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Genotoxicity studies on benzimidazole retinoids

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Retinoids consist of a family of naturally occuring compounds including all-*trans* retinoic acid (ATRA), retinal, retinol (vitamin A), 9-*cis* retinoic acid, 13-*cis* retinoic acid as well as a large number of synthetic derivatives. Retinoids are known to elicit diverse pharmacological profiles such as controlling cell differentiation/proliferation and modulating specific premalignant lesions and reducing second primary tumors in patients. Clinical use of retinoids is limited due to their toxicity. Three benzimidazole retinoid derivatives (BITN, BITNm, BITNe) were synthesized and were examined in terms of genotoxicity towards human lymphocyte cultures by sister chromatid exchange (SCE) analysis. It has been found that BITN decreased the number of SCEs 20% at 10^{-6} M, but had no effect at 10^{-5} M. No significant effect on SCEs was observed for BITNm and BITNe at both concentrations. ATRA increased the SCEs (35%) at 10^{-5} M but had no effect at 10^{-6} M. The results have shown that benzimidazole retinoids did not induce SCE significantly. Besides this, BITN reduced the SCEs and had a protective effect at low concentration. Since the induction of glutathione S-transferase (GST) is associated with anticancer drug resistance, the effects of BITN, BITNm, BITNe and ATRA on human lymphocyte GSTs were also investigated using CDNB as substrate. BITN and BITNm induced GST activities 54% and 49% respectively at 10^{-5} M, but had no effect at 10^{-6} M. BITNe induced GST activity 62% at 10^{-5} M and 35% at 10^{-6} M. ATRA had no effect on GST activity at 10^{-5} M.

1. Introduction

Natural retinoids are derivatives of vitamin A (retinol) which has a critical role in normal and abnormal cellular functions (Sporn and Roberts 1983). Both natural and synthetic retinoids modulate differentiation, inhibit growth and induce programmed cell death in a wide variety of cancer cell lines (Dragnev et al. 2000; Costa et al. 2001; Dawson et al. 2001). Recent data suggest that the biologic effects of retinoids result from modulation of gene expression (Lampen et al. 2001; Yoshikawa et al. 2001), mediated through two complex families of nuclear receptors (RARs and RXRs) (Mangelsdorf et al. 1993; Pfahl et al. 1994; Almasan et al. 1994). The relationship between specific RAR alterations and all-*trans*-retinoic acid (ATRA) activity has been elegantly demonstrated by translational molecular studies in acute promyelocytic leukemia (Douer 2000).

Studies have shown that various retinoid analogues can reverse or suppress epithelial carcinogenesis at many sites, including the breast, head and neck, lung, bladder, skin and cervix (Teplitzky et al. 1999; Thaller et al. 2000; DiGiovanna 2001; Klaassen et al. 2001; Um et al. 2000). New retinoids such as fenretinide (alone and combined with tamoxifen) are now in clinical trials and have generated promising early results (Camerini et al. 2001; Fabian 2001). Preclinical data indicate that retinoid-interferon combinations have significant activity in modulating malignant cell growth differentiation and programmed cell death in certain hematologic and solid tumor systems (Konta et al. 2001). ATRA is widely involved in the control of cell proliferation and embryonic development (Chambon 1996). It is a potent inducer of cell differentiation (Petkovich 1992). For example, retinoic acid influences the clonal growth of normal human myeloid cells and induces the differentiation of both HL-60 cells (human promyelocytic leukemia cell line) and fresh human acute promyelocytic leukemia (APL) cells into normal granulocytes in a morphological study (Miyatake 1997). On the other hand, antioxidants and antioxidative enzymes are the major protective systems of the organism and the use of ATRA in many biological models of carcinogenesis has also suggested that their action may be dependent on their antioxidant activity (Hiramatsu and Packer 1990). Several studies have shown that the antioxidant activity of retinoids remains unclear. It is reported that their potential efficacy is studied on granulocytes with regard to their role in inflammatory dermatoses and that the *in vivo* effect of acitretin and isotretionin on the generation of reactive oxygen species (ROS) is different (Bohne et al. 1997). In our previous study, we have found that a benzimidazole compound (BITN) caused inhibitions on EROD and

PROD with activities higher than those of butylated hydroxytoluen (BHT) and ATRA (Ates et al. 1997). The molecular mechanisms underlying effects are known to involve specific receptors, which can be grouped into classes and isoforms whose functions still remain obscure, and one of our research projects is to develop new retinoids which may have low levels of side effects, being especially free from genotoxic properties and having optimal retinoidal activities.

