al.* (1987)

RA, retinoic acid; S9 mix, 9000 x *g* microsomal fraction used as exogenous metabolic system; ID_{50} = dose of retinol required to inhibit *umu* C gene expression by 50%

[a] Doses of retinoids and mutagens are given as reported by the authors.

[b] +, inhibition of genotoxicity; –, no inhibition of genotoxicity

[c] LED, lowest effective (inhibitory) dose; HID, highest ineffective dose

[d] Probably all-*trans*-RA but could be 13-*cis*-RA, since, 13-*cis*-RA was listed in Sigma catalogue from 1988 and 9-*cis*-RA from 1996.

[e] Presumed to be all-*trans*-RA since 13-*cis*-RA was listed in Sigma catalogue only from 1988 and 9-*cis*-RA from 1996.

Table 5. Inhibition by all-*trans*-retinoic acid of genetic and related effects in cultured mammalian cells

Retinoid (tested dose)[a]	Genotoxic agent (tested dose)[a]	Cells/system	Investigated effect	Result[b]	LED/HID[c]	Reference
all-*trans*-RA (0.25–4 µg/ml)	Mitomycin C (0.03 µg/ml)	Chinese hamster V79 cells	Sister chromatid exchange	–	4 µg/ml	Sirianni *et al.* (1981)
all-*trans*-RA[d] (25–50 nmol/ml)	Cyclophosphamide (1 mmol/plate)	Chinese hamster epithelial liver cells	Sister chromatid exchange	+	(25 nmol/ml)	Cozzi *et al.* (1990)
all-*trans*-RA[d] (25–50 nmol/ml)	7,12-Dimethylbenz-[*a*]anthracene (0.078 µmol/plate)	Chinese hamster epithelial liver cells	Sister chromatid exchange	+	37 nmol/ml	Cozzi *et al.* (1990)
all-*trans*-RA (0.2–0.8 µg/ml)	Aqueous extract of *pan masala* (13.5, 12.5 µg/ml water-soluble *masala* with and without tobacco, respectively)	Chinese hamster ovary cells	Sister chromatid exchange	+	0.2 µg/ml	Patel *et al.* (1998)
all-*trans*-RA (0.2–0.8 µg/ml)	Aqueous extract of *pan masala* (13.5, 12.5 µg/ml water-soluble *masala* with and without tobacco, respectively)	Chinese hamster ovary cells	Chromosomal aberrations	+	0.4 µg/ml	Patel *et al.* (1998)
all-trans-RA[d] (0.1–10 mol/ml)	None	C127 mouse cells transformed by bovine papilloma-virus DNA	Chromosomal instability (chromatid bridges and fragments)	+	5 nmol/ml	Stich *et al.* (1990)
RA (Sigma)[e] (1–25 nmol/ml)	Ethyl methane-sulfonate (100 µg/ml)	Chinese hamster ovary cells	*Hprt* mutation	+	25 nmol/ml	Budroe *et al.* (1988)
RA (Sigma)[e] (1–25 nmol/ml)	7,12-Dimethylbenz-[*a*]anthracene (1.25–5 µg/ml)	Chinese hamster ovary cells	*Hprt* mutation	–	5 nmol/ml	Budroe *et al.* (1988)
RA (Sigma)[e] (1–50 nmol/ml)	Ethyl methanesul-fonate (200 µg/ml)	Rat primary hepatocytes	Unscheduled DNA synthesis	–	50 nmol/ml	Budroe *et al.* (1987)
RA (Sigma)[e] (1–25 nmol/ml)	Ultraviolet light (32 J/m^2)	Rat primary hepatocytes	Unscheduled DNA synthesis	–	25 nmol/ml	Budroe *et al.* (1987)
RA (Sigma)[e] (1–50 nmol/ml)	7,12-Dimethyl-benz[*a*]anthracene (2.5, 5 µg/ml)	Rat primary hepatocytes	Unscheduled DNA synthesis	+	1 nmol/ml	Budroe *et al.* (1987)
all-*trans*-RA (1–100 µg/ml)	Dimethylbenz[*a*]-anthracene (71 nmol/ml)	Mouse epidermal cells	Binding to DNA *in vitro*	+	10 µg/ml 22% decrease	Shoyab (1981)

Table 5. (contd)

Retinoid (tested dose)[a]	Genotoxic agent (tested dose)[a]	Cells/system	Investigated effect	Result[b]	LED/HID[c]	Reference
all-*trans*-RA (50,100 µg/ml)	^{3}H-Benzo[*a*]pyrene	Cultured human bronchial explants	Binding to DNA	+	100 µg/ml; decreased binding	Bodo *et al.* (1989)
all-*trans*-RA (≤ 500 nmol/ml)	Aflatoxin B_1 (2 nmol/ml)	None	Binding to calf thymus DNA in presence of microsomes	+	60 nmol/ml; 50% inhibition	Firozi *et al.* (1987) (see also Bhattacharya *et al.*, 1984)
all-*trans*-RA (≤ 500 nmol/ml)	Aflatoxin B_1 (43 nmol/ml)	None	Metabolism of aflatoxin B_1 to Tris-diol complex in presence of microsomes	+	75 nmol/ml; 50% inhibition	Firozi *et al.*, (1987)
all-*trans*-RA (≤ 200 nmol/ml)	Benzo[*a*]pyrene (60 nmol/ml)	None	Binding to calf thymus DNA	+	84 nmol/ml; 50% inhibition	Shah *et al.* (1992)
all-*trans*-RA ≤ 200 nmol/ml)	Benzo[*a*]pyrene (60 nmol/ml)	None	B[a]P-7,8-diol formation	–	200 nmol/ml;	Shah *et al.* (1992)
all-*trans*-RA (0.1–30 nmol/ml)	None	Primary rat hepatocytes	Cytochrome p450C7 mRNA expression	#	0.1 nmol/ml	Westin *et al.* (1993)
all-*trans*-RA (40 nmol/ml)	None	Primary rat hepatocytes	Cytochrome P450 RNA activity			Jurima-Romet *et al.* (1997)
			Cyp1A1	#	40 nmol/ml	
			Cyp1A2	–	40 nmol/ml	
			Cyp3A	#	40 nmol/ml	

RA, retinoic acid; B[*a*]P, benzo[*a*]pyrene

[a] Doses of retinoids and mutagens are given as reported by the authors.

[b] +, inhibition of the investigated end-point; –, no effect on the investigated end-point; #, enhancement of the investigated end-point

[c] LED, lowest effective dose that inhibits or enhances the investigated effect; HID, highest ineffective dose

[d] Personal communication from author

[e] Presumed to be all-*trans*-RA since 13-*cis*-RA was listed in Sigma catalogue only from 1988 and 9-*cis*-RA from 1996

inhibited the induction of the *umu* C gene by the heterocyclic amine 3-amino-3,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in the presence of hepatic metabolizing enzymes derived from a 9000 x *g* liver supernatant (S9), but it did not prevent the induction of *umu* C expression by two directly acting mutagens, adriamycin and mitomycin C (Okai *et al.*, 1996).

Four of the studies conducted with the standard Ames' *Salmonella*/microsome test tested the ability of all-*trans*-retinoic acid to inhibit the effects of directly acting mutagens. It did not prevent the induction of reverse mutation by hydrogen peroxide in *S. typhimurium* TA104, a strain sensitive to oxidative mutagens (Han, 1992), and it had no effect on the mutagenic action of MNNG in strain TA100 or of 4-nitroquinoline 1-oxide in strain TA98 (Shetty *et al.*, 1988; Camoirano *et al.*, 1994). It significantly inhibited the mutagenicity of three nitroarenes: 2-nitrofluorene, 3-nitrofluoranthene and 1-nitropyrene in *S. typhimurium* TA98 (Tang & Edenharder, 1997).

The other studies, conducted with compounds and mixtures that require the presence of S9, showed mixed results. all-*trans*-Retinoic acid did not affect the mutagenicity of unfractionated cigarette smoke (Camoirano *et al.*, 1994) or of cigarette-smoke condensate in *S. typhimurium* TA98 (Wilmer & Spit, 1986) and did not affect the induction of mutation by benzo[*a*]pyrene, but it significantly reduced reverse mutation induced by aflatoxin B_1 in the same tester strain (Qin & Huang, 1985). The ability of all-*trans*-retinoic acid to inhibit aflatoxin B_1-induced mutation in strain TA98 has been reported in two other studies (Whong *et al.*, 1988; Raina & Gurtoo, 1985), while contradictory results were reported with TA100, one showing a protective effect (Bhattacharya *et al.*, 1987) and the other no inhibition (Raina & Gurtoo, 1985). [The Working Group noted that the protocol used by Raina and Gurtoo was different from that in the other five studies with Ames' test, in that a 5-min preincubation assay was used rather than the standard plate incorporation approach as described by Maron and Ames (1983). In addition, the S9 mixtures were prepared from the livers of C57BL/6Ha mice or Wistar rats without pretreatment, whereas in the other studies S9 liver fractions were prepared from Arochlor 1254-induced rats.]

(b) Mammalian cells

The ability of all-*trans*-retinoic acid to inhibit sister chromatid exchange and chromosomal breakage has been tested in carcinogen-treated cultures of mammalian cells (Table 5). all-*trans*-Retinoic acid did not affect the induction of sister chromatid exchange by the directly acting carcinogen mitomycin C in Chinese hamster V79 cells (Sirianni *et al.*, 1981). [The Working Group noted that the retinoid was not present concomitantly with the carcinogen in this study but was added after the carcinogen.], whereas another study showed that all-*trans*-retinoic acid protected against the induction of sister chromatid exchange when present at the same time as cyclophosphamide or DMBA, which are indirect mutagens or carcinogens. In the latter study an epithelial liver cell line of Chinese hamster cells was used which is known to activate promutagens and procarcinogens (Cozzi *et al.*, 1990). In Chinese hamster ovary cells exposed to aqueous extracts of *pan masala*, a complex mixture of areca nut, catechu, lime and cardamom, with or without tobacco, a lower frequency of sister chromatid exchange was observed in the presence of all-*trans*-retinoic acid. The frequencies of chromatid-type and chromosome-type aberrations were also reduced (Patel *et al.*, 1998).

C127 mouse cell lines created by transformation with bovine papillomavirus DNA carry 20–160 copies of the DNA and increased frequencies of chromatid bridges and fragments (27–59%) and of micronuclei (6.6–35%). Three-day exposure to all-*trans*-retinoic acid significantly reduced this instability, but the effect was transient since the instability reappeared after cessation of treatment with the retinoid (Stich *et al.*, 1990).

