RESEARCH ARTICLE

Cytogenetic study of *Ascaris* trypsin inhibitor in cultured human lymphocytes with metabolic activation

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Abstract

The trypsin inhibitor (ATI) isolated from gastrointestinal nematode *Ascaris suum* was tested *in vitro* for induction of chromosome aberrations and sister chromatid exchanges (SCE). Genotoxicity assessment of purified ATI was carried out on metaphase plates received from peripheral blood lymphocyte macroculture (48 h test of structural chromosome aberrations and 72 h test of SCE) with exogenous metabolic activation. ATI was tested in dose of 25, 50 and 100 μg per ml of culture. Kinetics of cell divisions were determined by the replication index (RI). The mitotic index (MI) was expressed as a number of metaphases per 1000 nuclei analysed. Analysis of chromosome aberrations showed that higher doses of ATI (50 and 100 μg/ml) significantly increased the frequency of chromosome aberrations (mainly of chromatid gaps and breaks) compared to the negative control. All concentrations of ATI caused a statistically significant reduction in the MI and RI. In comparison with the negative control, a significant increase in the SCE frequency was observed in all applied doses of ATI. Thus, in the presence of S9 activation, the *Ascaris* trypsin inhibitor showed potential clastogenic activity and inhibition of the dynamics of lymphocyte divisions.

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Introduction

It has been commonly accepted that exposure of mammals to mutagenic chemicals may induce pre-implantation losses of non-viable zygotes, early fetal deaths, sterility, abortion, and congenital malformations (Epstein *et al*. 1972). In some previous studies, it was observed that proteolysis inhibitors isolated from *Ascaris suum* disturbed the embryonic development of laboratory animals. It was found that *Ascaris* trypsin and chymotrypsin inhibitors acted embryotoxically and teratogenically on white leghorn chick embryo (Blaszkowska 1998a, 2001). The *Ascaris* trypsin inhibitor injected during organogenesis produced congenital malformations in mice (Blaszkowska 1998b, 2001). The mechanism by which this inhibitor disturbed prenatal development is not clear; its influence on pregnancy is not connected with its antiproteolytic properties but with maternal toxicity (Blaszkowska 2003).

The *Ascaris* trypsin inhibitor is one of the proteins secreted during migration of *Ascaris* larvae. Although the physical and chemical properties of *Ascaris* trypsin inhibitors as well as their effect on mammalian host's enzymes have been extensively studied, their biological significance is still insufficiently known (Pudles and Rola 1967; Dzik 2006; Knox 2007). It has been demonstrated that most of the symptoms of larval and intestinal ascariosis are the result of the host responding to toxic secretory/excretory products of *Ascaris* or to peptides that cause release of histamine or allergens (O'Lorcain and Holland 2000). Currently, only single reports provide information on the influence of *Ascaris* metabolites on the host's somatic and generative cells (Bekish 2000, 2001). It has been established that different antigens from *Ascaris suum* change the chromosome apparatus of human blood lymphocytes *in vitro*, and increase the number of aneuploid and aberrant cells

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(Bekish 1999). Moreover, during experimental-migrating ascariosis, *Ascaris* metabolites have been found to exert a mutagenic effect on somatic cells of bone marrow, spermatogonia and spermatids in mice. Recently, a study demonstrated that the chymotrypsin inhibitor isolated from *Ascaris suum* induced dominant lethal mutations in mice, mainly at the post-meiotic stage of spermatogenesis (Blaszkowska 2008). Another study has shown that the extract from whole worms contains a mitogenic factor, which stimulates human T-lymphocytes (Sasagawa *et al*. 1987). Lee and Xie (1995) also described the presence of a B cell mitogen in the *Ascaris* body fluid, which stimulated G_0 B lymphocytes to enter the cell cycle. This *Ascaris* fluid did not exert such effect on T cells. In contrast to the immunostimulatory activity of the *Ascaris* compounds reported above, other investigators have demonstrated immunosuppressive properties of extracts and secretions of this helminth (Souza *et al*. 2002; Oshiro *et al*. 2004). Previously we reported the results on chromosome aberrations and sister chromatid changes in human lymphocytes exposed to *Ascaris* trypsin inhibitor in the absence of metabolic S9 activation (Blaszkowska *et al*. 2004). In these experimental conditions, no clastogenic effect of this inhibitor was found but significant decrease in the mitotic index values for all tested doses was noted. Moreover, the experiment with human lymphocytes without exogenous activation revealed sister chromatid exchange (SCE)-inducing effect of ATI.