Administration of excessive amounts of retinoids may cause toxicity (Badr et al. 1998). Toxicity usually results from abusively high intakes of vitamin A supplements and rarely high consumption of products including vitamin A, such as liver (Gerster 1997). Early signs of chronic retinoid toxicity include loss of appetite, dryness of the skin, hair loss, weakness, headache, enlarged liver and spleen, vomiting, anorexia, blurred vision, pain and tenderness in extremities (Gerster 1997; Barber and Harris 1994; Hinds et al. 1997). One of the most serious toxicity produced by retinoid exposure is teratogenicity (Levin 1995). Normal embryonic development is dependent on the presence of retinoids but excess as well as deficiency of retinoids has been found to be teratogenic (Nau 1995). The teratogenic potency of a retinoid depends on placental transfer, plasma clearance and metabolic detoxification. It was found that 13-*cis*-RA is a potent teratogen in monkeys, but not in rat and mice. The high teratogenic activity of this retinoid in monkey is the result of high placental transfer, slower plasma clearance and low metabolic detoxification (Nau 1995). Retinoids in excess amounts also have cytotoxic effects. The cytotoxic effects of retinoids have been investigated in human lymphocyte cultures. It has been found that the frequency of lymphocytes with chromosomal aberrations in blood cultures treated with high doses of all-*trans*-retinol is 3–5 fold greater than the untreated control ones (Badr et al. 1998). The principal limiting factor in clinical use of retinoids is their toxicity (Barber and Harris 1994). For example, etretinate (ethyl 3-methoxy-2-methyl-17-nor-1,2,3,4-tetradehydroretinoate) and arotinoids have been found to be potent retinoids and could be used clinically. However, these compounds, as well as ATRA, posses the clinical disadvantages of high toxicity (hypervitaminosis A) and teratogenicity. Their toxicity and teratogenicity are long-lasting because the compounds are highly lipophilic and difficult to eliminate from the body (Hashimoto 1991). Therefore, it is desirable to develop novel retinoids that are less toxic and can be eliminated promptly from the body. For this reason the novel retinoids 2-[5,5,8,8-tetramethyl,5,6,7,8-tetrahydronaphthyl] benzimidazol 5-carboxylic acid (BITN), 2-[5,5,8,8-tetramethyl,5,6,7,8-tetrahydronaphthyl] benzimidazol 5-methyl carboxylate (BITNm), 2-[5,5,8,8-tetramethyl,5,6,7,8-tetrahydronaphthyl] benzimidazol 5-ethyl carboxylate (BITNe) (Ates et al. 1997; Ates-Alagoz and Buyukbingol 2001), have been synthesized and their biochemical and genotoxic effects have been investigated in comparison to ATRA in human lymphocyte cultures.

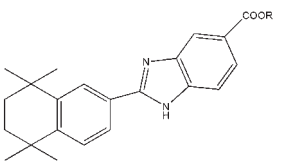
The most common test systems used to assess if humans are exposed to genotoxic compounds are scoring of chromosome aberrations (chromatid breaks and chromatid fragments; interchange and intrachange between chromatids, and interchange and intrachange between chromosomes, an increase or decrease in the chromosome number, ring chromosomes etc.) and sister chromatid exchanges (SCEs) which is a sensitive and well established cytogenetic indicator of DNA damage (Wulf 1990). The genotoxicity of these compounds have been examined using Sister Chromatid Exchanges (SCEs) analysis. SCEs with its high sensitivity, is a widely accepted genotoxicity test which is used as an indicator of mutagenic and carcinogenic potency of a given chemical. It yields few false positive or false negative results *in vivo* and *in vitro* and thereby provides valuable information on the genotoxic potential of a compound. Glutathione S-transferases (GSTs) posses many biological functions, the most important of which is detoxification, conjugation of reduced glutathione with a large number of electrophiles (Wolkof 1980). The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is less reactive than the parental compound and therefore the actions of GST generally result in detoxification. The covalent binding of electrophiles derived from carcinogens to macromolecules, especially DNA, has been considered as an initial step in chemical carcinogenesis. Such electrophilic compounds were shown to be detoxified by enzymatic or, in some cases, spontaneous conjugation with glutathione (Mannervik and Danielson 1988). In carcinogenesis, GSTs are of interest because they may confer resistance to chemotherapeutic drugs and also may have diagnostic value as tumor markers. The glutathione system is the most extensively studied drug resistance mechanism identified in resistance to alkylating agents. In a variety of experimental models, increased tolerance of drugs is associated with increased expression of GSTs (Hayes and Wolf 1990). Since the induction of GSTs activity is associated with drug resistance, the effects of BITN, BITNm, BITNe and ATRA on human lymphocyte GSTs have also been investigated using CDNB as substrate.