The effect of all-*trans*-retinoic acid on mutation in mammalian cells has been addressed in only one study. It had no effect on cytotoxicity or mutation expression at the hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) locus in Chinese hamster ovary cells exposed to the directly acting mutagen ethyl methanesulfonate, but it significantly reduced DMBA-induced cytotoxicity and mutation when metabolic activation was provided by either uninduced Sprague rat liver S9, Arochlor 1254-induced Sprague-Dawley rat liver S9 or co-cultivation with primary Sprague-Dawley rat hepatocytes (Budroe *et al.*, 1988). This retinoid also inhibited unscheduled DNA synthesis in primary

rat hepatocytes induced by the procarcinogen DMBA, but not that induced by two directly acting mutagens, ethyl methanesulfonate and ultraviolet light. The inhibitory effect on DMBA activity occurred at nontoxic concentrations, and the authors hypothesized that the effect was due to a reduction in DMBA-induced DNA damage through alterations of the DNA adduct load in cells treated concurrently with retinoid and carcinogen (Budroe *et al.*, 1987).

This hypothesis is supported by the results of five studies of the effect of all-*trans*-retinoic acid on the formation of carcinogen–DNA adducts. Three were carried out with cultured cells and the remaining two with calf thymus DNA co-incubated with rat liver microsomes (Table 5). When murine epidermal cells from newborn NIH Swiss mouse skin were exposed to [^{3}H]DMBA in the presence of all-*trans*-retinoic acid, a significant reduction was observed in the binding of the carcinogen to DNA, in the absence of a significant effect on the number of cells. This finding was particularly striking because the actual uptake of the radiolabelled carcinogen was higher in retinoid-treated cultures (Shoyab, 1981). In a similar study of the uptake and binding of [^{3}H]benzo[*a*]pyrene to DNA in cultured bronchial mucosa explants from 10 patients (all smokers) with bronchial cancer, the amount of DNA-bound carcinogen was significantly reduced when all-*trans*-retinoic acid was added. As in the previous study, binding to DNA was decreased even though the actual uptake of the carcinogen was increased in retinoid-treated cells. The authors concluded that since incorporation of [^{3}H]thymidine into DNA (as an index of the number of cells) did not change during treatment with the retinoid and the carcinogen, the increased cellular uptake of the carcinogen was not due to an increase in the number of cells in the explants (Bodo *et al.*, 1989). [The Working Group noted that incorporation of [^{3}H]thymidine into DNA was found to be altered in other studies with retinoids.]

The ability of all-*trans*-retinoic acid to potentiate the action of the chemotherapeutic drug, cisplatin, was assessed in human ovarian carcinoma cell lines. The number of DNA adducts formed was increased in NIHOVCAR$_3$ cells, a line known to be sensitive to all-*trans*-retinoic acid, but not in IGROV1 cells, which are insensitive to this retinoid (Caliaro *et al.*, 1997).

Both of the studies of the effect of all-*trans*-retinoic acid on the binding of carcinogens to calf thymus DNA showed a protective effect. Binding of [^{3}H]aflatoxin B$_1$ to calf thymus DNA, activated by liver microsomes from phenobarbital-induced Wistar rats, was significantly reduced by all-*trans*-retinoic acid. This effect was ascribed to a reduction in the formation of the reactive intermediate aflatoxin B$_1$-8,9-epoxide in the presence of the retinoid, measured by quantifying its hydrolysis product aflatoxin B$_1$-8,9-dihydrodiol as Tris-diol complex in the reaction mixtures (Firozi *et al.*, 1987). all-*trans*-Retinoic acid suppressed the formation of adducts on calf thymus DNA by the carcinogen benzo[*a*]pyrene in a reaction catalysed by liver microsomes from Arochlor 1254-treated rats. The inhibitory effect did not appear to be associated with the enzymatic activation step (i.e. generation and further activation of the proximate carcinogen, benzo[*a*]pyrene-7,8-diol in reaction mixtures). Instead, the retinoid accelerated the rate at which the ultimate carcinogenic metabolite, benzo[*a*]pyrene-7,8-diol-9,10-epoxide, disappeared from the reaction mixture containing all-*trans*-retinoic acid (Shah *et al.*, 1992).

In studies of the role of all-*trans*-retinoic acid in controlling the expression of genes involved in metabolism, a 19-fold induction of cytochrome P450 (CYP) 2C7 mRNA levels was found in primary rat hepatocytes exposed to all-*trans*-retinoic acid, this effect being exerted at the transcriptional level, as shown in nuclear run-on experiments (Westin *et al.*, 1993). In a similar study, CYP3A mRNA levels were shown to increase by approximately eightfold in primary hepatocytes exposed to all-*trans*-retinoic acid. The levels of CYP1A1 in messenger RNA were nonsignificantly increased, whereas no effect was observed on CYP1A2 levels (Jurima-Romet *et al.*, 1997).

(c) Experimental animals

These studies are summarized in Table 6.

In rats, the activity of enzymes involved in the metabolism of *N*-acetylaminofluorene and *N*-hydroxyacetylaminofluorene was enhanced by feeding all-*trans*-retinoic acid. The glucuronyl transferase activity in microsomal preparations prepared from the livers of treated animals was enhanced by 37%, thus increasing detoxification of the carcinogen, and the activity of *para*-nitro-

Table 6. Effect of exposure to all-*trans*-retinoic acid on metabolic activity in rodents *in vivo*

Retinoid (tested dose and administration route)[a]	Carcinogen (tested dose and administration route)[a]	Animal strain and species	Investigated effect	Result[b]	LED/HID[c]	Reference
RA (0.25% in diet for 3 days)[d]	Acetylaminofluorene or *N*-hydroxyacetylaminofluorene (17 mg/kg bw ip for 3 days)	Sprague-Dawley rats	Liver enzyme activity: • glucuronyltransferase • sulfotransferase • AAF-deacylase AAF-deacylase	 # + _	 37% increase 50% decrease No effect	Daoud & Griffin (1978)
all-*trans*-RA (30 mg/kg bw per day by gavage for 4 days)	None	Male Sprague-Dawley rats	Liver cytochrome P450 levels CYP1A2 CYP2B1/2 CYP2C11 CYP2E CYP3A CYP4A P450 metabolism Glucuronidation	 – – + + – – (+) +		Howell *et al.* (1998)
all-*trans*-RA (single topical dose of 0.1% applied to skin)	None	Human	P450-mediated mediated metabolism of all-*trans*-RA to 4-hydroxyretinoic acid	#	0.1% (4.5-fold increase)	Duell *et al.* (1992)
all-*trans*-RA (single topical dose of 0.05% applied to skin)	None	Human	Basal P4501A1 expression	+	0.05% (68% decrease in P4501A1 mRNA levels; 75% decrease in P4501A1 protein levels)	Li *et al.* (1995)
all-*trans*-RA (single topical dose of 0.05% applied to skin)	None	Human	Basal P4501A2 expression	+	0.05% (93% reduction in P4501A2 mRNA levels)	Li *et al.* (1995)
all-*trans*-RA (single topical dose of 0.05% applied to skin)	10% crude coal-tar	Human	Induced P4501A1 expression	+	0.05% (46% decrease in P4501A1 mRNA levels)	Li *et al.* (1995)
all-*trans*-RA (single topical dose of 0.05% applied to skin)	0.025% clobetasol propionate	Human	Induced P4501A1 expression	+	0.05% (69% decrease in P4501A1 mRNA levels)	Li *et al.* (1995)

RA, retinoic acid; ip, intraperitoneally; AAF, acetylaminofluorene

[a] Doses of retinoids and carcinogens and routes of administration are given as reported by the authors.

[b] +, inhibition of the investigated end-point; (+), weak inhibition of the investigated end-point, not significantly different; –, no effect on the investigated end-point, #, enhancement of investigated end-point; (#) enhancement but statistically only approaching significance, $p < 0.06$

[c] LED, lowest effective dose that inhibits or enhances the investigated effect; HID, highest ineffective dose

[d] Source and type not given; presumed to be all-*trans*-RA because of date

phenol-sulfotransferase, the enzyme involved in activation of *N*-acetylaminofluorene and *N*-hydroxyacetylaminofluorene to reactive states, was inhibited; however, it had no effect on *N*-acetylaminofluorene-deacylase activity (Daoud & Griffin, 1978).

In a comprehensive study of the effect of five retinoids, one of which was all-*trans*-retinoic acid, on hepatic microsomal metabolism and CYP activity in Sprague-Dawley rats, the animals received daily oral doses of 30 mg/kg bw for 4 days, and liver microsomes were prepared on day 5. The activity of CYP isoenzymes was assayed by western blot immunoanalysis. The activities of CYP1A2, CYP2B1/2 and CYP3A were reduced by roughly 27%, 20% and 27% respectively, in animals receiving all-*trans*-retinoic acid, although these effects were not statistically significant. The activity of CYP2E was reduced by 30% and that of CYP2C11 by 40% (both $p < 0.05$), whereas that of CYP4A remained unchanged. The effect of this drug on metabolic activity was limited to a study of its own phase I and II metabolism. A decrease was observed in the CYP-mediated metabolism of all-*trans*-retinoic acid by microsomal preparations from treated animals, although the effect only approached significance ($p = 0.06$). In contrast, a significant decrease was found in the glucuronidation capacity of the microsomal preparation. The authors noted that the patterns of alterations in metabolism and isozyme profiles differed significantly among the retinoids studied and suggested that the effect could be related to binding selectivity of the different retinoids for either RAR or RXR receptors, although the data are not conclusive (Howell *et al.*, 1998).

Retinoids may also modulate the metabolism of carcinogens in CYP-independent pathways. In a study of the effect of all-*trans*-retinoic acid on DNA adduct formation, female CD-1 mice were given a topical application of the proximate carcinogen (7*S*,8*S*)-dihydroxy-7,8-dihydrobenzo[*a*]pyrene ((+)-BP-7,8-diol), which is further metabolized by epoxidation to 7,8-dihyroxy-9,10-epoxy-7,8,9,10 tetrahydrobenzo[*a*]pyrene (BPDE). When the BP-7,8-diol was applied by itself to the animals, it was metabolized mainly by CYP systems, resulting in (+)-*syn*-BPDE–DNA adducts; however, when the animals were pretreated with TPA 24 h before administration of TPA and (+)-BP-7,8-diol, the pattern of adducts changed to include (–)-*anti*-BPDE adducts, thought to be derived from a CYP-independent pathway that probably involves peroxyl radical-dependent epoxidation. When all-*trans*-retinoic acid was administered with the second TPA treatment, formation of the (–)-*anti*-BPDE-DNA adducts was significantly inhibited. Administration of the retinoid with the first TPA treatment had no effect. The authors speculated that the first TPA dose recruited neutrophils to the treatment site and the second dose triggered the release of oxidants from these neutrophils. They further suggested that the retinoic acid acts as a radical scavenger, thus preventing peroxyl radical-dependent epoxidation (Marnett & Ji, 1994).

