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Genotoxic effects of *Karwinskia humboldtiana* toxin T-514 in peripheral blood lymphocytes

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Toxicity by *Karwinskia humboldtiana*, a Rhamnaceae plant, has been assessed in a number of studies. Four dimmeric anthracenones, named T-496, T-514, T-516 and T-544 for their molecular weight, have been isolated from this plant. T-514, in particular, has been shown to be toxic to liver and lung as well as to tumoral cell lines, preferentially to those from liver tumors. For this reason it has been suggested that the toxin could be used as an antineoplastic agent. The present study was performed to characterize the biological activity of T-514 as a potential cytostatic and genotoxic agent. Peripheral blood lymphocytes in culture were used as a test system, where chromosomal aberrations and sister chromatid exchanges were scored in order to evaluate genotoxicity, and mitotic index and cell proliferation kinetics were used as parameters for cytostatic and cytotoxic ability. Genotoxicity to lymphocytes was negative. However, proliferation was affected by the toxin, demonstrating a cytostatic activity independent of genotoxic damage.

Key words: Anthracenones, cytostatic effects, human lymphocytes, genotoxicity, *Karwinskia humboldtiana*, Rhamnaceae plants, T-514.

Introduction

Karwinskia humboldtiana is a poisonous plant of the Rhamnaceae family that is distributed in Mexico, Southwestern US and several localities in Central America. The toxic principle has been found in the seeds of the fruit from which four substances identified as dimmeric anthracenones have been isolated and named by their molecular weight as T-496, T-514, T-516 and T-544.[1]

When accidentally ingested the dark purple fruits produce intoxication characterized by ascending systemic paralysis similar to Guillian–Barre syndrome.[2–4] Comparable symptoms have also been described in experimental and accidental intoxication of animals,[5,6] and other studies revealed that besides the neurological damage, purified toxins T-514 and T-544 are also able to affect lung and liver, especially toxin T-514;[7] this toxicity has been correlated with findings in primary liver and skin cell cultures[8] where a greater sensitivity of hepatocytes was observed. Selective toxicity of T-514 (Figure 1) has been further described in studies performed with several human tumor cell lines, thus suggesting a potential use as an anti-neoplastic agent; in order to evaluate this capacity, the drug was assayed in comparison with other known anticancer agents, in normal and tumoral cells: interestingly, it displayed a greater toxicity in liver cancer cells as compared with lung and colon, at the same time that normal cell lines were about 20 times less sensitive.[9]

In order to more fully understand the biological activity of T-514, experiments on its cytostatic and cytotoxic capacity were performed using the lymphocyte culture system as proposed by Ostrosky *et al.*,[10] Rojas *et al.*[11] and Montero *et al.*;[12] the genotoxic potential of this drug was also tested.

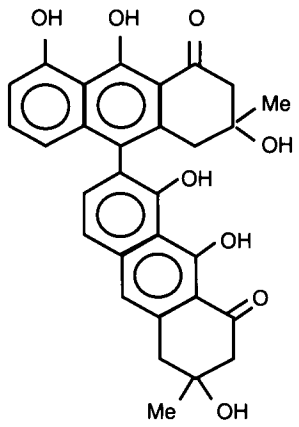

Figure 1. Chemical structure of toxin T-514.

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Materials and methods

Lymphocyte cultures

Heparinized peripheral blood from healthy donors, males and females averaging 30.7 years of age, was used for *in vitro* determinations. All donors were non-smokers and had not taken any drugs for at least 1 month before sampling. Culture conditions and treatments were used according with those of Montero *et al.*[12] Briefly, cultures were set-up in duplicate, containing: 0.5 ml of blood in supplemented RPMI 1640 medium (Flow-Lab; Mc Lean, Virginia, USA) (with 0.1 mM non-essential amino acids and 2 mM L-glutamine; Gibco; Grand Island, New York, USA), 32 μM bromodeoxyuridine (BrdU; Sigma; St. Louis, Missouri, USA) and stimulated with phytohemagglutinin (PHA) (Microlab; México, D.F., México) in a final volume of 7 ml. No antibiotics were used. At 70 h of incubation 2 mg of colcemid (Demecolcine; Sigma) was added to each culture and incubation continued for a further 2 h. Cultures were stopped with 0.075 M KCl and cells fixed with Carnoy solution. Slides were prepared and stained by the fluorescence plus Giemsa method[13] and used to evaluate cytotoxicity and cytostatic effect by scoring: *mitotic index* (MI) as the number of metaphases in 2000 cells analyzed; *inhibition of the mitotic index* produced by the drug, calculated as: MII = 100 − (MI treated × 100/MI control); and *lymphocyte proliferation kinetics* (LPK) evaluated in 100 consecutive metaphases separately scoring the cells in first (M1), second (M2) or third (M3) division, the proportions obtained were used to calculate the *replication index* according to the formula RI = (M1 + 2M2 + 3M3)/100. Two parameters for genotoxicity were used: the mean frequency of *sister chromatid exchanges* (SCE) established by analyzing 25 second-division cells and the frequency of *chromosomal aberrations* (CA) determined in 100 first-division cells.