2. Investigations and results

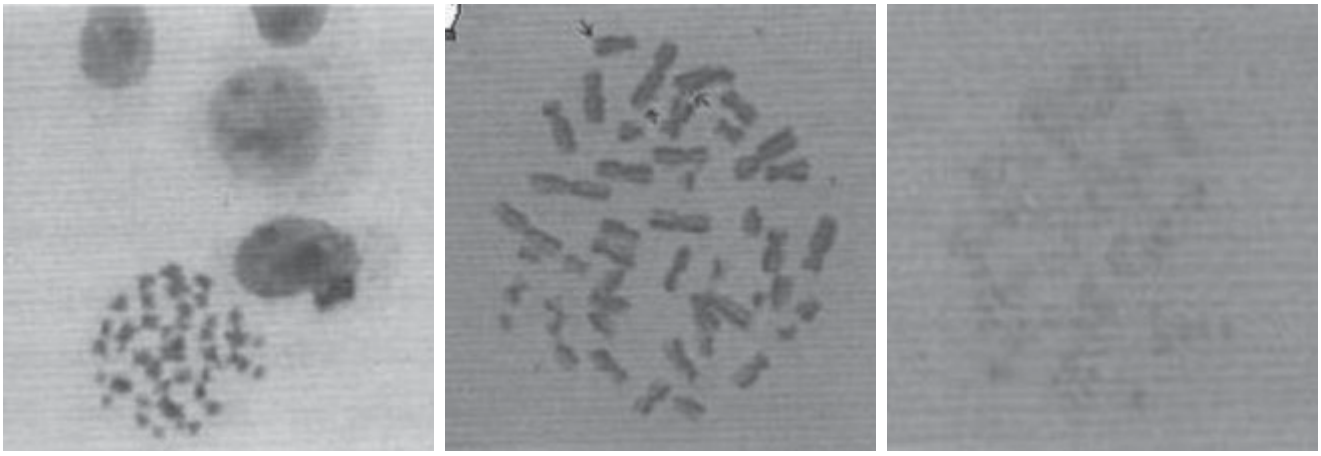
2.1. Observation and analysis

The slides prepared in staining the chromozomes with FPG technique, were examined by a Prior (James Switt) binocular compound microscope. A 10× objective was used to determine the location of metaphase spreads and a 100× oil immersion objective was used to analyze the metaphase chromosomes (Fig. 1a, b, c). During analysis, metaphase chromosomes were divided into three groups as M1, M2 and M3. M1 metaphase chromosomes were those having unifiliarly substituted BrdUrd on their DNA strands and their both sister chromatids were darkly stained with the above staining procedure (Fig. 1a). The M2 metaphase chromosomes had bifiliarly substituted BrdUrd on one sister chromatid and unifiliarly substituted BrdUrd on the other, thus one sister chromatid stained darkly whereas the other stained lightly (Fig. 1b). Sister chromatid exchanges were scored from M2 metaphase spreads having 46 chromosomes. M3 metaphases were those having bifiliarly substituted BrdUrd on most of their DNA (Fig. 2c). So chromatids are either uniformly lightly stained or mixed with Harlequin chromosomes.

In order to analyze cell cycle kinetics, more than 100 mitotic cells were scored for each treatment and categorized as being M1, M2 and subsequent division metaphase chro-



R=H	BITN
$R=CH_3$	BITNm
$R=CH_2CH_3$	BITNe



A B C

Fig. 1: a) M1 metaphase chromosomes, b) M2 metaphase chromosomes (200× enlargements, black arrows show SCEs), c) M3 metaphase chromosomes under 100× objective

mosomes. An increase in the portion of M1 cells with concomitant decrease in M2 and M3 cells as reflected by a decrease in proliferation index indicated a delay in the cell cycle progression in response to culture conditions. The proliferation index (PI) was calculated using the following formula to study the cell cycle delays following the treatments (Saplakoglu and Iscan 1998).

$$PI = (1 \times M1 + 2 \times M2 + 3 \times M3)/M_{total}$$

2.2. Effects of BITN, BITNm, BITNe and ATRA on the frequency of sister chromatid exchanges in human lymphocytes

In vitro induction of sister chromatid exchanges (SCEs) in cultured peripheral blood lymphocytes (PBL) allows for the assessment of individual susceptibility to genotoxic effects of compounds (Landi et al. 1996). In order to examine the genotoxic effects of BITN, BITNm, BITNe and ATRA in human lymphocytes, the whole blood cultures were prepared as described in section 4.3.1. Human lymphocytes were treated with four different doses of ATRA ranging between 10^{-3} and 10^{-6} M concentrations. Concentrations equal to and higher than 10^{-4} M were found to be lethal to human lymphocytes under our experimental conditions. In order to compare the effects of ATRA and benzimidazole retinoids 10^{-5} M and 10^{-6} M concentrations were used. Human lymphocytes were treated with 10^{-5} M and 10^{-6} M of BITN, BITNm, BITNe and ATRA after 24 h initiation of culture, which happened to be the early S phase and cells were harvested after 72 h of incubation. Since these retinoids are nonpolar compounds, BITN, BITNm and BITNe were dissolved in ethanol, and ATRA, which is less soluble in ethanol, was dissolved in ether before addition into the culture medium. Therefore, eleven whole blood culture tubes were set up for each experiment. One untreated culture tube served as control (Control I). Two culture tubes were treated with 0.4% ethanol (final concentration) and 0.4% ether (final concentration) and served as solvent controls (Control II) for newly synthesized retinoid derivatives and ATRA, respectively, and the remaining 8 culture tubes were treated with 10^{-5} M and 10^{-6} M of BITN, BITNm, BITNe and ATRA. When SCEs were counted in Control I and Control II of the whole blood cultures, a significant difference was observed. Both ethanol and ether showed a statistically significant ($p < 0.01$ and $p < 0.005$, respectively) decrease in the number of SCEs compared to Control I, but no significant difference was found between them. Since ethanol and ether decreased the number of SCEs, the SCEs value of ethanol and ether treated cultures were taken as control value (Control II) for the comparison of SCE frequencies caused by BITN, BITNm, BITNe and ATRA, respectively. As 10^{-5} M of BITN was added to human lymphocytes, it had no significant effect on SCE frequencies when compared to control. However, an effective decrease ($p < 0.05$) in the number of SCEs was observed at 10^{-6} M of BITN. Table 1 gives the response of human lymphocytes to BITN.

At both concentrations of BITNm, there appeared to be no statistically significant difference in the SCE values when compared to control (Table 2).

Table 1: Response of human lymphocytes to differing doses of BITN

	Total number of metaphases	Number of M_2 cells	% of M_2 cells	Number of M_2 cells containing SCE	% of M_2 cell containing SCE	Mean SCE/cell[b] ± SD	PI
Control I	321	89	28	50	56	2.8 ± 1.7	1.37
Control II[a]	460	201	44	60	30	2.1 ± 1.15**	1.85
10^{-5} M	590	132	22	50	38	1.93 ± 1.01	1.57
10^{-6} M	1033	199	20	48	24	1.69 ± 0.97*	1.43

[a] Et-OH (solvent, 0.4% final concentration), [b] Mean of SCE among M_2 cells contaning SCE
* $p \leq 0.05$, significantly different from Control II, ** $p \leq 0.01$, significantly different from Control I