There have been few studies of the effect of all-*trans*-retinoic acid on metabolism in humans, but there is some indication that metabolic activity and the CYP enzyme profiles in tissues are altered by all-*trans*-retinoic acid. A single topical dose of 0.1% all-*trans*-retinoic acid cream or cream vehicle was applied to adult human skin and the region was occluded for four days with Saranwrap. After four days, the test area was washed and sliced off, and microsomal fractions were prepared and assayed for their capacity to metabolize [^{3}H]all-*trans*-retinoic acid *in vitro*. Microsomes from treated sites had a 4.5-fold increase in their capacity to form 4-hydroxyretinoic acid in comparison with microsomes from vehicle-treated sites. This metabolism appeared to be CYP-mediated since the inclusion of ketoconazaole, an inhibitor of CYP-mediated activity, in the reaction mixtures resulted in inhibition of metabolism in microsomal fractions isolated from retinoic acid-treated sites (Duell *et al.*, 1992). In a similar study, the basal level of expression of CYP1A1 and CYP1A2 was quantified in skin samples from volunteers receiving topical 0.05% all-*trans*-retinoic acid in cream or cream without retinoic acid. The authors also examined the effect of all-*trans*-retinoic acid on the expression of these two isoenzymes in patients receiving the cream with either 10% crude coal-tar or 0.025% clobetasol proprionate, a potent glucocorticoid. all-*trans*-Retinoic acid reduced the basal activities of CYP1A1 mRNA and protein by 68% and 75% respectively, and the basal activity of CYP1A2 mRNA by 93%. Coal-tar and clobetasol increased the activity of CYP1A1 but not that of CYP1A2 mRNA expression. all-*trans*-Retinoic acid inhibited the CYP1A1 mRNA expression induced

by coal-tar by 46% and that induced by clobetasol by 69% (Li *et al.*, 1995).

4.3 Mechanisms of cancer prevention

all-*trans*-Retinoic acid may prevent or delay carcinogenesis by several mechanisms, which depend on the cell type used *in vitro*, on the carcinogen and animal strain *in vivo* and on the tissue affected. Most of the studies indicate that retinoids inhibit the promotion step of the multistage carcinogenesis process, although there are indications that it also affects initiation. Several reports described in section 4.2.3.2 support the hypothesis that retinoids such as all-*trans*-retinoic acid can inhibit initiation induced by carcinogens that require metabolic activation, whereas they have little effect on directly acting carcinogens. The mechanisms of the suppression of initiation include: induction of the transcription of certain CYP enzymes that can detoxify carcinogens, decreased binding of carcinogens to DNA and decreased carcinogen-induced DNA damage. The activity of several CYP enzymes was found to be regulated at the level of transcription by direct binding of nuclear RARs to retinoic acid response elements in the gene promoters, as shown for CYP1A1 (Vecchini *et al.*, 1994).

Most studies on the effects of all-*trans*-retinoic acid on carcinogenesis indicate that the main mechanism is inhibition of promotion, which is related to the ability of retinoids to antagonize the activity of tumour promoters and affect the proliferation, differentiation and apoptosis of premalignant and malignant cells. The ability of all-*trans*-retinoic acid to alter intercellular adhesion and inhibit host responses such as angiogenesis may also play a role in its chemopreventive activity.

4.3.1 Antagonism of tumour promotion and activator protein 1 activity

all-*trans*-Retinoic acid inhibits the action of tumour promoters by antagonizing tumour promoter signalling. For example, it inhibited the induction of ornithine decarboxylase by TPA in cultured tracheal cells (Jetten & Shirley, 1985) and mouse skin (Connor *et al.*, 1983). The mechanism by which ornithine decarboxylase expression is controlled by all-*trans*-retinoic acid appears to be suppression of gene transcription and involves liganded nuclear RAR (Mao *et al.*, 1993). all-*trans*-Retinoic acid can also affect other down-stream events of TPA signalling. TPA activates protein kinase C and eventually activates the transcription activator protein 1 (AP-1). all-*trans*-Retinoic acid can exert anti-AP-1 activity by *trans*-repressing the AP-1 function and thereby inhibiting TPA-induced transformation of mouse epidermal JB6 cells (Li *et al.*, 1996). Furthermore, in DMBA-initiated skin of transgenic mice carrying an AP-1-luciferase transgene, inhibition of papilloma formation by all-*trans*-retinoic acid and by retinoids with anti-AP-1 activity appeared to be mediated by suppression of AP-1 activation. Retinoids capable of RAR element *trans*-activation but devoid of anti-AP-1 activity failed to inhibit papilloma formation (Huang *et al.*, 1997).

4.3.2 Inhibition of cell proliferation

The formation of a premalignant lesion requires that initiated cells proliferate to expand the initiated clone. Clearly, inhibition of such proliferation would be an important mechanism for cancer prevention. There is no direct evidence that all-*trans*-retinoic acid can block the proliferation of initiated cells *in vivo* because they cannot be identified at an early stage as such, but there is ample evidence that all-*trans*-retinoic acid can inhibit cell proliferation in a number of settings. It inhibited cell proliferation by regulating cell cycle progression from G1 to S phase by altering the levels of cell cycle controlling proteins.

4.3.2.1 Cyclins and cyclin-dependent kinase inhibitors

The inhibition of DNA synthesis and the G1 arrest in SV-40-T-immortalized human bronchial epithelial BEAS-2B cells and their NNK-transformed derivative cell lines was found to occur as a result of suppression of the protein levels of cyclins D1 and E at a post-translational level. all-*trans*-Retinoic acid promoted the degradation of cyclin D1 by targeting it for proteolysis in proteasomes via increased ubiquitinylation which depended on the C-terminal PEST[proline (P), glutamate (E), serine (S) and threonine (T)] sequence (Langenfeld *et al.*, 1996, 1997; Boyle *et al.*, 1999). Specific nuclear RARs have been implicated in this effect because the receptor-selective retinoids that activated RARβ or RXR pathways caused a greater decrease in the amount of cyclin D1 protein and corresponding

inhibition of DNA synthesis (Boyle *et al.*, 1999). Studies with lymphoblastoid B cell lines immortalized with Epstein-Barr virus have shown that all-*trans*-retinoic acid-induced accumulation in the G0/G1 phase is associated with multiple effects on G1 regulatory proteins, including p27Kip1 up-regulation, decreased levels of cyclins D2, D3 and A and inhibition of cyclin-dependent kinase (CDK)2, CDK4 and CDK6 activity, which ultimately resulted in reduced pRb phosphorylation and G0/G1 growth arrest (Zancai *et al.*, 1998). p21, which has been shown to induce G1 arrest by inhibiting CDK and proliferating cell nuclear antigen dependent DNA replication, was recently found to possess an RAR element in its promoter and to be regulated by retinoic acid. This could be another mechanism for cell cycle regulation (Liu *et al.*, 1996).

4.3.2.2 trans-*Repression of activator protein 1*

As activated AP-1 is the ultimate mediator of signalling by many mitogens, the antagonistic effects of all-*trans*-retinoic acid on AP-1 may suppress growth. This was found to occur in normal bronchial epithelial cells in which growth had been inhibited by all-*trans*-retinoic acid but not in the all-*trans*-retinoic acid-resistant, tumorigenic derivatives of SV-40-T-immortalized BEAS-2B cells (Lee *et al.*, 1997).

4.3.2.3 Suppression of the human papillomavirus oncogenes E6 and E7

HPV-16 immortalized keratinocytes were very sensitive to growth inhibition by all-*trans*-retinoic acid because the retinoid inhibited the expression of HPV-16 *E6* and *E7* oncogenes, which are required for maintenance of the continuous proliferation of these immortalized cells (Pirisi *et al.*, 1992).

4.3.2.4 Modulation of autocrine and paracrine loops

Premalignant cells often have a growth advantage over normal cells, as they overexpress growth factor receptors that can mediate paracrine or autocrine growth stimulation. One example of such a receptor is the epidermal growth factor receptor (EGFR) which is overexpressed in various premalignant lesions and can enhance cell growth by autocrine or paracrine routes in conjunction with epidermal growth factor (EGF) or transforming growth factor α (TGF-α). all-*trans*-Retinoic acid suppressed the expression of TGF-α and EGFR mRNA in head-and-neck squamous-cell carcinoma cells by decreasing gene transcription (Grandis *et al.*, 1996).

HPV immortalization increased EGF receptor levels in ectocervical cells, increasing their sensitivity to growth stimulation by EGF. all-*trans*-Retinoic acid reduced both EGF binding and EGF receptor protein levels in immortalized cells but not in normal ectocervical cells. Thus, it can attenuate the increased responsiveness to EGF by decreasing the EGFR level (Sizemore & Rorke, 1993). all-*trans*-Retinoic acid inhibition of the growth of HPV-immortalized ectocervical cells and cervical carcinoma cell lines has been proposed to result from an increase in insulin-like growth factor binding protein 3 mRNA and protein levels and a reduced extracellular concentration of free insulin-like growth factor I (Andreatta-Van Leyen *et al.*, 1994).

Transforming growth factor-β (TGF-β) plays a complex role in the regulation of proliferation and differentiation of many cell types, including cells of epithelial origin, for which TGF-β is usually a growth inhibitory factor. In the immortal mouse epidermal cell line JB6, TPA caused progression to anchorage independence and tumorigenicity, partly by decreasing the level of TGF-β receptor expression. all-*trans*-Retinoic acid counteracted both the promoting effect of TPA and its suppression of TGF-β receptor (De Benedetti *et al.*, 1991).

4.3.3 Restoration of normal differentiation

Carcinogenesis is characterized by aberrant differentiation, which is manifested by either blockage of cells at an early stage of differentiation or redirection of differentiation towards an abnormal pathway. Several reports described in the General Remarks (section 4) have demonstrated the ability of all-*trans*-retinoic acid to suppress squamous-cell differentiation and enhance mucociliary differentiation in epithelial cells as well as stimulate differentiation of numerous tumour cell lines. It is thought that restoration by all-*trans*-retinoic acid of normal differentiation in premalignant cells might be accompanied by restoration of normal growth control mechanisms as well, but there is no clear experimental evidence that the effect of all-*trans*-retinoic acid on differentiation of premalignant cells is the cause of either cell growth inhibition or cell apoptosis.

The clear demonstration that all-*trans*-retinoic acid can induce terminal differentiation *in vivo* even in a fully malignant condition, acute promyelocytic leukaemia, lends strong support to this concept (Castaigne *et al.*, 1990; Warrel *et al.*, 1993).

4.3.4 Inhibition of prostaglandin production

An excessive production of prostaglandins has been correlated with tumour promotion. More recently, it was found that expression of the enzyme cyclooxygenase-2 (Cox-2), which catalyses the synthesis of prostaglandins, can be induced by growth factors and tumour promoters and is up-regulated in transformed cells and tumours. Therefore, it has become a target for chemoprevention. Treatment of oral epithelial cells with either EGF or TPA enhanced transcription of Cox-2 and increased production of prostaglandin-2. These effects were inhibited by all-*trans*-retinoic acid (Mestre *et al.*, 1997). [The Working Group noted that the molecular mechanism of this effect of all-*trans*-retinoic acid has not been elucidated.]

