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Cell cycle kinetics, apoptosis rates, DNA damage and *TP53* gene expression in bladder cancer cells treated with allyl isothiocyanate (mustard essential oil)

André Luiz Ventura Savio [a,*], Glenda Nicioli da Silva [b], Elaine Aparecida de Camargo [a], Daisy Maria Fávero Salvadori [a]

[a] UNESP—Universidade Estadual Paulista, Faculdade de Medicina de Botucatu, Departamento de Patologia, Botucatu, SP18618-970, Brazil
[b] UFOP – Universidade Federal de Ouro Preto, Escola de Farmácia, Departamento de Análises Clínicas, Ouro Preto, MG35400-000, Brazil

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ABSTRACT

Allyl isothiocyanate (AITC) is present in plants of the cruciferous family and is abundant in mustard seed. Due to its high bioavailability in urine after ingestion, AITC has been considered a promising antineoplastic agent against bladder cancer. Because *TP53* mutations are the most common alterations in bladder cancer cells and are frequently detected in *in situ* carcinomas, in this study, we investigated whether the AITC effects in bladder cancer cells are dependent on the *TP53* status. Two bladder transitional carcinoma cell lines were used: RT4, with wild-type *TP53*; and T24, mutated *TP53* gene. AITC was tested at concentrations of 0.005, 0.0625, 0.0725, 0.0825, 0.0925, 0.125 and 0.25 μM in cytotoxicity, cell and clonogenic survival assays, comet and micronucleus assays and for its effects on cell cycle and apoptosis by flow cytometry and on *TP53* gene expression. The data showed increased primary DNA damage in both cell lines; however, lower concentrations of AITC were able to induce genotoxicity in the mutant cells for the *TP53* gene. Furthermore, the results demonstrated increased apoptosis and necrosis rates in the wild-type cells, but not in mutated *TP53* cells, and cell cycle arrest in the G2 phase for mutated cells after AITC treatment. No significant differences were detected in *TP53* gene expression in the two cell lines. In conclusion, AITC caused cell cycle arrest, increased apoptosis rates and varying genotoxicity dependent on the *TP53* status. However, we cannot rule out the possibility that those differences could reflect other intrinsic genetic alterations in the examined cell lines, which may also carry mutations in genes other than *TP53*. Therefore, further studies using other molecular targets need to be performed to better understand the mechanisms by which AITC may exert its antineoplastic properties against tumor cells.

1. Introduction

Bladder cancer is the fourth most common neoplasm diagnosed in men and the ninth most common in women in the Western world [1]. Approximately 90% of malignant bladder tumors are represented by urothelial cell carcinomas (UCC), which present as small papillae or invasive lesions [2]. Due to the high recurrence rates, progression to muscle invasive disease and increased aggressiveness, bladder cancer is considered a highly morbid disease [3]. The majority of cases are associated with cigarette smoking and occupational exposure to aromatic amines [4]. It has been reported that tobacco metabolites excreted into the urine of smokers are responsible for approximately 50% of bladder tumors. Furthermore, smokers have a four-fold higher incidence of bladder cancer than non-smokers [5].

Therapies for UCC include surgical procedures (partial or radical cystectomy), radio and chemotherapy. However, currently, natural substances found in fruits, vegetables and essential oils have been investigated as alternative approaches for treating diseases such as cancer [6–8]. Allyl isothiocyanate (AITC), known as mustard essential oil, is abundant in mustard seed and wasabi (horseradish) and is a natural compound with chemopreventive potential. An epidemiological study has demonstrated that raw cruciferous vegetables containing AITC may reduce the risk of bladder cancer [9]. In fact, it was shown that approximately 80% of AITC oral doses are selectively delivered to the bladder tissue through urinary excretion and can potently inhibit cancer development and muscle invasion [10,11]. Furthermore, it has been suggested that this compound

* Corresponding author. Tel.: +55 14 38807263; fax: +55 14 38807263.
E-mail address: savio.alv@gmail.co (A.L.V. Savio).

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has promising activity for treating and preventing bladder cancer because of its capacity for interfering with mitosis, increasing ubiquitination and tubulin degradation and inducing apoptosis [12].

TP53 mutations are the most common alterations in bladder cancer cells and are frequently detected in *in situ* carcinomas (CIS) and in advanced disease states [13–15]. These mutations are related to cellular transformation, malignancy and the high recurrence rate of urinary bladder cancers [13,16]. *TP53* is a critical gene in the G1 checkpoint and is evolved in the tumor response to several anticancer drugs [17–19]. Its encoded protein, p53, can activate G1 cell cycle arrest in response to DNA damage, extending the time available for DNA repair before entry into the S phase, and can play an important role in the apoptosis pathways [20,21]. Therefore, *TP53* mutations can affect the p53DNA-binding activity, abolishing the transcriptional activation of *TP53* target genes and apoptosis [22].