Table 2: Response of human lymphocytes to differing doses of BITNm

	Total number of metaphases	Number of M_2 cells	% of M_2 cells	Number of M_2 cells containing SCE	% of M_2 cell containing SCE	Mean SCE/cell[b] ± SD	PI
Control I	321	89	28	50	56	2.8 ± 1.7	1.37
Control II[a]	460	201	44	60	30	2.1 ± 1.15*	1.85
10^{-5} M	1040	223	21	117	52	2.00 ± 1.11	1.64
10^{-6} M	886	199	23	87	44	1.94 ± 1.02	1.39

[a] Et-OH (solvent, 0.4% final concentration), [b] Mean of SCE among M_2 cells contaning SCE
** $p \leq 0.01$, significantly different from Control I

We also observed no significant difference between the number of SCEs of 10^{-5} M and 10^{-6} M of BITNe treated cultures and control, the results are given in Table 3.
All-*trans*-retinoic acid (ATRA) induced a statistically significant ($p < 0.05$) increase in SCE rates at a concentration of 10^{-5} M when compared with control, on the other hand 10^{-6} M of all-*trans*-retinoic acid had no effect on SCE frequencies (Table 4).
In order to compare the effects of newly synthesized retinoids (BITN, BITNm, BITNe) and all-*trans*-retinoic acid on SCEs frequencies, percent inhibition of the mean of SCE per cell was calculated. Table 5 indicates the results as percent of Control II SCEs produced by benzimidazole retinoids and all-*trans*-retinoic acid.
20% Inhibition of SCEs was observed by BITN at 10^{-6} M, the decrease is also significant statistically ($p < 0.05$). On the other hand 10^{-5} M of BITN showed a slight inhibition in the SCEs. Also, **BITNm** and **BITNe** inhibited the SCE frequencies, but inhibition was not statistically significant. ATRA did not inhibit the SCE frequencies at both concentrations. It caused a significant increase in the number of SCEs at a concentration of 10^{-5} M.

Table 3: Response of human lymphocytes to differing doses of BITNe

	Total number of metaphases	Number of M_2 cells	% of M_2 cells	Number of M_2 cells containing SCE	% of M_2 cell containing SCE	Mean SCE/cell[b] ± SD	PI
Control I	321	89	28	50	56	2.8 ± 1.7	1.37
Control II[a]	460	201	44	60	30	2.1 ± 1.15*	1.85
10^{-5} M	806	167	21	50	30	1.98 ± 1.27	1.85
10^{-6} M	652	176	27	50	29	1.89 ± 1.1	1.35

[a] Et-OH (solvent, 0.4% final concentration), [b] Mean of SCE among M_2 cells contaning SCE
** $p \leq 0.01$, significantly different from Control I

Table 4: Response of human lymphocytes to differing doses of ATRA

	Total number of metaphases	Number of M_2 cells	% of M_2 cells	Number of M_2 cells containing SCE	% of M_2 cell containing SCE	Mean SCE/cell[b] ± SD	PI
Control I	321	89	28	50	56	2.8 ± 1.7	1.37
Control II[a]	436	36	8	20	56	1.85 ± 0.96**	1.13
10^{-5} M	363	57	16	17	30	2.5 ± 1.19*	1.47
10^{-6} M	343	77	23	25	32	2.0 ± 1.06	1.21

[a] Et-OH (solvent, 0.4% final concentration), [b] Mean of SCE among M_2 cells contaning SCE
* $p \leq 0.05$, significantly different from Control II, ** $p \leq 0.05$, significantly different from Control I

Table 5: Effects of BITN, BITNm, BITNe and ATRA on the number of SCEs expressed as percent of Control II

Compounds	Control SCEs (%) 10^{-5} M	10^{-6} M
Control ethanol	100	100
Control ether	100	100
BITN	92	80*
BITNm	95	92
BITNe	94	90
ATRA	135**	108

* $p < 0.05$, significantly different from Control II
** $p < 0.05$, significantly different from Control II

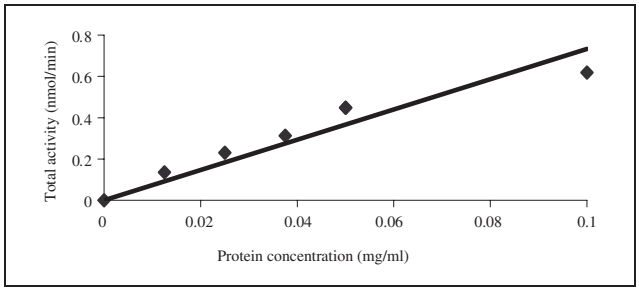


Fig. 2: The effect of enzyme amount on human lymphocyte GST activity. The reaction mixture was prepared with 1.0 mM GSH, 1.0 mM CDNB, 0.1 M potassium phosphate buffer, pH = 6.5, and the reaction was started by the addition of human lymphocyte cytosol in a final volume of 1 ml. The reaction was carried out at room temperature for 3 minutes. Each point was the mean of duplicate determinations. This experiment was repeated three times

2.3. GSTs activities in cultured human lymphocytes

GSTs activities in human lymphocyte cultures were also studied spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate by monitoring the thioether formation at 340 nm as described by Habig et al. (1974). The whole blood cultures were prepared as described in section 4.3.1 and for each experiment eleven culture tubes were set up as mentioned in section 2.2 and the experiments were repeated three times. The average specific activity of cultured human lymphocyte GSTs was determined under optimized conditions as 8.96 ± 0.1 nmol/min/mg.