4.3.5 Induction of apoptosis

Several studies summarized in the General Remarks (section 4) demonstrated the ability of all-*trans*-retinoic acid to induce apoptosis in various tumour cell lines. The mechanism by which apoptosis is induced is not clear, but several studies have implicated nuclear RARs and RXRs in the process on the basis of the results of experiments with receptor-selective retinoids and transfection (Nagy *et al.*, 1995; Melino *et al.*, 1997; Monczak *et al.*, 1997). It is still not clear which target genes are induced that trigger apoptosis. It has been suggested that induction of tissue transglutaminase by all-*trans*-retinoic acid may be related to apoptosis induction (Melino *et al.*, 1997).

4.3.6 Increased gap-junctional communication

Most normal cells are directly coupled via intercellular gap junctions formed by the assembly of connexin proteins. Loss of gap-junctional communication is an early event in neoplasia both *in vitro* and *in vivo*. Restoration of this communication by transfection of connexin genes into tumour cells results in enhanced growth control and/or suppression of the neoplastic phenotype (Trosko & Ruch, 1998). all-*trans*-Retinoic acid has been shown to increase the expression of connexin 43 (Cx43) in C3H/10T1/2 cells at the levels of mRNA and protein (Rogers *et al.*, 1990); the resulting increase in gap-junctional communication is highly correlated with the ability of all-*trans*-retinoic acid to inhibit neoplastic transformation in these cells (Hossain *et al.*, 1989) and the suppression of proliferation in normal and transformed cells (Mehta *et al.*, 1989). Inhibition of proliferation by all-*trans*-retinoic acid was dependent on cell–cell contact and was not seen in sparsely seeded cells, again implicating gap-junctional communication in this response (Hossain & Bertram, 1994). The relevance of induced gap-junctional communication to the chemopreventive activity of all-*trans*-retinoic acid *in vivo* is suggested by the demonstration that the retinoid upregulates Cx43 expression in human skin after topical application (Guo *et al.*, 1992). The mechanism by which it increases Cx43 expression is not clear, as the promoter region does not contain a known RAR element. In one study, stabilization of Cx43 mRNA by all-*trans*-retinoic acid was suggested (Clairmont *et al.*, 1996).

4.3.7 Modulation of cell adhesion and motility

Cell invasion through the basement membrane is the ultimate step that distinguishes a carcinoma *in situ* from a malignant tumour. As this process requires changes in cell adhesion and detachment, local proteolysis and migration, inhibition of invasion may suppress malignant conversion. It is noteworthy that all-*trans*-retinoic acid can up-regulate the function of the invasion-suppressor complex E-cadherin/catenin (Vermeulen *et al.*, 1995) and inhibit the expression of various enzymes that degrade the basement membrane, such as collagenase and stromelysine, at the level of transcription by antagonizing AP-1 (Nicholson *et al.*, 1990; Schüle *et al.*, 1991). In addition, all-*trans*-retinoic acid suppressed the level of autocrine motility factor receptor and decreased cell motility (Lotan *et al.*, 1992). These findings suggest that if it can suppress the expression of the invasive phenotype in carcinoma *in situ*, it may prevent the progression of a premalignant lesion to a malignant one.

4.3.8 Inhibition of angiogenesis

The progressive growth of solid tumours depends on the development of new blood vessels, a

process known as neovascularization or angiogenesis. Tumour cells secrete factors that induce the directed migration and proliferation of endothelial cells from capillaries in normal tissue, which eventually differentiate and form vessels around and within tumours. It is likely that some premalignant lesions also depend on angiogenesis for conversion into invasive cancer. all-*trans*-Retinoic acid has demonstrated antiangiogenic effects in several systems. In one study, it caused the endothelial cells of large and small vessels to become refractory to stimulation of migration by tumour-conditioned media or purified angiogenic factors (α-fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor, platelet-derived growth factor, TGFβ-1, and interleukin-8) without affecting cell proliferation (Lingen *et al.*, 1996). Rats given all-*trans*-retinoic acid were unable to mount a neovascular response to tumours implanted in their corneas. These results indicated that all-*trans*-retinoic acid can affect directly both tumour cells and endothelial cells and thereby suppress the formation of new blood vessels *in vivo*. Treatment of mice xenotransplanted with a human squamous-cell carcinoma reduced the level of a binding protein for fibroblast growth factor. This inhibited angiogenesis and led to a decrease in the tumour growth rate.

5. Other Beneficial Effects

all-*trans*-Retinoic acid is of benefit to patients suffering from a variety of dermatological disorders (see section 2.3).

6. Carcinogenicity

6.1 Humans

No data were available to the Working Group.

6.2 Experimental models

No studies of the carcinogenicity of all-*trans*-retinoic acid were available to the Working Group, but all-*trans*-retinoic acid can act as a tumour promoter in mouse skin and can enhance liver carcinogenesis induced by NDEA in mice (for details, see section 4.2.1).

7. Other Toxic Effects

7.1 Adverse effects

7.1.1 Humans

At the recommended oral dose of all-*trans*-retinoic acid, 45 mg/m^2 per day as two evenly divided doses, virtually all patients experience some drug-related toxicity. The most frequent adverse events are similar to those described in patients taking high doses of retinol and its esters (IARC, 1998), especially headache, fever, weakness and fatigue. Rare adverse events associated with use of all-*trans*-retinoic acid in patients with acute promyelocytic leukaemia include hypercalcaemia, bone-marrow necrosis, bone-marrow fibrosis, acute pancreatitis, thromboembolic events, acute neutrophilic dermatosis, erythema nodosum, basophilia, hyperhistaminaemia, granulomatous proliferation and necrotizing vasculitis (reviewed by Hatake *et al.*, 1997; Paydas *et al.*, 1998). The toxicity associated with topically applied all-*trans*-retinoic acid at 0.025% or 0.01% is generally localized, dermal and reversible after discontinuation of treament (Arky, 1998).

7.1.1.1 Retinoic acid syndrome

About 25% of patients with acute promyelocytic leukaemia who have been treated with the recommended oral dose of all-*trans*-retinoic acid have experienced 'retinoic acid syndrome', a life-threatening disorder reported to be related to leukocyte activation (Fenaux & De Botton, 1998). The syndrome usually occurs within the first month of treatment, sometimes after the first dose, and is characterized by fever, dyspnoea, weight gain, radiographic pulmonary infiltrates and pleural or pericardial effusion. The syndrome has occasionally been accompanied by impaired myocardial contractility, and episodic hypotension and has been observed with or without concomitant leukocytosis. Glucocorticoids given at high doses when symptoms of the syndrome appear may reduce the morbidity and mortality; in the absence of prevention by chemotherapy, 60% or more of patients taking oral all-*trans*-retinoic acid may require this treatment (Arky, 1998).

Retinoic acid syndrome has been ascribed to infiltration of leukaemic or maturing myeloid cells

into the lung parenchyma. Diffuse alveolar haemorrhage with pulmonary capillaritis was reported in one woman who was treated with all-*trans*-retinoic acid for acute promyelocytic leukaemia (Nicolls *et al.*, 1998). Thromboembolic complications have also been reported, including acute renal failure due to occlusion of renal vessels (Pogliani *et al.*, 1997). A study of the development of retinoic acid syndrome showed that clinical signs generally develop within a median of seven days. Respiratory distress (89%), fever (81%), pulmonary infiltrates (81%), weight gain (50%), pleural effusion (47%), renal failure (39%), pericardial effusion (19%), cardiac failure (17%) and hypotension (12%) were the main clinical signs, and almost all subjects with the syndrome experienced at least three of these events. Nine of 64 cases of retinoic acid syndrome resulted in death. No significant predictive factors of the syndrome were found, but its occurrence was associated with shorter event-free survival and shorter overall survival (De Botton *et al.*, 1998).

7.1.1.2 Relapse of acute promyelocytic leukaemia at unusual sites

Although the combination of all-*trans*-retinoic acid and chemotherapy with daunorubicin and cytosine arabinoside has become the standard therapy for acute promyelocytic leukaemia, concern has been raised that all-*trans*-retinoic acid in combination with the other agents may increase the risk for relapse at unusual sites. Myelodysplastic syndrome and central nervous system relapse, although rare, have been seen after treatment of acute promyelocytic leukaemia with all-*trans*-retinoic acid. Whether the frequency of such events is truly increased among these patients is being addressed in clinical trials (Bseiso *et al.*, 1997; Evans *et al.*, 1997).

7.1.1.3 Metabolic, nutritional and haematological toxicity

Up to 60% of patients given the prescribed oral dose of all-*trans*-retinoic acid experience hypercholesterolaemia and/or hypertriglyceridaemia, and 50–60% show increased activity of liver enzymes. These abnormalities can sometimes lead to more severe complications such as acute pancreatitis, hepatotoxicity, hyperleukocytosis, worsening of coagulopathy, renal dysfunction, myocardial infarct and thrombocytosis (Cull *et al.*, 1997; Kentos *et al.*, 1997; Yutsudo *et al.*, 1997; Arky, 1998). Hypercalcaemia has also been observed after oral administration of all-*trans*-retinoic acid; in one case, the hypercalcaemia was controlled and complete remission of acute promyelocytic leukaemia was achieved after the dose of all-*trans*-retinoic acid was reduced from 45 to 27 mg/m^2 per day (Lemez, 1995).

A phase-I trial of orally administered all-*trans*-retinoic acid was conducted to establish the maximum tolerated dose when given once daily to patients with solid tumours. In 49 patients who received doses ranging from 45 to 309 mg/m^2 per day, hypertriglyceridaemia was the dose-limiting effect at 269 mg/m^2 per day; grade 3 hypertriglyceridaemia developed in one patient each at 110, 138 and 269 mg/m^2 per day, and grade 4 hypertriglyceridaemia developed in one patient each at 88, 215 and 269 mg/m^2 per day. Grade 3 hypercholesterolaemia was seen in one patient at 269 mg/m^2 per day. Grade 3 transient elevations of transaminase activity were seen in four patients treated at doses ≥ 56 mg/m^2 per day. Toxic effects at grade 3 and 4 occurred sporadically and included thrombocytopenia, dehydration and transient renal failure, localized desquamous rash, staphylococcal bacteraemia, probable pseudotumour cerebri, shortness of breath, severe cough, myalgia, severe exacerbation of neck pain, headache, anorexia, fatigue, dysphagia, scleral haemorrhage, anaemia, nausea and vomiting. Severe toxicity tended to occur when the initial peak plasma concentration of all-*trans*-retinoic acid was 0.5 μg/ml, and the frequency of toxicity did not change as the plasma concentration decreased. The daily dose of all-*trans*-retinoic acid in patients with solid tumours recommended on the basis of these findings is 215 mg/m^2 (Conley *et al.*, 1997). [The Working Group noted that the symptoms were not necessarily related to treatment.]