Treatments

Without metabolic activation. T-514 dissolved in 80% ethanol was added to the cultures after 48 h of incubation in concentrations of 1, 5 and 10 μM; they were left in culture until the end of the incubation time, 24 h after. Mytomicin C (MMC) was employed as a positive control. Other controls were cultures with solvent alone.

With coupled metabolic activation system. After 48 h of incubation, 0.5 ml of S9 mixture (in a proportion of 18% of the S9 fraction according to White and Hesketh[14]) together with the same concentrations of T-514 used in treatments without metabolic activation were added to cultures and incubation continued for 2 h; samples were then washed twice with medium, the supernatant replaced with fresh growth medium containing PHA and BrdU, and cells returned to incubation for a further 24 h. Cyclophosphamide was the drug used as positive control. Negative controls were cultures with S9 mixture and solvent. MI, RI and SCE were the parameters evaluated.

Statistical analysis

The χ^2-test was used for CA analysis, and a *t*-test was employed for MI, RI and SCE.

Results and discussion

Tested for its mutagenicity, T-514 did not induce structural CA and neither caused SCE in peripheral blood lymphocytes (Tables 1 and 2a); when S9 was used, no modification was observed with respect to SCE (Table 2b); emodin, a monomeric anthracenone, behaved similarly when studied in V79 Chinese hamster cells.[15] Another hydroxyanthraquinone, luteoskyrin (dimmeric, polyhydroxylated and methylated molecule), similar to T-514, appeared to be highly mutagenic, inducing hyperploidy and chromosome aberrations in Ehrlich ascite tumor cells.[16] It must be said, however, that this damage was induced only in resistant cells which had survived a toxic treatment by the drug and then were expanded for months in the presence of the toxin, that they were already transformed cells and apparently the selection with the drug induced a further process of transformation, and that whether the damage was produced by the treatment or by genome instability of the cells is an issue that was not adequately assessed since no control of resistant cells expanded without the toxin was performed.

In this study, T-514 affected the proliferative ability of lymphocytes, measured both as the MII or as the cell cycle kinetics (Figure 2), indicating a toxic effect. A concentration related inhibition of both parameters was obtained, very similar to that observed with MMC, although less toxic (Figure 2). When microsomal enzymes were added to the culture, treatment had to be reduced to 2 h due to

Table 1. Frequency of chromosomal aberrations in lymphocytes treated with T-514

Treatments (μM)	No. of cells	Type of structural aberration						Aberrant cells (%)	Chromosomal aberrations (%)	
		G'	*G"*	*B'*	*B"*	*R*	*D*		Gaps	Without gaps
Donor 1										
0	100	0	0	0	0	0	0	0	0	0
1	100	0	0	1	0	0	0	1	1	1
5	100	0	0	0	0	0	0	0	0	0
10	100	nm	nm	nm	nm	nm	nm	nm	nm	nm
Donor 2										
0	100	2	0	0	0	0	0	1	2	0
1	100	0	0	0	0	0	0	0	0	0
5	100	5	1	0	0	0	0	2	6	0
10	100	nm	nm	nm	nm	nm	nm	nm	nm	nm
Donor 3										
0	100	0	0	1	0	0	0	1	1	1
1	100	0	3	0	0	0	0	1	3	0
5	100	3	0	1	0	0	0	2	4	1
10	100	0	0	1	0	0	0	1	1	1
Donor 4										
0	100	2	1	0	0	0	0	2	3	0
1	100	0	0	1	0	1	0	2	2	2
5	100	0	0	1	0	0	2	2	3	3
10	100	nm	nm	nm	nm	nm	nm	nm	nm	nm

nm, no mitosis to analyze.
G, gaps; *B*, breaks; *R*, rearrangements; *D*, dicentrics; 'chromatidic; "chromosomic.