2.3.1. Effect of enzyme amount on GSTs activities

Effect of enzyme amount on the GST activity was measured by changing the final protein concentration in the 1.0 ml reaction mixture between 12.5 μg and 100 μg protein. Fig. 2 shows the effect of protein concentration on GST activity. It was found that the activity was proportional to enzyme amounts up to 100 μg protein in 1.0 ml reaction mixture. In order to obtain a sufficient quantity of product for spectrophotometric determinations, 50 μg human lymphocyte protein was routinely used throughout this study.

2.3.2. Effects of BITN, BITNm, BITNe and ATRA on GSTs activities in cultured human lymphocytes

The average specific activities for ethanol and ether treated lymphocyte cultures were calculated as 5.9 ± 0.62 nmol/min/mg and 4.99 ± 0.75 nmol/min/mg, respectively. So, both ethanol and ether decreased the GSTs activities and the decrease was statistically significant ($p < 0.001$ and $p < 0.01$, respectively). As 10^{-5} M of BITN was added into the culture medium, it induced the GST activity significantly when compared to its respective Control II (0.4% ethanol) ($p < 0.001$). However, 10^{-6} M of BITN had no effect on GST activity. Specific activities were found as 9.09 ± 1.4 nmol/min/mg and 5.85 ± 1.28 nmol/min/mg for 10^{-5} M and 10^{-6} M of BITN, respectively (Table 6).
The average specific activities were determined as 8.8 ± 1.53 nmol/min/mg and 6.78 ± 1.4 nmol/min/mg for 10^{-5} M and 10^{-6} M of BITNm, respectively. Although 10^{-5} M of BITNm induced the GST activity significantly ($p < 0.001$), 10^{-6} M of BITNm had no effect on GST activity. Table 7 indicates the results of BITNm on cultured human lymphocytes GST activity.

Table 6: Effect of BITN on human lymphocyte GST activity

	Specific Activity ± SD (nmol/min/mg)	% of Control II
Control I	8.96 ± 0.0	152
Control II	5.9 ± 0.62**	100
10^{-5} M	9.09 ± 1.4*	154
10^{-6} M	5.85 ± 1.28	99

* $p < 0.001$, significantly different from Control II
** $p < 0.001$, significantly different from Control I

Table 7: Effect of BITNm on human lymphocyte GST activity

	Specific Activity ± SD (nmol/min/mg)	% of Control II
Control I	8.96 ± 0.0	152
Control II	5.9 ± 0.62**	100
10^{-5} M	8.8 ± 1.53*	149
10^{-6} M	6.78 ± 1.4	115

* $p < 0.001$, significantly different from Control II
** $p < 0.001$, significantly different from Control I

Table 8: Effect of BITNe on human lymphocyte GST activity

	Specific Activity ± SD (nmol/min/mg)	% of Control II
Control I	8.96 ± 0.0	152
Control II	5.9 ± 0.62**	100
10^{-5} M	9.57 ± 0.79*	162
10^{-6} M	7.97 ± 1.26***	135

* $p < 0.001$, significantly different from Control II
** $p < 0.001$, significantly different from Control I
*** $p < 0.05$, significantly different from Control II

Table 9: Effect of ATRA on human lymphocyte GST activity

	Specific Activity ± SD (nmol/min/mg)	% of Control II
Control I	8.96 ± 0.0	180
Control II	4.99 ± 0.75**	100
10^{-5} M	5.8 ± 1.15	162
10^{-6} M	ND	–

** $p < 0.001$, significantly different from Control I
ND: Not detectable

Table 10: Effect of BITN, BITNm, BITNe and ATRA on human lymphocyte GST activity expressed as percent of control II

Compounds	Specific activity % of control II	
	10^{-5} M	10^{-6} M
BITN	154*	99
BITNm	149*	115
BITNe	162*	135**
ATRA	116	–

* $p < 0.001$, significantly different from Control II
** $p < 0.05$, significantly different from Control II

BITNe caused a statistically significant increase in GST activities at both concentrations ($p < 0.001$ for 10^{-5} M, $p < 0.05$ for 10^{-6} M) when compared to control. The average specific activity for 10^{-5} M of BITNe was calculated as 9.57 ± 0.79 nmol/min/mg and for 10^{-6} M of BITNe was 7.97 ± 1.26 nmol/min/mg (Table 8).

It has been found that ATRA had no significant effect on GST activity at 10^{-5} M with a specific activity of 5.8 ± 1.15 nmol/min/mg, the specific activity of 10^{-6} M of ATRA was not detectable (Table 9).

Table 10 gives the summary of results expressed as percent of Control II. It can be easily seen that BITN, BITNm and BITNe induced the GST activities at 10^{-5} M, BITNe also showed significant induction at 10^{-6} M, but BITN and BITNm had no effect at 10^{-6} M.

3. Discussion

Within the last decade both *in vivo* and *in vitro* studies have shown that retinoids are one of the most important agents used for chemoprevention and chemotherapy of cancer. In recent years it became increasingly evident that retinoids can inhibit growth of neoplastic cells and reduce the effects of carcinogens. Borek et al. have found that all-*trans*-retinoic acid (ATRA) inhibited the cell growth of radiation (3 Gy min^{-1}) induced cell transformation in hamster embryo cells (Borek et al. 1981). Although retinoids are useful agents for therapy and prevention of cancer, their clinical use is limited because of their toxicity. One of the most serious toxicity produced by retinoids is the malformation of developing embryos. An important consideration in the selection of agents for clinical chemotherapy and chemoprevention trials is their toxicity (McCormick et al. 1999; Hashimoto 1991). In this study, we investigated the genotoxic effects of three benzimidazole retinoid derivatives (BITN, BITNm and BITNe) and also all-*trans*-retinoic acid (ATRA) in human lymphocyte cultures by using SCE analysis. Sister chromatid exchanges are indicators of SCEs frequency provide a basis for evaluating whether these compounds are genotoxic or not.