In a clinical study of the treatment of chronic myeloid leukaemia, 18 patients were given 12 courses of 80 mg/m^2 per day, divided into two equal doses, each course consisting of one week of drug followed by one week's rest. Eleven patients left the study before the sixth course because of progressive hyperleukocytosis, thrombocytosis or refusal. Five additional subjects were withdrawn before the twelfth course because of elevated leukocyte counts, and one withdrew for other

reasons. Hepatic toxicity of grades 1–2 was observed in 5% of participating subjects (Russo *et al.*, 1998). Conversely, there were no documented instances of increased liver function or triglyceride concentration in 13 patients with chronic myeloid leukaemia who carried the Philadelphia chromosomal translocation and were treated for a median duration of 56 days with 175 mg/m^2 per day, divided into two equal daily doses (Cortes *et al.*, 1997).

7.1.1.4 Mucocutaneous toxicity

(a) Topical application

The most frequent adverse effects of topically applied all-*trans*-retinoic acid are dermal. Local peeling, dry skin, burning, stinging, erythema and pruritus were reported by almost all of 179 patients who applied 0.05% all-*trans*-retinoic acid cream to their faces. The reactions were usually of mild to moderate severity, occurred early during therapy and recurred after an initial 24-week decline. Patients using topical all-*trans*-retinoic acid have a heightened sensitivity to sunlight and extreme wind or cold. Temporary hyper- or hypopigmentation has also been reported (Arky, 1998). Skin irritation caused by topically applied all-*trans*-retinoic acid does not appear to involve injury to the skin barrier but may be caused by dilatation of the cutaneous vasculature (Fullerton & Serup, 1997). Modified delivery systems such as polymer complexes and microsponge particles have been developed to reduce the irritating effects of topical all-*trans*-retinoic acid (Leyden, 1998).

The effectiveness of topical all-*trans*-retinoic acid (0.05.%) in reversing signs of photoageing was investigated under conditions of extended use (48 weeks). As expected, the adverse events were generally mild and were essentially dermal (dry skin, peeling, burning/stinging, erythema, irritation, pruritis and papules). The frequency of the adverse effects decreased with time (Olsen *et al.*, 1997).

Topically applied all-*trans*-retinoic acid has been investigated as an intravaginal treatment for cervical intraepithelial neoplasia. In one study, doses of 0.05–0.2% applied to a sponge and inserted in a cervical cap for four days produced vaginal irritation, ulceration and discharge, with no evidence of systemic toxicity (Surwit *et al.*, 1982). A study of doses increasing from 0.05% for four days showed that the maximum tolerated dose was 0.372% (Meyskens *et al.*, 1983). In a subsequent study, 1 mL of 0.375% all-*trans*-retinoic acid induced only local, mild side-effects consisting primarily of increased vulvar burning, itching and irritation; again, no systemic side-effects were observed (Meyskens *et al.*, 1994).

(b) Oral dosing

Mucocutaneous reactions are also common after treatment with oral all-*trans*-retinoic acid and include mucocutaneous dryness, xerostomia, dry skin, xerophthalmia, erythema and pruritis (Conley *et al.*, 1997; Sutton *et al.*, 1997; Budd *et al.*, 1998; Russo *et al.*, 1998). An oral dose of 230 mg/m^2 in combination with 20 mg/day tamoxifen resulted in unacceptable dermatological toxicity (moist desquamation) in patients with advanced breast cancer (Budd *et al.*, 1998). Six of 13 patients with chronic myeloid leukaemia experienced dry skin or dry mucous membranes after administration of 175 mg/m^2 per day in two equal doses (Cortes *et al.*, 1997).

7.1.1.5 Headache and general toxicity

Moderate to severe headaches are commonly experienced during oral therapy with all-*trans*-retinoic acid. Cases of pseudotumour cerebri with clinical signs of headache, nausea, double vision and bilateral papilloedema have been reported (Sano *et al.*, 1998). General pain, bone pain, malaise and myalgia are also associated with such therapy (Conley *et al.*, 1997; Budd *et al.*, 1998; Russo *et al.*, 1998).

Headache was the most common side-effect (54%) in a pilot study of orally administered all-*trans*-retinoic acid in patients with chronic myelogenous leukaemia who carried the Philadelphia chromosome and were given a dose of 175 mg/m^2 per day in two equal doses (Cortes *et al.*, 1997). In a phase-II trial in patients with metastatic breast cancer, all-*trans*-retinoic acid was given orally at a dose of 50 mg/m^2 three times a day for 14 days of a 21-day cycle, which was repeated until progression of the disease, unacceptable toxicity or patient withdrawal. Of 17 subjects, 14 completed at least one course of therapy; the median time to progression for the remaining 14 subjects was six weeks. Two patients experienced grade 3 headache during the first cycle; the symptoms resolved after discontinuation of the drug, and returned after re-challenge. Six other subjects experienced grade 2 headache during treatment (Sutton *et al.*, 1997).

The combination of all-*trans*-retinoic acid and interferon-α was investigated in a phase-I trial in children with refractory cancer. Pseudotumour cerebri or dose-limiting headache was observed in two of five patients over 12 years of age who had been treated at a dose of 120 mg/m² per day, and in one of six patients under 12 years who had been given 90 mg/m² per day (Adamson *et al.*, 1997).

A phase-I/II trial of all-*trans*-retinoic acid and tamoxifen was conducted in patients with potentially hormone-responsive advanced breast cancer. The patients received 20 mg/day tamoxifen and all-*trans*-retinoic acid on alternating weeks at doses of 70–230 mg/m² per day, divided into two equal doses per day. At all doses, headache, nausea, bone pain and skin changes were noted. The headaches were most severe during the first week of therapy and peaked at the end of the first week. The symptoms recurred in subsequent weeks of all-*trans*-retinoic acid therapy, although their severity tended to wane with each subsequent cycle. all-*trans*-Retinoic acid at a dose of 230 mg/m² produced unacceptable headache. Grade 3 headache, grade 2 nausea and vomiting and dermatological effects also occurred in some subjects given all-*trans*-retinoic acid at 190 mg/m². The maximum tolerated dose that could be given in alternating weeks with 20 mg/day tamoxifen was 190 mg/m² per day (Budd *et al.*, 1998).

7.1.1.6 Gastrointestinal toxicity

Gastrointestal toxicity ranging from constipation to severe nausea has been reported after oral administration of all-*trans*-retinoic acid. A dose of 80 mg/m² per day divided into two equal doses produced constipation (11%) and nausea (5%) in patients with chronic myeloid leukaemia (Russo *et al.*, 1998). When all-*trans*-retinoic acid at doses of 70–230 mg/m² per day was combined with 20 mg/day tamoxifen, grade 1 nausea and vomiting were observed in most subjects; grade 2 nausea and vomiting occurred in some subjects receiving doses ≥ 190 mg/m² per day (Budd *et al.*, 1998). Two of 17 patients with metastatic breast cancer experienced grade 3 nausea and vomiting at a dose of 50 mg/m² three times a day (Sutton *et al.*, 1997). Nausea was also experienced by four of 13 patients with chronic myelogenous leukaemia given 175 mg/m² per day in two equal doses; one of the cases was described as severe (Cortes *et al.*, 1997).

7.1.2 Experimental models

The LD_{50} of orally administered all-*trans*-retinoic acid is approximately 2200 mg/kg bw in mice and 2000 mg/kg bw in rats (Kamm, 1982). The maximum tolerated dose in athymic nude mice was 20 mg/kg bw per day when given five times per week for four weeks. The dose was selected on the basis of acceptable weight loss and symptoms of hypervitaminosis A; a weight loss of 10–20% and mild to severe mucocutaneous reactions, such as dry or red skin, were seen at doses ≥ 30 mg/kg bw per day (Shalinsky *et al.*, 1995).

Rats given all-*trans*-retinoic acid orally at 5 or 50 mg/kg bw per day for 13 weeks showed hair loss, dermal and mucosal alterations, inhibition of spermatogenesis and weight loss at the low dose, and increased serum transaminase and alkaline phosphatase activities at the high dose. Similar signs were seen in dogs, but 50% of those at the high dose died. In mice, doses of 150–250 mg/kg bw per day caused alopecia, weight loss and skin and membrane changes after five days (Kamm *et al.*, 1984). In other studies in mice, the main dose-related findings included bone rarefaction, bone fracture, elevated serum alkaline phosphatase activity, testicular degeneration, dermal and epidermal inflammation and hyperkeratosis. In rats, decreases in plasma albumin and in haemoglobin concentration and an increase in serum alkaline phosphatase activity were seen. In a comparative study of all-*trans*-retinoic acid, etretinate and retinol palmitate/retinol acetate in rats, doses of 15, 7.5 and 40 mg/kg bw per day, respectively, had about the same negative effect on body weight. After four weeks of administration at these doses, bone fractures and increased serum alkaline phosphatase activity were observed with all agents, but all-*trans*-retinoic acid and retinol palmitate/retinol acetate were more lethal than etretinate. Retinol palmitate/retinol acetate resulted in the lowest frequency of bone fractures, and the increase in serum alkaline phosphatase activity was greatest with all-*trans*-retinoic acid, indicating that retinoids are not equipotent with respect to their short-term toxicity (reviewed by Kamm, 1982).

In male mice treated topically with 0.1% all-*trans*-retinoic acid in gel microspheres for 90 days at 2 or 5 mg/kg bw per day, testicular weight was reduced, with no pathological changes. This effect was not seen at 0.5 mg/kg bw per day. Females

showed a reduction in ovarian weight at 5 mg/kg bw per day, with no pathological changes. A dose-related increase in the plasma concentration of all-*trans*-retinoic acid was seen 4 h after the first dose. Male and female dogs treated for 90 days with 0.1% all-*trans*-retinoic acid in gel microspheres at 0.2, 0.5 or 1 mg/kg bw per day showed no evidence of reduced testicular or ovarian weights or other pathological changes. The systemic exposure observed in preclinical studies of toxicity with topically applied all-*trans*-retinoic acid is probably the result of incidental ingestion (Arky, 1998). [The Working Group noted that topical administration of high doses of all-*trans*-retinoic acid gives rise to severe skin irritation, which is dose-limiting.]

7.2 Reproductive and developmental effects

7.2.1 Humans

7.2.1.1 Reproductive effects

Oral administration of all-*trans*-retinoic acid at 20–30 mg/day (~ 0.4 mg/kg bw per day) to 12 patients with acne for 90–300 days was associated with dry oral and nasal mucous membranes. The treatment had no measurable effect on sperm count, sperm density, total and progressive motility or morphology other than an increase in total ejaculate volume (Plewig *et al.*, 1979).

7.2.1.2 Developmental effects

(a) Topical exposure

The available case reports and epidemiological studies do not provide evidence for developmental effects after administration of the standard dose of all-*trans*-retinoic acid (Rosa, 1992). In a retrospective study of 215 pregnant women with a history of dermal use of all-*trans*-retinoic acid, no evidence was found for an increased risk of major malformations (Jick *et al.*, 1993). In United States Medicaid data on 1120 cases of topical use of all-*trans*-retinoic acid during pregnancy, the frequency distribution of abnormal outcomes was no different from that expected for the country as a whole (Rosa, 1992).