Based on the fact that most chemicals need metabolic activation to bind with DNA and to manifest their genotoxic effects, we also investigated potential mitostatic and clastogenic activity of this *Ascaris* inhibitor after metabolic activation by liver microsomal enzymes (fraction S9). The objective of the present study was to evaluate the ability of the *Ascaris* trypsin inhibitor to induce chromosome aberrations and sister chromatid changes in cultured human lymphocytes in the presence of the rat liver S9 fraction. Additional variables, the reduction of mitotic (MI) and replication (RI) indices, were included for assessment of the cell cycle inhibition.

Materials and methods

Chemicals

Ascaris suum obtained from a slaughterhouse were washed thoroughly in physiological saline and distilled water. The *Ascaris* trypsin inhibitor (ATI) was isolated from homogenate of musculocutaneous sacs of mature female and male of *Ascaris suum* according to the method of Pudles and Rola (1967). The procedure of purification of the inhibitor and its inhibitory activity against crystalline trypsin had been described elsewhere (Blaszkowska *et al*. 2004).

Lymphocyte macrocultures

Human peripheral blood lymphocytes were obtained from 12 healthy adult male non-smoker donors who have not received any relevant drug nor had any relevant medical records. Blood samples were cultured and then harvested by standard procedures (Preston *et al*. 1987). Lymphocyte (whole lymphocyte population) cultures were prepared from heparinized blood according to the method of Moorhead *et al*. (1960). For each assay, 20 ml of venous blood was allowed to sediment for 2 to 3 h. After red cell sedimentation, the supernatant (1 ml) was added to 8 ml of Eagle's fluid 1959 (MEM; Wytwórnia Surowic i Szczepionek, Lublin, Poland), 1 ml of fetal serum (Gibco, Paislay, Scotland), crystalline penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were stimulated with 1% phytohaemagglutinin M-PHA (Difco Laboratories, Detroit, USA) at the beginning of incubation.

The liver homogenate (S9 fraction) from Aroclor 1254-treated male rats was purchased from MolTox (Molecular Toxicology, Boone, USA). The S9 mix solution was prepared immediately before the use by mixing 0.8 ml of S9 with 0.2 ml of 0.2M glucose-6-phosphate, 0.2 ml of 0.2 M of NADP, 0.2 ml of 1.65 M KCl, 0.2 ml of 0.4 M $MgCl_2$, 5 ml of 0.2 M phosphate buffer (pH 7.4) and 3.15 ml of distilled water (each from Sigma, St Louis, USA). The final concentration of S9 used was 1% (v/v).

The experiment was carried out with the same doses of ATI as were used in an earlier study (Blaszkowska *et al*. 2004), in the absence of metabolic S9 activation. For selection of the dose of ATI, in the preliminary experiment, the highest concentration due to cytotoxicity, solubility in the culture medium and changes in pH were determined according to OECD protocol (1997, OECD 1997 Guidelines for testing of chemicals, guideline 473, genetic toxicology: *in vitro* mammalian chromosome aberration test, Paris). The *Ascaris* trypsin inhibitor was dissolved in MEM and added to cultures in dose of 25, 50 or 100 μg/ml for 2 h with the S9 mix (0.4 ml of S9 mix/5 ml of culture medium). The time for the treatment ATI and S9 was selected according to Preston *et al*. (1987) and Kirkland (1998).

For every culture of lymphocytes with the tested compound, the negative control was performed. Negative controls consisted of all compounds of the treated medium except for tested *Ascaris* inhibitor and treated in the same way as treatment cultures. Chlormethine hydrochloride (nitrogranulogen, Polfa; 0.1 μg/ml) was used as the positive control.