We observed that 10^{-5} M (3 μg/ml) of ATRA induced the SCEs, but 10^{-6} M of ATRA had no effect on SCEs frequency. It has been indicated by Dozi-Vassiliades et al. (1985) that 4 μg/ml all-*trans*-retinol induced the SCEs frequency in human lymphocytes culture. They also investigated the synergistic effect of retinol and melphalan, which is a direct-acting cytostatic agent, on SCEs. The same amount of retinol also increased the SCEs frequency of melphalan induced SCEs. It has been suggested that the SCEs induction by retinol in human lymphocytes may be due to its ability to bind to chromatin. The synergistic induction of SCEs by retinol in combination with melphalan may be attributed to the ability of retinol to produce sublethal injury to cellular membranes, thus facilitating the action of melphalan upon DNA. Another possible explanation is that in human lymphocytes undertaking DNA repair after being damaged by melphalan, retinol may interfere with DNA repair and this might lead to an increase in the number of unrepaired lesions at the S-phase. If damage to DNA is left unrepaired it can be linked causally to the formation of SCEs by a process that resides in semiconservative DNA replication. Retinol has been reported to give 50% repair inhibition of DNA replication which normal human lymphocytes undertake after being damaged by UV radiation (Dozi-Vassiliades et al. 1985). It has also been found that 4 μg/ml of all-*trans*-retinol had enhancing effect on chromosomal aberrations induced by indirect-agents ben-

zo(a)pyrene (BP) and dimethlybenzo(a)anthrene (DMBA) in V79 cell line (Chinese hamster lung cells) (Qin et al. 1985). They claimed that these increases may be explained by the well-known surface-active properties exhibited by retinol. The chemical structure of retinol consists of a lipophilic cyclic ring attached to a polyene chain with a polar terminus. This structural feature determines the surface-active nature of the molecule and makes it interact with cell membranes in a detergent-like way and it was possible that at high levels of retinol, the enhanced clastogenic effects of BP or DMBA were simply due to the fact that the partially damaged membrane made it easier for the carcinogens or their active metabolites to enter into the cell and resulted in more chromosome damage (Qin et al. 1985). It can be suggested that ATRA at high doses in the same way with retinol can disrupt the nucleus membrane and excess retinoic acid diffuses into the nucleus. As a result, ATRA may interfere with DNA repair and suppress the repair enzymes, and cause induction of SCEs. Retinoids (retinoic acid, an aromatic retinoic acid analogue and vitamin A palmitate) have been reported to enhance SCEs in human fibroblasts (Juhl et al. 1978). Borek et al. showed that a retinoic acid analogue (TMMP-ERA-trimethlymethoxyphenyl analogue of N-ethyl retinamide) slightly increased the SCEs frequency of radition (3 Gy min^{-1}) induced SCEs in the mouse fibroblast cells at 7.1 μM concentration (Borek et al. 1981). On the other hand, there are many studies either *in vitro* or *in vivo* indicating that retinoids do not induce genotoxic effects *per se* (De Flora et al. 1999). It has been reported by Sirianni et al. (1981) that retinoic acid and retinol did not significantly affect the SCEs frequency in V79 cells and also in mitomycin C (direct-acting cytostatic agent) induced V79 cells. Cozzi et al. (1990) observed that both retinoic acid and retinol had no effect on the frequency of SCEs in CHEL cells (Hamster tracheal epithelial cells). Besides this, even in the absence of any genotoxic agents, retinoids appeared to display protective effects towards some mechanism, reproduced *in vitro*, which are involved in tumor promotion and progression to malignancy (De Flora et al. 1999). It is well known that a large proportion of genotoxicants require bioactivation via metabolic pathways which occur in mammalian cells, mainly in the endoplasmic reticulum. Retinoids were much more effective in inhibiting the genotoxicity of this category of compounds, as compared with direct-acting agents. However, efficacy of inhibition and consistency of results varied depending on the chemical nature of the genotoxic compounds. For example, both *in vivo* and *in vitro* studies provided evidence for the efficiency of retinoids in inhibiting the induction by aflatoxin B1 of progenotoxic and genotoxic effects, including DNA binding, SCEs and chromosomal aberrations (De Flora et al. 1999). Qin et al. suggested that retinoids may exert their anticarcinogenic effects by inhibiting the enzyme activities required for activation of certain precarcinogens (1985). Generally many precarcinogens are activated to ultimate carcinogenic metabolites by the multiforms of microsomal cytochrome P450 mixed function oxidases. Retinoids appear to inhibit the cytochrome P450 system, which is involved in the metabolism of a broad variety of genotoxicants. It should be kept in mind that the cytochrome P450 system is not only involved in activation pathways but also in detoxification of genotoxicants and therefore different effects may be seen depending on the metabolic pathways of genotoxicants (Gradelet et al. 1997). Some conflicting findings reported in different studies can be ascribed to several variability factors, such as the type of nutrient, its solubility, mode of application, dosage, schedule of administration, cellular substrate, investigated end-point, interlaboratory variations, etc.