Eighteen case reports of anomalies in children of mothers who had used all-*trans*-retinoic acid during pregnancy included four cases of holo-prosencephaly but which did not share all or most of the characteristic features of retinoid embryopathy, such as small or absent ears. The authors reviewed data to 1988 and concluded that any effect of topical all-*trans*-retinoic acid on human development would be weak (Rosa, 1992).

At least two other cases of congenital malformation have been associated with cosmetic or therapeutic topical application of all-*trans*-retinoic acid, which have features in common with retinoid embryopathy (Camera & Pregliasco, 1992; Lipson *et al.*, 1993).

(b) Percutaneous absorption

The absence of teratogenic effects after topical application of all-*trans*-retinoic acid is compatible with its limited percutaneous absorption. Classical pharmacokinetics analyses were used to compare the circulating concentrations of all-*trans*-retinoic acid and its metabolites after topical application of 0.05% all-*trans*-retinoic acid cream. Topical application of all-*trans*-retinoic acid did not appreciably alter the endogenous plasma concentration of retinoids (Nau, 1993; Johnson, 1997). A physiologically based pharmacokinetics method for interspecies scaling of internal dose showed that the effective concentration of parent all-*trans*-retinoic acid and its metabolites across species was of the same order of magnitude (Clewell *et al.*, 1997). After topical application of 0.05% all-*trans*-retinoic acid to the face, arms and chest, the circulating levels of the compound and its metabolites were unchanged (Clewell *et al.*, 1997; Willhite & Clewell, 1999). The low dose that was absorbed, metabolized and delivered after topical application to intact human skin is consistent with the findings *in vivo* in humans (Jensen *et al.*, 1991; Buchan *et al.*, 1994; Latriano *et al.*, 1997), rodents (Willhite *et al.*, 1990) and lagomorphs (Christian *et al.*, 1997).

(c) Oral exposure

Although all-*trans*-retinoic acid is an isomer of 13-*cis*-retinoic acid, which is responsible for retinoid embryopathy (Creech-Kraft & Willhite, 1997), clinical experience with all-*trans*-retinoic acid at the customary dose of 45 mg/m^2 per day as treatment for acute promyelocytic leukaemia in early or in late gestation has shown no evidence of fetotoxicity.

Three case reports have been published of oral administration of all-*trans*-retinoic acid during early pregnancy. Treatment at 45 mg/m² per day with prednisone at 1 mg/kg bw per day from day 36 through week 30 did not induce terata, and the infant showed neither retinoid dermatitis nor cheilitis, although it developed jaundice and respiratory distress shortly after delivery. These conditions resolved without sequelae after supporting therapy, at 7 and 11 days, respectively, and were considered not to be treatment-related. At 15 months, the child's growth and development were normal (Simone *et al.*, 1995). Similar treatment beginning at week 13 had no adverse effect on delivery, survival or early neonatal development (Morton *et al.*, 1995), and no effects were seen of treatment beginning at 14 weeks of gestation and continued for 60 days (Lin *et al.*, 1996).

Exposure late in pregnancy (week 30 until parturition) was not associated with fetotoxicity in four case reports. Oral administration of all-*trans*-retinoic acid at 45 mg/m² per day to patients at 23–28 weeks' gestation and continued for 30 days (Harrison *et al.*, 1994; Watanabe *et al.*, 1995) or reduced after remission of the leukaemia had been achieved after 28 days to 22 mg/m² per day for 14 days (Stentoft *et al.*, 1994) produced treatment-related changes in the mothers (gingival hypertrophy and increased activity of circulating liver enzymes). Spontaneous delivery at weeks 30–32 in each case was uneventful; evaluation of the children at five to eight months gave no evidence of ophthalmological or neurological abnormalities; growth and development were considered normal (Harrison *et al.*, 1994; Stentoft *et al.*, 1994; Watanabe *et al.*, 1995). Treatment with all-*trans*-retinoic acid at 45 mg/m² per day from week 34 through week 38 increased the concentration of 13-*cis*-retinoic acid in cord blood to 0.44 ng/mL and that of its 4-oxo metabolite to 1.3 ng/mL, but neither all-*trans*-retinoic acid nor all-*trans*-4-oxo-retinoic acid was detected. Exposure to all-*trans*-retinoic acid did not induce fetotoxicity, and delivery was uneventful; follow-up at nine months showed no complications (Lipovsky *et al.*, 1996).

Physical and mental development were normal in eight infants born to mothers given all-*trans*-retinoic acid orally at 70 mg/kg bw from week 30 until birth for treatment of acute promyelocytic leukaemia (Maeda *et al.*, 1997).

7.2.2 Experimental models

7.2.2.1 *Reproductive effects*

In rats given all-*trans*-retinoic acid by dietary supplementation at 0.03–0.75 mg/kg bw per day or by oral intubation at 0.4, 5, 10 or 50 mg/kg bw per day for 12 or 13 weeks, decreased testicular weights were observed, and there was histological evidence of decreased spermatogenesis. Similar results were found in dogs fed all-*trans*-retinoic acid by capsule at a dose of 5 or 50 mg/kg bw per day for 13 weeks (Kamm, 1982).

7.2.2.2 *Developmental effects*

Numerous studies have shown that embryos of mammalian species are susceptible to the embryotoxic effects of excess all-*trans*-retinoic acid (Table 7) at doses that do not cause overt maternal toxicity (Geelen, 1979; Agnish & Kochhar, 1993). The embryotoxic effects of retinoids can also be induced in other vertebrate classes, such as birds (Tickle *et al.*, 1982), amphibians (Durston *et al.*, 1989) and fish (Holder & Hill, 1991).

Since the initial studies, more than 70 types of anomalies affecting almost every organ system related to excess intake of all-*trans*-retinoic acid have been described in more than 100 reports (Soprano & Soprano, 1995). The anomalies described in mammalian embryos (monkeys, rabbits, rats, mice and hamsters) are microcephaly, holoprosencephaly, spina bifida, encephalocele, exencephaly, hydrocephaly, facial nerve palsy, cranial abnormalities, maxillary hypoplasia, mandibular hypoplasia, cleft palate, cleft lip, micrognathia, absent or deformed ear, exophthalmus, microphthalmus, anophthalmus, coloboma, nasal defects, facial clefting, oesophageal atresia, cardiovascular malformations, hypoplastic aorta, rib fusions, vertebral transformations, omphalocoele, multiple limbs, shortened limbs, ectrodactyly, syndactyly, anal atresia, thymic hypoplasia, amastia and defects of the uterus, testis, kidney, thyroid and pituitary. The important features of the teratogenicity of all-*trans*-retinoic acid are the wide spectrum of its effects on virtually every organ system of the body and the impressive stage-specificity of the induced effects, as described classically by Shenefelt (1972). For example, treatment of mice embryos before implantation (days 4.5–5.5 of gestation) resulted in a bizarre multiple hindlimb phenotype in which the pelvic girdle is

Table 7. Teratogenic effects of all-*trans*-retinoic acid

Species	Dose (mg/kg bw)	Effects	Reference
Mouse	70; GD 12	100% cleft palate; alteration of mesenchyme	Degitz *et al.* (1998)
		Thymic defects, increased expression of *Hoxa 3*	Mulder *et al.* (1998)
Chick embryo	Soaked bead, stage 20	Face and wing patterning; Sonic hedgehog expression	Helms *et al.* (1997)
Cynomolgus monkey	5; GD 16–26 2 x 5; GD 26–31	No teratogenic effects	Tzimas *et al.* (1996)
Rat	20–30; GD 10	Cranial facial defects	Tembe *et al.* (1996)
Rabbit	5–25; GD 10	Cranial facial defects; deformed tail	
NMRI mouse	20 mmol/kg bw GD 11	all-*trans*-Retinyl-β-D-glucuronide more teratogenic than all-*trans*-retinoic acid	Nau *et al.* (1996)
C57BL mouse	7.5; GD 7 1.25; GD 7	Holoprosencephaly, anophthalmia, microphthalmia	Sulik *et al.* (1995)
CD1 mouse	2.5; GD 7	Exencephaly	Sulik *et al.* (1995)
CD1 mouse	20; GD 9	Fused ribs and vertebrae tail; spina bifida increased by stress	Rasco & Hood (1995a,b)
Rat	40; GD 13, 14	Cleft palate	Ikemi *et al.* (1995)
Rat, rabbit	6; GD 12 6; GD 7–12	Decreased concentrations and effects after multiple applications	Collins *et al.* (1995)
Rat	6; GD 6–15	Axial skeletal defects	Collins *et al.* (1994)
Rabbit	6; GD 6–18	Low teratogenic response	Tzimas *et al.* (1994a)
C57BL mouse	28; GD 8 (3 x)	Spina bifida aperta	Alles & Sulik (1990)
Cynomolgus monkey	10; GD 10–20 2 x 10; GD 21–24	Ear defects, mandibular cleft palate	Hendrickx & Hummler (1992)
Rat	10; GD 6–15	Not specified	Kamm (1982)
Rabbit	6; GD 6–18	Not specified	
Mouse	3; GD 6–15	Not specified	
NMRI mouse	10; GD 11	Cleft palate, limb defects	Creech Kraft *et al.* (1989)
C57BL mouse	0–200; GD 10, 12	Cleft palate, synergistically increased by TCDD	Birnbaum *et al.* (1989)
Xenopus embryo	Stage 10; 10^{-5} mol/L	Microcephaly	Durston *et al.* (1989)

GD, gestation day; TCDD, 2,3,7,8-tetrachlordibenzo-*para*-dioxin

duplicated (Rutledge *et al.*, 1994; Niederreither *et al.*, 1996). Exposure of postimplantation embryos (days 7–9.5 of gestation) typically results in the craniofacial, central nervous system, ear, cardiovascular and thymus defects listed above; and exposure at later stages of development (days 9.5–12 of gestation) is associated with defects of the limbs, palate and genitourinary tract (Kalter & Warkany, 1961; Shenefelt, 1972; Kistler, 1981; Sulik *et al.*, 1995). Even during the later phase, forelimb defects appear at slightly earlier times of treatment than hindlimb defects.

Studies have also been conducted on the effects of all-*trans*-retinoic acid on the viability and behaviour of offspring. For example, after administration of all-*trans*-retinoic acid on days 8–10, 11–13 and 14–16 of gestation in rats, hyperactivity in the open field and in running wheels was observed at doses > 4 mg/kg bw. Performance in the Morris maze was poorer than in the controls after a dose of 2.5 mg/kg bw on days 11–13. The scores on active avoidance tests were lower after a dose of 6 mg/kg bw. It was concluded that all-*trans*-retinoic acid induced functional defects at doses that did not cause morphological defects (Nolen, 1986).