Chromosome aberration (CA) assay

The CA assay was conducted using standard procedures (Preston *et al*. 1987). The metaphase plates were obtained in the conventional manner from the above described macroculture of peripheral blood lymphocytes after 48 h of incubation. The lymphocytes were treated for 2 h with ATI in the presence of S9 mix, 24 h after initiation of the cultures. After an incubation period of 2 h, the inhibitor and S9 mix were removed from the cultures. The lymphocytes were centrifuged and washed twice with Eagle's fluid, and

resuspended in fresh complete medium. Cultures were incubated for further 22 h. Colcemid (Serva 0.15 μg/ml, Heidelberg, Germany) was added 2 h before harvesting. Hypotonic shock was induced by 0.075 M potassium chloride solution. Cell suspension was fixed in a mixture of methanol and glacial acetic acid (v/v 3:1). Preparations were stained with Giemsa. For each donor in each concentration 40–50 metaphases were analysed (depending on metaphase quality). Chromosome aberrations were classified according to the International Nomenclature convention (ICSN 1995).

For each dose of the inhibitor, the MI was calculated. To evaluate the MI, cytogenetic preparations were analysed in a light microscope at 400× magnification. MI was expressed as a number of metaphases per 1000 nuclei analysed. The inhibition of the MI was calculated as 100 − (MI treated × 100/MI control) (Rojas *et al*. 1993).

Sister chromatid exchange (SCE) assay

Lymphocyte cultures used for SCE studies were from the same donors and were incubated for 72 h. Bromodeoxyuridine (BrdU, 10 μg/ml, Sigma, St Louis, USA) was added at the beginning of incubation. The lymphocytes were treated for 2 h with ATI in the presence of the S9 mix, 44 h after initiation of the cultures. Following an incubation period of 2 h, the cells were removed from the culture medium washed twice and replaced in a completely fresh culture medium. The cultures were incubated for further 26 h. The slides were coded and stained for sister chromatid differentiation according to Perry and Wolff (1974). A total 50-second metaphases were screened for each donor on coded slides in a blind study. In some cultures for which the quality of metaphases was poor, the numbers of analysed cells were lower (but not less than 25 metaphases). The mean of SCE frequency was calculated as SCE per cell for each dose. Only well spread, good quality metaphases with 46 chromosomes were analysed.

Cell division kinetics was determined by RI values. One hundred metaphases per donor and concentration were analysed for determination of first, second and third generations of mitotic cells: M_1 (darkly stained chromatids), M_2 (one darkly and one lightly stained chromatids) and M_3 (part of metaphase with darkly (1/3) and lightly (2/3) stained chromatids). RI was calculated according to the formula of Rojas *et al*. (2002) as:

$$\mathrm{RI} = \frac{1\mathrm{M}_1 + 2\mathrm{M}_2 + 3\mathrm{M}_3}{100}.$$

Statistical analysis

Parametric statistical procedures were applied to selected measures in these studies. Frequency of SCE per cell and RI values were evaluated by the Bartlett's test for equality of variances. Based upon the outcome of the Bartlett's test, the parametric analysis of variance (ANOVA) was performed. Continuous data were compared among the single treated group and negative control using Student's t-test. The Fisher's extact test or the chi-square test was used to compare aberration frequencies and the MI values. Both statistical calculations for total chromosomal aberrations, including and excluding gaps, were done according to Preston *et al*. (1987). Probability values less than 0.05 were considered to indicate significant differences.

Results

In these experiments, two methods for evaluation of genotoxic activity of ATI were used in the presence of metabolic S9 activation. Frequencies in structural chromosome aberrations induced by ATI are presented in table 1. The number of aberrations observed after incubation of ATI with lymphocytes in the presence of S9 mix increased together with increasing ATI concentration. The analysis of chromosome aberration showed that ATI induced aberrations mainly of chromatid type (gaps and breaks). The total structural aberrations including gaps were significantly higher than the negative control (for concentrations of 50 and 100 μg/ml, $P < 0.005$ and $P < 0.0001$, respectively), but they were significantly lower in comparison with respective positive

Table 1. Frequency of structural chromosome aberrations induced by the *Ascaris* trypsin inhibitor (ATI).