Nevertheless, the bulk of the data is consistent with the conclusion that interference with the metabolism of xenobiotics is the major mechanism involved in modulation of genotoxicity by retinoids (De Flora et al. 1999). The three benzimidazole retinoids used in our study, BITN, BITNm and BITNe gave different results when compared with ATRA. We thought that difference may be related with their chemical structures. BITN, BITNm and BITNe have two nitrogen atoms within the intermediate ring system and according to Hashimoto (1991) hetero-atoms like nitrogen gives polarity to molecules. It may be concluded that the polarity of these compounds regarding the intermediate structure causes the compounds less affinity to enter the nucleus resulting the lack of DNA repair inhibition. The only difference in structures of these molecules is at the carboxyl moiety in which BITN has free carboxyl group whereas in BITNm and BITNe the carboxyl group is esterified by methyl and ethyl groups, respectively. As mentioned before at high concentrations ATRA induced the SCEs. We may suggest that it can disrupt the nuclear membrane and excess retinoic acid enters the nucleus and may cause inhibition of DNA repair. However, at the same concentration BITN, BITNm and BITNe had no effect on SCE frequency. It can be claimed that they cannot damage the nucleus membrane and excess of these compounds stay in the cytosol. They appear not to interfere with repair of DNA and also not induce the repair of DNA. At low concentration (10^{-6} M) ATRA, BITNm and BITNe had no effect on SCEs. But BITN had protective effects. We observed 20% of decrease in SCEs frequency, so it can be concluded that at this concentration BITN may induce the repair of DNA. We do not know the exact mechanism how these compounds affect the SCEs. They may have different affinity to nuclear retinoid receptors than that of retinoic acid. Therefore, binding capacity of these compounds to their receptors should be investigated as a further study. In addition to the modulation of cytochrome P450 isozymes involved in phase I reactions, studies in cultured cells or whole animals have indicated that retinoids can induce phase II enzymes activities such as glutathione S-transferase, GSH peroxidase and uridine diphosphate glucuronyl transferase (De Flora et al. 1999). Again although these enzymes bear a predominantly detoxifying role, under certain circumstances they may trigger opposite effects.

We observed that while 10^{-5} M of ATRA had no effect on GST activity, the same amounts of BITN, BITNm and BITNe induced GST activity. However, this induction was not 2 or 3 times of the control GST activity and GSTs may not confer resistance to these compounds. On the other hand, it can be concluded that excess amount of these compounds may be detoxified by GSTs so that they do not exert genotoxic effects. At low concentration (10^{-6} M) among three benzimidazole retinoids only BITNe induced GST activity, the other two retinoids had no effect. However, we must consider that GST activity was determined using a common GST substrate. The GSTs are a great family of isozymes each exhibiting a different substrate specificity. Amongst them, the GST-Theta isozyme even does not show any activity towards CDNB. In the future the effects of these compounds on GSTs activity must be determined using specific substrates of respective isozymes, so that each isozyme might be individually affected which could not be pronounced among the others using CDNB only as substrate.

4. Experimental

4.1. Chemicals

RPMI 1640 HEPES modified, Histopaque 1077–1, sodium pyruvate, L-glutamine-penicillin-streptomycin solution, 5-bromo-2-deoxyuridine (BrdUrd), bisbenzimide Hoechst No: 33258 [23491-44-3], colchicine [64-86-8], bovine serum albumine (BSA), reduced glutathione (GSH) and all-*trans*-retinoic acid [302-79-4] were obtained from Sigma Chemical Company, Saint Louis, Missouri, USA. Phytohemagglutinin L (PHA-L lymphocyte type) and fetal calf serum were obtained from Seromed, Biochrom K.G., Berlin, Germany. Giemsa stain, glacial acetic acid, extra pure methanol and ether were purchased from E. Merck, Darmstadt, Germany. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

4.2. Sister chromatid exchange analysis

The optimum culture conditions for human whole blood were adopted from Hill and Wolff (1983) as set previously in our laboratory with some modifications (Topsoy 1997). Three hundred microliters (1×10^5 cells/ml) of freshly drawn heparinized (10 units/ml) human blood (obtained from a non-smoking, 25 year-age-female donor) was inoculated into 2.5 ml culture medium in 15 ml Falcon polypropylene tubes containing RPMI 1640 HEPES modified, 2.6% sodium pyruvate, 1% L-glutamine-penicillin-streptomycin solution (2 mM L-glutamine and 100 units/ml penicillin-streptomycin) and 12% heat inactivated (incubated at 56 °C for 30 min) fetal calf serum. PHA-L was added at a final concentration of 5 μg/ml to the culture medium as mitotic agent for initiation of growth. The cultures were grown in an incubator at 37 °C under humidity. Twenty-four hours after inoculation of the cells, bromodeoxyuridine (BrdUrd) was added to a final concentration of 10 μM (3 μl from 100 mM stock) and the cultures were continued to be incubated for additional 48 h under the same conditions in the dark. Complete darkness is necessary to protect the BrdUrd substituted DNA from degradation by light. The protection was achieved by wrapping the culture tubes with aluminum foil and working under dim yellow light. Four hours before harvesting the cell, 0.5 μg/ml colchicine (final concentration) was added to entrap the cells at the metaphase of their mitotic division. Then, the cells were collected by centrifugation at 600 g for 15 min using a bench top centrifuge (IEC clinical centrifuge DAMON/IEC Division) at room temperature. After discarding the supernatant, 0.075 M hypotonic KCl, prewarmed to 37 °C, was added on to the pellet and mixed well with gentle shaking (without vortexing). Culture tubes were than put back to the incubator for further 20 min. Swollen lymphocytes were collected by centrifugation at 600 g for 15 min. Supernatant was discarded leaving approximately 0.5 ml of 0.075 M KCl on the pellet. Then the cells were fixed by adding freshly prepared chilled methanol: acetic acid (3 : 1) on to the pellet drop by drop while mixing with vortex at low speed in order to prevent the aggregation of cells and leaving at 4 °C overnight. The next day the fixation step was repeated two more times by adding fresh fixative and collecting the cells by centrifugation at 600 g for 15 min between the fixations. Finally, cells were suspended in 1 ml of fresh fixative. The cell suspension was dropped (5–7 drops/slide) on chilled wet slides left to dry at room temperature. The aged slides were then stained using the fluorescent plus Giemsa (FPG) technique (Perry and Wolff 1974) with some modifications in order to visualize Harlequin chromosomes. The slides were soaked in PBS for 3 min, and then, in Hoechst 33258 solution (5 μg/ml PBS) for 15 min. The slides were first rinsed in PBS and then in distilled water. The slides were placed on a tray and the slide surfaces were covered with McIlvanes buffer (pH 8.0) prewarmed to 60 °C. The edges of slides were sealed with Parafilm® to prevent the evaporation of McIlvanes buffer from the surface of the slides. Then, the slides were irradiated with short-wave UV (Ultraviolet Products Inc., Mineral Light Lamp Model C-81) for 25 min in a tray placed on a water bath at 60 °C. The distance between UV-source and the slides was approximately 5 cm. After irradiation, the slides were rinsed with distilled water and incubated two times with SSC (sodium saline citrate) for 10 min at 60 °C. Then, the slides were rinsed with distilled water thoroughly. Finally, the slides were stained with 2% Giemsa in 0.1M phosphate buffer, pH 6.8, for 15 min and rinsed well with distilled water again for few seconds and dried at room temperature.