Table 2. SCE frequencies in lymphocytes treated with T-514

Treatments (μM)	Donor 1	Donor 2	Donor 3	Donor 4
(a) After 24 h exposure to T-514 without S9				
0	5.28 ± 1.92	5.26 ± 1.25	4.12 ± 1.81	4.48 ± 1.35
1	5.22 ± 1.78	5.28 ± 1.50	4.08 ± 1.11	5.12 ± 2.06
5	4.95 ± 1.39	5.74 ± 1.75	5.12 ± 1.53	5.50 ± 1.63
10	5.70 ± 2.4	5.00 ± 1.30	nm	4.36 ± 1.25
MMC 1 μM	22.52 ± 3.62			
(b) After 2 h exposure to T-514 with S9				
0	6.14 ± 1.76	4.56 ± 0.91	4.96 ± 0.97	5.24 ± 1.42
1	5.92 ± 1.80	5.08 ± 1.03	4.68 ± 0.94	5.11 ± 0.83
5	5.48 ± 1.22	4.85 ± 0.89	4.78 ± 0.99	5.75 ± 1.48
10	5.71 ± 1.14	5.44 ± 1.41	5.36 ± 1.72	nm
cyclophosphamide 40 μM	17.0 ± 2.5			

S9, metabolic activation system.
nm, no mitosis for analysis.
Values are given as mean SCE/cell ± SD.

the toxicity to cells; in these conditions, MI was equally inhibited, but the effect seen on the RI was moderated. This effect was analyzed to establish if the different time of treatment had affected the results, hence cultures without S9 metabolic activation were set in which T-514 was allowed to act for only 2 h; inhibition of proliferation (measured as RI) obtained was very similar to that observed with S9 treatment (Figure 3) and, again, MI was strongly inhibited with the different concentrations of the drug. For this reason it cannot be said that hepatic metabolism affects T-514 activity (Figure 3).

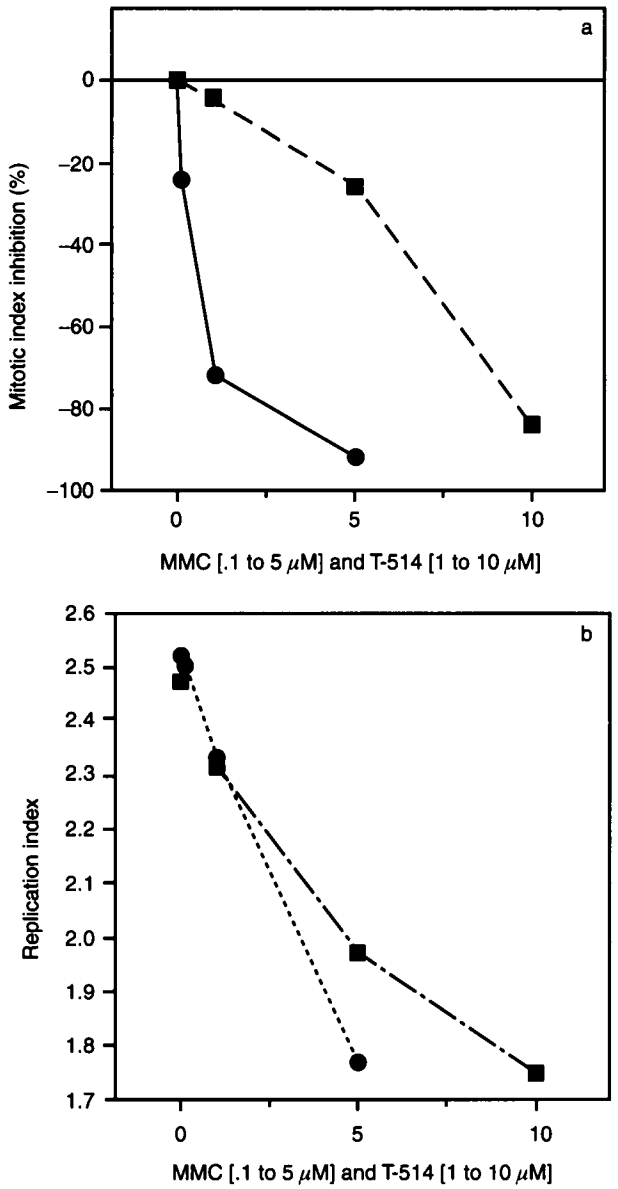

Figure 2. Effects of MMC (circles) and T-514 (squares) on the proliferative ability of lymphocytes, measured as (a) the MII and (b) the RI. MMC exerts a toxic effect at smaller concentrations than T-514 as seen from these data. (MII was plotted with negative numbers in order to facilitate understanding of the negative effect it represents.)

These results coincide with those obtained by Garza-Ocañas *et al.*[8] who tested T-514 in primary hepatocyte cultures for its ability to affect plasma membrane integrity, lysosomal function and mitochondrial metabolic activity; at 2 h of culture with concentrations of 6–50 mM (compared to 1–10 mM used by us) no damage to the cell membrane was observed; however, it was present at all the concentrations tested at 6 and 24 h of treatment. The other parameters, i.e. tetrazolium blue reduction by mitochondrial function and neutral red uptake to measure lysosomal function, were inhibited at all the concentrations tested, after 4 h of exposure of hepatocytes to the toxin. MI of lymphocyte cultures is an indicator of the proliferative ability of cells; hence, as we could detect an effect due to the T-514 action as early as 2 h of treatment, this parameter could be considered as more sensitive to assess cell damage at low concentrations of drugs. It also shows that the toxin affects cell function in many ways and early in the interaction with them.