	No. of cells	No. of aberrations							Total aberrations per cell	
Compounds/dose (μg/ml)	scored	G′	G″	B′	B″	AF	DIC	EF	(+ Gaps)	(− Gaps)
Negative control (MEM)	500	1	1	1	0	0	0	0	0.006	0.002
ATI:										
25	520	5	0	2	0	1	0	1	0.017	0.008
50	540	9[a]	1	5	0	2	0	0	0.31[b]	0.013
100	480	11[b]	3	6	1	1	0	0	0.046[c]	0.017[a]
Positive control (chloromethine hydrochloride) 0.1	110	9	5	9	4	0	2	5	0.309	0.227

Statistically significant differences in relation to the negative control: [a]$P < 0.05$; [b]$P < 0.005$; [c]$P < 0.0001$; G′, chromatid gap; G″, chromosome gap; B′, chromatid break; B″, chromosome break; AF, acentric fragment; DIC, dicentric; EF, exchange figure.

control. Moreover, human lymphocytes cultures treated with ATI at the highest concentration (100 μg/ml) showed a statistically significant increase in the total chromosome aberrations excluding gaps ($P < 0.05$).

Mean values of the MI for all doses of the inhibitor were significantly lower ($P < 0.005$) than in the negative control group (figure 1). Differences between mitotic index values for the three different doses of this inhibitor were insignificant. The reduction in mitotic activity of lymphocytes cultivated in the presence of ATI was demonstrated by the percentage of the MI inhibition. All doses of this inhibitor reduced the MI value of the negative control by over 49% (49.2% for 25 μg/ml, 52.1% for 50 μg/ml and 55.5% for μg/ml).

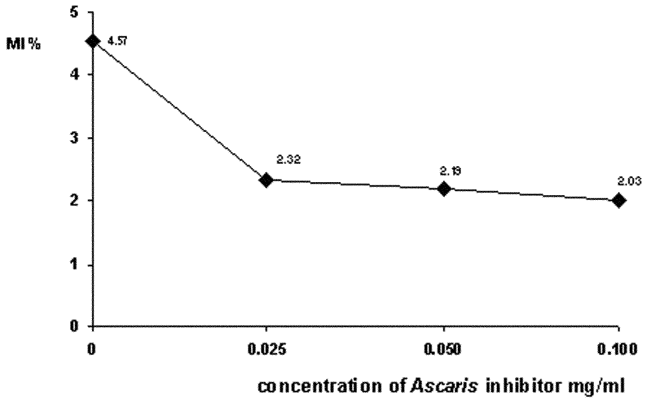

Figure 1. Effect of the *Ascaris* trypsin inhibitor on the mitotic index (MI) of cultured human lymphocytes. Statistically significant differences in relation to the negative control for all doses $P < 0.005$.

Mean SCE frequency per cell (table 2) for all concentrations of the inhibitor was higher and differed significantly from the negative control (for 25 and 50 μg/ml, $P < 0.001$; for 100 μg/ml, $P < 0.0001$). No significant differences were observed between the mean values of SCE frequency/cell for the tested doses of the inhibitor. Mean values of the RI calculated for all tested inhibitor doses were significantly lower than in the negative control (table 2). Comparison of mean RI values between three examined doses of ATI did not reveal significant differences.

Discussion

Chromosome aberrations have been recognized to be an important bio-marker of human exposure to genotoxic chemicals. Some genotoxic chemicals can produce their effects directly, whereas others require metabolism to mutagenically active intermediates. Our previous study demonstrated that trypsin inhibitor isolated from *Ascaris* did not exhibit clastogenic activity in the absence of exogenous metabolic activation (Blaszkowska *et al*. 2004). Chromosome aberrations occuring with a low frequency, and increase in the number of aberrations per cell in comparison with the negative control group, were not statistically significant. Until now there have been no data on clastogenic and genotoxic activity of ATI. The present results of chromosome aberrations assay conducted with metabolic activation revealed potential clastogenicity of *Ascaris* trypsin inhibitor. The compound tested by us induced mainly chromatid gaps and chromatid breaks. ATI in doses 25, 50, 100 μg/ml caused a 5–10-fold increase in the number of cells with chromatid gaps. The validity of analysing gaps in human biomonitoring is controversial; some studies have included gaps in the analysis of chromosomal aberrations, while others have not (Brogger and Waksvik 1978; Brogger 1982). At the moment, there is the tendency to consider gaps as a non-staining region of less than the width of a chromatid or true chromosome discontinuities with no biological significance. However, results of comet assay (Paz-y Mino *et al*. 2002) support the hypothesis that the gaps constitute a type of chromosome aberration, and suggest that these events should be scored in this type of analysis. Since the genetic significance of gaps is not clearly understood, it is appropriate to analyse the data both excluding and including gaps. If the aberration data, with no gaps, are borderline for statistical significance, but a positive response is obtained with gaps included, the test compound should be considered as potentially clastogenic (Preston *et al*. 1987). In our experiment, both statistical calculations for total chromosomal aberrations, including and excluding gaps were done. In the cultured human

Table 2. Frequency of sister chromatid exchanges (SCE) induced by the trypsin inhibitor from *Ascaris* and corresponding replication index (RI) values.