The pharmacokinetics of all-*trans*-retinoic acid in pregnant animals has been studied extensively, and the findings shed light on the concentrations of this retinoid and its metabolites at the target site that are necessary to induce effects, the critical pharmacokinetics and the reasons for the species similarity in teratogenic responses. all-*trans*-Retinoic acid is rapidly eliminated from the circulation of mice, rats, hamsters, rabbits, monkeys and humans, with apparent half-lives < 3 h (Siegenthaler & Saurat, 1989; Allenby *et al.*, 1993; Fiorella *et al.*, 1993). The authors suggested that the presence of CRABPs in tissues of all species examined to date and their capacity to facilitate the metabolism of all-*trans*-retinoic acid may explain why it is rapidly eliminated. Studies in mice, rats and rabbits revealed that the embryonic concentrations of all-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid were similarly high or higher than those in plasma after administration of all-*trans*-retinoic acid (Tzimas *et al*, 1994b; Kochhar *et al.*, 1995). Binding of all-*trans*-retinoic acid to embryonic CRABPs or other as yet unidentified retinoid binding proteins in the embryo is likely to contribute to the greater concentrations in the serum of embryos than dams (Dencker *et al.*, 1987, 1990).

The duration of exposure to retinoids is another major determinant of teratogenic outcome. For example, when 5 mg/kg bw of all-*trans*-retinoic acid were administered orally to rats on gestation day 9, no specific embryotoxicity was seen, whereas subcutaneous treatment induced a high incidence of embryolethality and skeletal anomalies. The AUC but not the C_{max} in maternal plasma correlated with the embryonic outcome (Tzimas *et al.*, 1997). Importantly, teratogenic outcomes in mice are observed at doses (10 mg/kg bw) well below those at which metabolism is saturated (100 mg/kg bw) (Tzimas *et al.*, 1995).

A common characteristic of the pharmacokinetics of all-*trans*-retinoic acid in rats, rabbits and cynomolgus monkeys is a diminution of the plasma concentrations after repeated dosing, although the extent and the time course of this phenomenon vary among species. In pregnant rats given all-*trans*-retinoic acid at a single oral dose of 6 mg/kg bw on day 12 of gestation or the last of six daily doses of 6 mg/kg bw on days 7–12 of gestation, multiple dosing resulted in a pronounced reduction in the exposure to this compound and to all-*trans*-4-oxoretinoic acid, since the plasma AUC values were ninefold and fivefold higher for the two compounds, respectively, after the single than after multiple administrations. In rabbits treated in the same way, the effect on the plasma concentration of all-*trans*-retinoic acid after repeated dosing was also evident, although it was less marked than in rats (Collins *et al.*, 1994). Similar results were found after rats were given all-*trans*-retinoic acid at 17 mg/kg bw three times at 3-h intervals (Tembe *et al.*, 1996) and in rats and rabbits given either a single dose of all-*trans*-retinoic acid on day 12 of gestation or multiple daily doses on days 7–12 (Collins *et al.*, 1995), although the change was smaller in rabbits. The decrease in plasma concentrations after repeated dosing has been attributed to up-regulation of all-*trans*-retinoic acid metabolism and excretion (Adamson *et al.*, 1993).

7.3. Genetic and related effects

7.3.1 Humans

No data were available to the Working Group.

7.3.2 Experimental models

Most of the information on the mutagenicity of all-*trans*-retinoic acid in the *Salmonella*/microsome test was generated in studies of the ability of this compound to modulate the mutagenic activity of known carcinogens (Table 8). The number of tester strains, the range of doses and the use of S9 mix is therefore somewhat incomplete. When the compound was tested at 2 and 4 µmol/plate in *Salmonella* strains TA101 and TA100 without S9 and TA98 with S9, no effect was observed on mutation frequencies (De Flora *et al.*, 1994). Treatment of *S. typhimurium* at doses of 2.5–40 µg/plate had no toxic effect and did not increase the mutation frequencies in TA98, TA100, TA102 and TA1535, with or without the addition of S9; however, the actual data were not shown (Qin & Huang, 1985). All the other studies were restricted to single tester strains. No effect was reported on the spontaneous mutation frequencies of TA104 tested only in the absence of S9 (Han, 1992), and the mutation frequencies in TA100 (Bhattacharya *et al.*, 1987) and TA98 (Wilmer & Spit, 1986; Whong *et al.*, 1988) were unchanged, in all cases tested only in the presence of S9.

In cultured mammalian cells, all-*trans*-retinoic acid did not induce unscheduled DNA synthesis in rat primary hepatocytes (Bhattacharya *et al.*, 1987), It did not alter the spontaneous mutation frequency at the *Hprt* locus in Chinese hamster ovary cells (Budroe *et al.*, 1988) and did not change chromosomal aberration frequencies in Chinese hamster ovary or human embryonic palatal mesenchymal cells (Patel *et al.*, 1998; Watanabe & Pratt, 1991). Although no effect on spontaneous sister chromatid exchange frequency was reported in three studies (Sirianni *et al.*, 1981; Cozzi *et al.*, 1990; Watanabe & Pratt, 1991), two other studies showed an effect. Juhl *et al.* (1978) reported a dose-dependent increase in sister chromatid exchange frequency in human diploid fibroblasts exposed to this retinoid. More recently, Patel *et al.* (1998) reported a statistically significant increase in sister chromatid exchange frequency in Chinese hamster ovary cells, but only after continuous treatment for 20 h and not after exposure for 3 h and only at the highest dose tested (0.8 µg/ml). S9 was used in neither of the latter two studies.

No reports were found of studies of the genotoxicity of all-*trans*-retinoic acid *in vivo*.

8. Summary of Data

8.1 Chemistry, occurrence and human exposure

all-*trans*-Retinoic acid is derived from retinol by oxidation of the C-15 alcohol group to a carboxylic acid. Like all members of the vitamin A family, it is lipohilic, sensitive to light, heat and oxygen and readily isomerized to a mixture of *cis* and *trans* isomers. Because of its acidic nature, it is slightly more soluble in water than retinol or retinal but still poorly so. Because of its conjugated tatraene structure, all-*trans*-retinoic acid has characteristic absorption spectra in the ultraviolet and visible, infrared and resonance Raman portions of the elctromagnetic spectrum.

all-*trans*-Retinoic acid is normally present in blood and tissues of animal species in smaller amounts than retinol and retinyl ester and is essentially absent from plant tissues. Human exposure occurs during topical or oral treatment for medical or cosmetic purposes.

all-*trans*-Retinoic acid has been used to treat dermatological disorders and several forms of cancer. The efficacious doses are 0.5–2 mg/kg of body weight per day when given orally and 0.1% as a cream or gel when administered topically.

all-*trans*-Retinoic acid is usually separated by high-performance liquid chromatography and detected by its absorption at 350 nm. After chemical formation of a suitable ester, it can also be separated and detected by gas–liquid chromatography and can be quantified by mass spectrometry.

8.2 Metabolism and kinetics

The metabolism, kinetics and distribution of all-*trans*-retinoic acid are complex but relatively well understood. It is present in most tissues of healthy animals, albeit at low concentrations. Most of the all-*trans*-retinoic acid in tissues is synthesized *in situ* from all-*trans*-retinol through the actions of two oxidoreductases (dehydrogenases) that catalyse the sequential oxidation of all-*trans*-retinol to all-*trans*-retinal and of all-*trans*-retinal to all-*trans*-retinoic acid. The identities and characteristics of these enzymes have not been established unequivocally. The low concentrations of all-*trans*-retinoic acid present in plasma (4–14 nmol/L) may be taken up by tissues.

Table 8. Genetic and related effects of retinoids in short-term tests *in vitro* and *in vivo*

End-point	Code	Test system	Result[a] Without exogenous metabolic system	Result[a] With exogenous metabolic system	LED/HID[b]	Reference
G	SA0	TA100, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA0	TA100, reverse mutation	0	+	0.5 µmol/plate	Bhattacharya *et al.* (1987)
G	SA2	TA102, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA4	TA104, reverse mutation	–	0	10 µmol/plate	Han (1992)
G	SA5	TA1535, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA8	TA98, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	TA98, reverse mutation	0	–	500 nmol/plate	Wilmer & Spit (1986
G	SA9	TA98, reverse mutation	0	–	2.6 µmol/plate	Whong *et al.* (1988)
D	URP	Unscheduled DNA synthesis, rat primary hepatocytes	0	–	50 mmol/L	Budroe *et al.* (1987)
G	GCO	Gene mutation, Chinese hamster ovary cells, *Hprt* locus	–	–	25 mmol/L (+S9) 100 mmol/L (-S9)	Budroe *et al.* (1988)
C	CIC	Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	0	1.6 mg/ml	Patel *et al.* (1998)
C	CIH	Chromosomal aberrations, human embryonic palatal mesenchymal cells *in vitro*	–	–	68 mmol/L (-S9) 140 mmol/L (+S9)	Watanabe & Pratt (1991)
S	SIC	Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	(+)	0	0.8 mg/ml	Patel *et al.* (1988)
S	SIC	[c] Sister chromatid exchange, Chinese hamster epithelial liver cells *in vitro*	–	0	50 µmol/L	Cozzi *et al.* (1990)
S	SIS	Sister chromatid exchange, Chinese hamster V79 cells *in vitro*	–	0	25 mg/ml	Sirianni *et al.* (1981)
S	SIH	Sister chromatid exchange, human embryonic palatal mesenchymal cells *in vitro*	–	–	68 µmol/L (–S9) 140 µmol/L (+S9)	Watanabe & Pratt (1991)
S	SHF	[d] Sister chromatid exchange, human fibroblasts *in vitro*	+	0	1 µg/ml	Juhl *et al.* (1978)

[a] Result: +, positive; (+) weak positive, only one dose showed significant increase; – considered to be negative; 0, not tested
[b] LED, lowest effective dose that inhibits or enhances the investigted effect; HID, highest ineffective dose
[c] Isomer type was all-*trans*-retinoic acid, personal communication from author
[d] Presumed to be all-*trans*-retinoic acid since, exception for Juhl *et al.*, all sources listed as Sigma. 13-*cis*-Retinoic acid listed only in Sigma catalogue from 1988 and 9-*cis*-retinoic acid from 1996. Budroe *et al.* (1988) probably used the same retinoic acid source as in 1987. The early study by Juhl *et al.* (1978) probably also involved all-*trans*-retinoic acid.
[d] Type of retinoid used most likely all-*trans*-retinoic acid, but it may have been13-*cis*-retinoic acid since publication date was after 1987.

Pharmacokinetic studies indicate that all-*trans*-retinoic acid is rapidly taken up from the gastrointestinal tract and rapidly cleared from the circulation. Repeated doses of all-*trans*-retinoic acid markedly lower both its maximal concentrations in plasma and the effective concentration in plasma integrated over time, implying that it induces its own catabolism *in vivo*. The recent identification and cloning of an all-*trans*-retinoic acid-inducible cytochrome P450 (CYP26) that catalyses oxidation of this retinoid adds to biochemical understanding of the observation. Although the metabolism of all-*trans*-retinoic acid in human beings and animal models is probably similar biochemically, the metabolite profiles and concentrations differ markedly among species. Although no animal model can be considered truly to reflect the human situation, the metabolite profiles and concentrations in monkeys most closely resemble those seen in humans.