Table 11: The constituents of a typical assay mixture for GST assay using CDNB as substrate

Components	Stock concentration	Added volume	Final concentration in 1ml cuvette
Potassium phosphate buffer, pH 6.5	200 mM	500 μl	100 mM
GSH	50 mM	20 μl	1.0 mM
CDNB	20 mM	50 μl	1.0 mM
Lymphocyte cytosol	0.5 mg/ml	100 μl	0.05 mg/ml

4.3. Determination of glutathione S-transferase (GSTs) activity in human whole blood cultures

4.3.1. Preparation of human whole blood cultures

Blood cultures contain six hundred microliters (1×10^5 cells/ml) of freshly drawn heparinized (10 units/ml) human blood, 5 ml culture medium containing RPMI 1640 HEPES modified, 2.6% sodium pyruvate, 1% L-glutamine-penicillin-streptomycin solution (2 mM L-glutamine and 100 units/ml penicillin-streptomycin) and 12% heat inactivated fetal calf serum. PHA-L was added as a final concentration of 5 μg/ml to the culture medium. Then the cells were cultured for 96 h to provide maximum growth at the same culture conditions as described above and 2 ml of fresh culture medium was added at 72 h. 24 h after the inoculation of the cells, they were treated with 10^{-5} M (≅ 3 μg/ml for ATRA and ≅ 4 μg/ml for BITN, BITNm, BITNe) and 10^{-6} M (≅ 0.3 μg/ml for ATRA and ≅ 0.4 μg/ml for BITN, BITNm, BITNe) BITN, BITNm, BITNe, ATRA, and 0.4% ether as a solvent for ATRA and 0.4% ethanol as a solvent for imidazole derivatives. At the end of the incubation period lymphocytes were isolated from whole blood cultures using the density gradient centrifugation technique according to Celis (1994), with some modifications. The cells were collected by centrifugation at 600 × g for 15 min in a bench top centrifuge (IEC Clinical Centrifuge DAMON/IEC Division) at room temperature. Cells were suspended in an equal volume of 0.9% NaCl. Then, 2 ml of suspension was layered on to the 1.2 ml of lymphocyte separation medium Histopaque 1077-1 and centrifuged at 800 × g for 15 min. The cloudy lymphocyte layer was transferred into an Eppendorf tube for centrifugation at 10000 rpm for 15 min using Heraeus INC,USA centrifuge, rotor no 3743. The lymphocyte pellet was suspended in 0.5 ml of 10 mM potassium phosphate buffer, pH 6.5, and stored at −74 °C until GSTs activities were measured. Before GST activities were measured, lymphocytes were sonicated and their protein concentrations were determined by the method of Lowry et al. (1951). The protein amount was found as 0.5–0.7 mg/ml.

4.3.2. Determination of GST activity using CDNB as subtrate

GSTs activity was determined spectrophotometrically by monitoring the thioether formation at 340 nm as described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. A typical reaction mixture included 0.1 M potassium phosphate buffer, 1.0 mM CDNB, 1.0 mM GSH and 50 μg human lymphocyte cytosolic protein in a final volume of 1 ml as shown in Table 11. The increase in optical density at 340 nm was determined by following the reaction for 3 min. The extinction coefficient used in the calculations was 0.0096 $\mu M^{-1}cm^{-1}$ at 340 nm (Habig et al. 1974). GST activities were expressed as units/mg. One unit of GST activity is defined as the amount of enzyme producing one nmole of CDNB conjugate in 1 min.

4.4. Viability test for isolated lymphocytes

The number of living cells in the isolated lymphocyte suspension were determined using Trypan blue staining. Fifty microliter lymphocyte cells were taken from the isolated lymphocyte cell suspension and diluted with 50 μl Trypan blue. This mixture was kept at room temperature for 5 min to permit the dye penetrate through the membranes of the dead lymphocytes. Then the counting was performed on a hemocytometer. The number of living cells/ml were calculated using the formula: Total counted living cells in 25 squares of hemocytometer × dilution factor (DF) × 10^4.

4.5. Statistical analysis

For the statistical evaluation of the experimental data Student's t test was performed to determine whether any values deviated significantly from the controls.

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