Dose (μg/ml)	No. of cells scored	Frequency of SCE per cell (mean ± SD)	Replication index* (± SD)
Negative control	510	6.20 ± 1.397	1.75 ± 0.179
25	470	8.59 ± 1.513[b]	1.45 ± 0.247[a]
50	490	8.36 ± 1.295[b]	1.39 ± 0.219[b]
100	480	9.19 ± 0.987[c]	1.32 ± 0.247[c]

*100 metaphases from each donor were scored. Statistically significant differences in relation to the negative control: [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$.

lymphocytes treated with higher concentrations of ATI (50 and 100 μg/ml), we observed statistically significant ($P < 0.005$ and $P < 0.0001$, respectively) increases in frequency of chromosome aberrations (including gaps). Moreover, at the highest dose of ATI (100 μg/ml), the total chromosomal aberrations excluding gaps were significantly ($P < 0.05$) higher as compared with the negative control. The present results indicate that ATI seems to be potentially clastogenic.

In this study, ATI with S9 mix was added to 24 h culture of lymphocytes (S-phase) and mainly chromatid-type aberrations were observed. These facts suggest that *Ascaris* trypsin inhibitor can be classified as S-dependent agent (Preston *et al*. 1987; Natarajan 2002; Bailey and Bedford 2006). The majority of chemical agents produce their maximum clastogenicity in cells that are in the S-phase at the time of treatment, and generally only produce detectable aberrations in cells that pass through an S-phase between treatment and observation at metaphase. In this case chromatid-type aberrations will be produced. The experiment in which a compound was added at the beginning of incubation (G_0 stage of the cell cycle) has been used for cytogenetic testing of S-independent compounds which can produce aberrations at all stages of the cell cycle. Our previous study conducted without exogenous metabolic activation revealed that ATI did not exhibit clastogenic activity following continuous exposure (addition of ATI to PHA-stimulated lymphocytes at the beginning of incubation) (Blaszkowska *et al*. 2004).

For the blood lymphocyte cultures, a determination of the MI inhibition has a practical meaning (Preston *et al*. 1987; Rojas *et al*. 1993; Sari *et al*. 1998; Sivicova and Dianovsky 2006). The MI is interpreted in terms of the cell death or arrest of the cell at any moment during the interphase. The latter case would lead to an increase in the first metaphases along with a delay in the cell cycle affecting the RI values. Our results demonstrated reductions (over 50%) in the MI and RI at the ATI tested concentrations both in the absence and presence of S9 metabolic activation. The significant decrease in the MI values of lymphocytes in the presence of the *Ascaris* trypsin inhibitor suggests that this polypeptide—serine protease inhibitor—delays PHA-induced human lymphocyte transformation *in vitro*. Some investigators (Guadano *et al*. 1998; Sivicova and Dianovsky 2006) suggested that a parallel dose-dependent decrease in the MI and RI indicates not only a cell-cycle delay but also the cytotoxic effects of tested chemicals. To differentiate cytotoxic and/or cytostatic effects of the chemical agent, Rojas *et al*. (1993) suggested using a correlation between the percentage of MI inhibition and RI values. In accordance with the proposed method, a negative slope of the regression line ($r = -0.859; P < 0.01$) was obtained in our present experiment, indicating a cytostatic effect of the ATI. The studies concerning the effect of alpha 1-antitrypsin (alpha trypsin inhibitor /1-AT) on human lymphocyte proliferation demonstrated that *in vitro* 1-AT could inhibit PHA-induced lymphocyte transformation (Bata *et al*. 1977; Baranova *et al*. 1980). The investigations conducted by Breit *et al*. (1983) also reported suppression of the PHA response by purified 1-AT. The addition of increasing amounts of 1-AT into culture medium resulted in an exponential decrease in the response. The results of these studies demonstrated that 1-AT levels of 2 g/l and higher in normal sera would achieve maximal inhibition of lymphocyte activation. It is worth emphasizing that in the available literature, there is no information on the quantitative analysis *in vivo* and *in vitro* of protease inhibitors secreted by parasites, including *Ascaris suum*. The concentrations of ATI used in the present experiments were about 10–80 times lower than 1-AT applied in the study of provide the specific reference. A decrease in mitotic activity of ATI-exposed lymphocytes was confirmed by significant reduction in the MI and RI at all applied concentrations of this inhibitor. Lipski *et al*. (1979) have reported that 1-AT may be expressed on surfaces of concanavalin-A (Con A) transformed human lymphocytes, and evidence has been presented for synthesis of 1-AT by human peripheral blood monocytes (Boldt *et al*. 1982). Other studies also demonstrated that cultivation of peripheral blood lymphocytes with T-cell mitogens (PHA) leads to the appearance of lymphoblasts with membrane-associated 1-AT (Lohrisch *et al*. 1981). Lohrisch and co-workers have shown that unstimulated lymphocytes neither have membrane-associated 1-AT nor possess a binding capacity for it. These data suggest that 1-AT may a play important role in lymphocyte blastogenesis and also immunoregulation.