In conclusion, T-514 did not display a mutagenic activity in any of the two parameters tested, CA or SCE, and, although there is evidence of the mutagenicity of other anthracenone derivatives, the structural differences seem to be crucial for the activity of each molecule. Finally, T-514 has been shown to stop active proliferating cells by toxicity: whether this is due to necrosis or induced apoptosis must be determined in order to assess its usefulness in therapeutic treatments.

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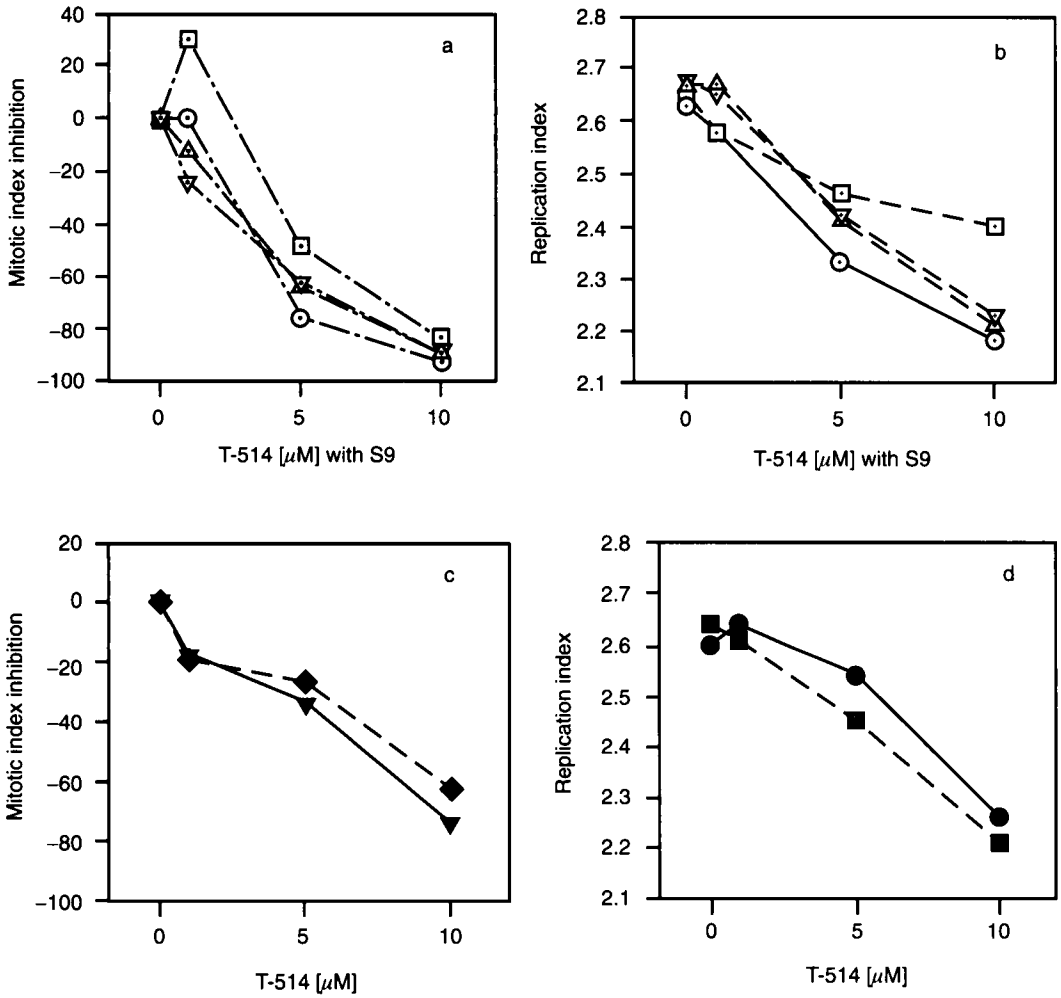

Figure 3. The toxic effect of T-514 was analyzed under the same regime of treatment (2 h) with and without metabolic enzymes in the S9 mixture. Panels (a) and (b) show the effect on MI and RI in the presence of S9, and panels (c) and (d) show the same parameters in cultures treated also during 2 h, but in the absence of the enzymes. Notice the delay in proliferation (RI) is very similar, although the combination of S9 and T-514 resulted in a slight increase in MII. (MII was plotted with negative numbers in order to facilitate understanding of the negative effect it represents; each curve corresponds to one donor.)

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