8.3 Cancer-preventive effects

8.3.1 Humans

No studies have been reported of the use of all-*trans*-retinoic acid for the prevention of invasive cancer in humans.

The results of one randomized, controlled trial indicate that topically applied all-*trans*-retinoic acid is effective in reversing moderate dysplasia of the uterine cervix (cervical intra-epithelial neoplasia-II) but not against more severe dysplastic lesions. It was reported to be efficacious against actinic keratosis of the skin in one randomized trial but not in another at a lower dose; the descriptions of the studies were insufficient for detailed evaluation. Two further trials suggest that topically applied all-*trans*-retinoic acid is effective against dysplastic naevi.

8.3.2 Experimental models

The preventive efficacy of all-*trans*-retinoic acid was evaluated in models of skin, liver and mammary gland carcinogenesis. The results of several experiments in mice indicated that all-*trans*-retinoic acid was effective against two-stage skin carcinogenesis when 7,12 dimethylbenz[*a*]-anthracene was used as the initiator, whereas it enhanced skin carcinogenesis induced by this carcinogen alone or with ultraviolet radiation. One study in mice indicated that all-*trans*-retinoic acid enhanced *N*-nitrosodiethylamine-induced liver carcinogenesis, but in one study in rats it was ineffective against *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis.

In vitro, all-*trans*-retinoic acid inhibited the transformation of normal cells by carcinogens and of immortalized cells by viral oncogenes. all-*trans*-Retinoic acid inhibited cell proliferation in monolayer cultures and modulated the differentiation of a large number of immortalized, transformed and tumorigenic cell types derived from trachea, skin and cervical epithelia. all-*trans*-Retinoic acid also suppressed the anchorage-independent growth of a variety of tumour cell lines. It suppressed abnormal squamous differentiation in immortalized and transformed cells and re-regulated growth control mechanisms by blocking cells in the G_1 phase of the cell cycle.

The inhibitory effects of all-*trans*-retinoic acid against carcinogen-induced genotoxicity were most often associated with agents that require bioactivation. Studies in animals and humans given topical applications of all-*trans*-retinoic acid have demonstrated alterations in enzymes that mediate carcinogen metabolism. all-*trans*-Retinoic acid can also act as a radical scavenger, suggesting that it acts on initiation by carcinogens. Carcinogen-induced neoplastic transformation *in vitro* was inhibited when all-*trans*-retinoic acid was added after removal of the carcinogen, while most studies in experimental animals in which inhibition of carcinogenesis was demonstrated involved treatment with all-*trans*-retinoic acid after exposure to the carcinogen. Thus, it can act in both the initiation and post-initiation phases of carcinogenesis.

8.3.3 Mechanisms of cancer prevention

Most studies of the effects of all-*trans*-retinoic acid on carcinogenesis indicate that inhibition of the post-initiation stage is the main mechanism of its putative preventive effects. The mechanisms of action may be related to the ability of retinoids to antagonize tumour promotion by *trans*-repressing the activity of AP-1. The ability of all-*trans*-retinoic acid to inhibit cell proliferation is associated with multiple effects on G_1 regulatory proteins which ultimately result in growth arrest in G_0 and G_1. Another possible mechanism is modulation of the autocrine and paracrine loops that mediate

positive and negative growth signals. Restoration of normal differentiation or enhancement of differentiation in cells with aberrant differentiation may contribute to the putative cancer-preventive effects of all-*trans*-retinoic acid. Restoration of controls on growth can also be mediated by enhancement of intercellular gap-junctional communication and suppression of prostaglandin biosynthesis. Induction of apoptosis provides the ultimate means for eradicating abnormal clones of premalignant cells. Enhancement of cell adhesion and inhibition of cell motility affect the invasive step in which carcinoma *in situ* is converted into a malignant tumour. Lastly, inhibition of angiogenesis may block the growth of advanced premalignant lesions and locally invasive malignancies.

8.4 Other beneficial effects

all-*trans*-Retinoic acid has been shown to be of benefit to patients suffering from a variety of dermatological disorders.

8.5 Carcinogenicity

No data were available to the Working Group.

8.6 Other toxic effects

8.6.1 Humans

The toxic effects associated with topical application of all-*trans*-retinoic acid are generally localized, cutaneous and reversible after treatment is discontinued. The most frequent adverse effects include peeling, dry skin, burning, stinging, erythema and pruritus. The reactions are usually of mild to moderate severity, occur early during therapy and recur after an initial decline. Patients using topical all-*trans*-retinoic acid have heightened sensitivity to sunlight and weather extremes, and temporary hyper- or hypopigmentation has been reported. Concentrations of 0.05–0.2% applied on a sponge inserted vaginally in a cervical cap for four days commonly produced vaginal irritation, ulceration and discharge, with no evidence of systemic toxicity.

all-*trans*-Retinoic acid administered orally at 45 mg/m^2 per day (the recommended daily dose) results in drug-related toxicity in virtually all patients with acute promyelocytic leukaemia. The most frequent adverse events are similar to those described in patients taking high doses of vitamin A (headache, fever, skin and mucous membrane disturbances, bone pain and inflammation, nausea and vomiting, mucositis, pruritus, increased sweating, visual disturbances and alopecia). Transient increases in the concentration of triglycerides and the activity of transaminases are common and can sometimes lead to severe complications such as pancreatitis and hepatotoxicity. Moderate to severe headaches are commonly experienced during oral therapy with all-*trans*-retinoic acid and may limit the dose that can be given. Cases of pseudotumour cerebri, with clinical signs of headache, nausea, double vision and bilateral papilloedema, have been reported. Concern has been raised that all-*trans*-retinoic acid may increase the risk for relapses at unusual sites in patients with acute promyelocytic leukaemia.

One of the most serious reactions to orally administered all-*trans*-retinoic acid is 'retinoic acid syndrome', an acute, life-threatening respiratory disorder experienced by about 25% of patients with acute promyelocytic leukaemia who are being treated with all-*trans*-retinoic acid. The syndrome is characterized by fever, dyspnoea, weight gain, radiographic pulmonary infiltrates and pleural or pericardial effusions and has been observed with and without concomitant leukocytosis. High doses of glucocorticoids given at the first sign of the syndrome appear to reduce the rates of morbidity and mortality, but as much as 60% of patients with acute promyelocytic leukaemia who are taking all-*trans*-retinoic acid orally require such treatment.

No signs of reproductive toxicity were seen in one study in which 12 fertile men were exposed to all-*trans*-retinoic acid for up to 300 days. There were no reports of adverse pregnancy outcomes after oral administration of all-*trans*-retinoic acid to one patient with acute promyelocytic leukaemia during early pregnancy, and no signs of retinoid embryopathy were seen in seven such patients treated with all-*trans*-retinoic acid later in pregnancy; however, there have been no retrospective or prospective follow-up studies of the childen of such patients. The available data do not indicate an increased risk for teratogenic effects after dermal application of all-*trans*-retinoic acid.

8.6.2 Experimental models

In mice given all-*trans*-retinoic acid orally, the major dose-related findings included bone rarefaction, bone fracture, elevated serum alkaline

phosphatase activity, testicular degeneration, dermal and epidermal inflammation and hyperkeratosis. In rats, decreased plasma albumin and haemoglobin concentrations and increased serum alkaline phosphatase activity were seen.

Few studies of genotoxicity with all-*trans*-retinoic acid *in vitro* are available, and the results of those that have been reported were largely negative. The frequency of sister chromatid exchange was increased in the presence of this retinoid in two studies but not in three others. No reports were available of the effects of all-*trans*-retinoic acid on mutation or chromosomal changes in animals *in vivo*.

all-*trans*-Retinoic acid applied topically at doses of 2–5 mg/kg of body weight per day for 90 days reduced testicular and ovarian weights in mice, with no pathological changes; no such effect was seen in dogs given doses up to 1 mg/kg of body weight per day for 13 weeks. all-*trans*-Retinoic acid was a potent teratogen in all experimental species examined; this finding is compatible with its extensive placental transfer, with consequent exposure of embryos, and its interaction with retinoic acid receptors. The extent of teratogenicity is similar in different mammalian species, reflecting its rate of elimination. The teratogenic effects, which involve nearly every fetal organ system, depend on the route and the time of administration during gestation. In general, administration during early organogenesis results in greater teratogenicity than administration during late organogenesis, partly because of decreasing placental transfer with advancing gestation. Behavioural defects have been observed in rodents exposed to all-*trans*-retinoic acid *in utero*. Topical application results in low bioavailability and little or no teratogenicity.

The extensive placental transfer of all-*trans*-retinoic acid is closely associated with its binding to the abundant embryonic cellular retinoic acid binding proteins. The effective concentration of all-*trans*-retinoic acid in plasma integrated over time that reaches the embryo during the sensitive stages of organogenesis defines the potency of the teratogenic effect. Multiple dosing results in a reduction in the concentration of the drug in maternal and fetal compartments, probably because induction of metabolic enzymes increases 4-oxidation and glucuronidation.

9. Recommendations for Research

9.1 General recommendations for research on all-trans-retinoic acid and other retinoids

The results of studies in which the protocols differed widely or in which proper controls and differences in the baseline conditions were neglected, omitted or inadequately described are difficult to evaluate and compare. Standard protocols are urgently needed to evaluate the cancer-preventive properties of retinoids. Such protocols should specify continuous quality control of supplements and the type of formulation to be used. Such a strategy should be developed for molecular, cellular, experimental animal and human studies, in order to:

- understand the role of nuclear retinoid receptors in cancer chemoprevention;
- develop more relevant models of chemoprevention with efficacious retinoids, including assays based on over-expression of oncogenes and ablation of tumour suppressor genes;
- identify suitable biomarkers that can serve as surrogate outcome measures of invasive cancer in assessing the efficacy of retinoids in chemoprevention trials;
- identify more precisely the structural properties of retinoids that affect their toxicity;
- identify the mechanisms of action by which retinoids effect their characteristic spectrum of toxicity;
- design and develop retinoids with improved therapeutic ratios for cancer chemoprevention;
- understand better the mechanisms by which retinoids target specific tissues;
- understand the factors that determine homeostatic mechanisms for retinoids in cells and whether these regulatory mechanisms are important in cancer chemoprevention;
- understand better the structural properties of retinoids that influence their pharmacokinetics;

- clarify the reasons underlying the loss of retinoid X receptors in the process of cellular transformation; and
- understand the interaction and potential chemopreventive efficacy of retinoids in combination with other drugs.

10. Evaluation

10.1 Cancer-preventive activity

10.1.1 Humans

There is *inadequate evidence* that all-*trans*-retinoic acid has cancer-preventive activity in humans.

10.1.2 Experimental animals

There is *inadequate evidence* that all-*trans*-retinoic acid has cancer-preventive activity in experimental animals.

10.2 Overall evaluation

all-*trans*-Retinoic acid has not been established to have cancer-preventive activity in humans. Therapy with this retinoid gives rise to significant toxic effects, and it is an established teratogen in experimental animals.

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