In the available literature we have found data on the effect of the *Ascaris* extract on lymphocyte subpopulation proliferation both *in vitro* and *in vivo*. It has been widely reported that nematodes or nematode products can stimulate or inhibit the generation of lymphocyte responses. For example *Ascaris suum* contains immunomodulatory factors capable of either stimulating (Sasagawa *et al*. 1987; Lee and Xie 1995) or inhibiting (Soares *et al*. 1992; Ferreira *et al*. 1995) T or B cell responses. Sasagawa and co-workers (1987) demonstrated that *Ascaris* extract stimulates predominantly T-lymphocytes. Moreover, the other investigators have shown that infection with *Ascaris* or the use of its soluble products or extracts at different stages of purification potentiates or suppresses the immune responses (Souza *et al*. 2002; Oshiro *et al*. 2004). Some of the discrepancies and apparent inconsistencies in reports regarding the modulation of proliferative response of human lymphocytes to *Ascaris* extract are undoubtedly due to variation in the profile of the helminths' modulatory factors, methods of isolation and different conditions of cell cultures.

There are limited data available describing genotoxic properties of substances coming from helminths (Shubber and Salin 1987; Bekish and Bekish 2000). Mutagenic activity of different antigens from *Ascaris suum* has been observed by Bekish (1999, 2000, 2001). The author demonstrated that the *Ascaris* compounds (whole *Ascaris*, musculocutaneous sac and cavity fluid) had mutagenic effects resulting in the

number of micronucleus erythrocytes in the mice bone marrow. Extracts from *Ascaris suum* changed the chromosome apparatus of human blood lymphocytes *in vitro* having provoked aneuploid and aberrant cells. On the other hand, it has been found that the nematode (*Trichinella spiralis, Ascaris suum* and *Toxocara canis*) metabolites secreted by migrating larvae in the host organism caused mutagenic effect on the somatic and the generative cells of mice (Bekish and Bekish 2000). In a recent study (Blaszkowska 2008), it has been found that exposure of male germ cells to the chymotrypsin inhibitor isolated from *Ascaris* resulted in the appearance of dominant lethal mutations in spermatids. Moreover, the *Ascaris* serine inhibitors (trypsin and chymotrypsin inhibitor) injected to mice females during organogenesis produced congenital abnormalities (Blaszkowska 1998b, 2001, 2003). In the present study ATI exhibited clastogenic effect in lymphocytes after exposure to higher concentrations of ATI in the presence of S9 micosomal fraction. Its genotoxic effect was demonstrated in the increased SCE frequency.

Considering biochemical properties of *Ascaris* trypsin inhibitor (serine inhibitor), and also its revealed *in vitro* inhibition of lymphocyte divisions, it is likely that the trypsin inhibitor may influence the activity of protein regulators of the cell cycle. The detection of potential clastogenic activity of this inhibitor in the presence of metabolic activation may contribute to explanation of the mechanism of host reproduction disturbances in parasitosis. However, more extensive studies are needed to establish the mechanisms of possible genotoxic activity of the *Ascaris* trypsin inhibitor.

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