Numerous studies have investigated the relationship between p53 and/or *TP53* mutations and the response to antineoplastic drugs, once both protein and gene are related to the DNA damage response pathway [17,18,23]. Recently, it was demonstrated that multiple myeloma cell death caused by the exposure to gemcitabine and clofarabine is p53-dependent [19]. Therefore, to investigate the antineoplastic potential of AITC for bladder carcinoma, we used two cell lines with different *TP53* status: one carrying the wild-type gene (RT4) and the other carrying a mutated *TP53* gene (T24). The cytotoxicity, mutagenicity, apoptosis rates, cell cycle alterations and toxicogenomic activities of AITC were evaluated in the two cell lines.

2. Materials and methods

2.1. Cell lines and test compound

The human urothelial carcinoma cell lines RT4 (from a low grade tumor with the wild type *TP53* gene) and T24 (from an invasive tumor with the *TP53* allele encoding an in-frame deletion of tyrosine 126) were purchased from the Cell Bank of the Federal University of Rio de Janeiro, Brazil, and maintained as previously described by da Silva et al. [24]. Allyl isothiocyanate (AITC) was purchased from Sigma-Aldrich (USA) and was diluted into 2% Tween 20 prior to use. The test compound concentrations were selected based on the data obtained in the clonogenic survival assay. All treatments with AITC were performed for 3 h, as described by Zhang et al. [25].

2.2. Cytotoxicity and cell proliferation

Cytotoxicity and cell proliferation rates were assessed using the Cell Proliferation Kit II (XTT) (ROCHE Diagnostics; Mannheim, Germany). Briefly, cells were seeded into 12-well culture plates (12×10^4 and 8×10^4 cells/well for cytotoxicity and cell survival, respectively). After 24 h, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825, 0.0925, 0.125 and 0.25 μM for 3 h. Untreated cells and cells treated only with Tween 20 were cultured as controls. Three hours after incubation, the cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L·H_2O). After washing, 50 μL of XTT test solution (1 mL XTT labeling solution/20 μL of electron-coupling reagent) was added to each well, and the absorbance was measured at 492 and 690 nm after 90 min (absorbance results are proportional to the percentage of viable cells). For evaluating cytotoxicity and cell proliferation, complete fresh medium was added, and the cells were incubated at 37 °C for 21 and 69 h, respectively. Then, a 50-μL aliquot of XTT solution was added to each well, and the absorbance was measured after 90 min. Both tests were conducted in triplicate.

2.3. Clonogenic survival

A clonogenic assay was used for evaluating the long-term effects of AITC. For clonogenic ability, cells were plated at a density of 1×10^6 cells/25 cm^3 culture flask; 24 h later, they were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 μM for 3 h. Cultures were rinsed with Hank's solution, trypsinized, and approximately 1000 cells were plated into 25-cm^3 culture flasks and allowed to grow for 15 days to form colonies. The cells were Giemsa stained, and the number of colonies with 50 or more cells was counted. The experiments were performed in triplicate.

2.4. Comet assay

Initially, 8×10^4 cells were seeded into 12-well plates for 24 h. Then, cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 μM for 3 h. Methyl methanesulfonate (0.006 M, 5 min, 37 °C; Sigma-Aldrich, Inc.; St. Louis, MO, USA) was used as positive control. The comet assay was conducted based on the methods described in Singh et al. [26] and Tice et al. [27]. Briefly, 10 μL of cells was added to 100 μL of 0.5% low-melting-point agarose at 37 °C. This mixture was layered onto pre-coated slides with 1.5% standard agarose and covered with a coverslip. After agarose solidification at 4 °C, the coverslip was gently removed. Then, the slides were immersed into lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl at pH 10, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) overnight, at 4 °C, which was followed by an incubation step in alkaline buffer (0.3 mM NaOH and 1 mM EDTA; pH > 13) for 20 min, to allow DNA unwind and alkali-labile site expression. Electrophoresis was conducted in the same alkaline buffer at 4 °C, for 20 min, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH 7.5) for 15 min, fixed with absolute ethanol and stored at room temperature until analysis. All steps were conducted in the dark to prevent additional DNA damage. The slides were stained with SYBR Gold (1:10,000; Invitrogen; Grand Island, NY, USA), immediately before analysis. Cell viability was assessed using the trypan blue exclusion test (0.4% trypan blue, Sigma-Aldrich, Inc.; St. Louis, MO, USA) exclusion test (viability was never lower than 90%). A total of 150 randomly selected nucleoids per treatment were analyzed under 400× magnification with a fluorescence microscope connected to an image analysis system (Comet Assay IV, Perceptive Instruments; Suffolk, Haverhill, UK). Tail intensity (% DNA in tail) was used to estimate DNA damage. The slides were prepared in duplicate from three independent experiments.

2.5. Cytokinesis-block micronucleus assay

The MN assay was performed based on the technique described by Fenech [28]. Briefly, 1×10^6 cells were seeded into a dish (100 mm × 20 mm). Twenty-four hours later, cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725, 0.0825 and 0.0925 μM, for 3 h. Then, cytochalasin B (3 μg/mL) was added and the cells were incubated at 37 °C and 5% CO_2 for 28 (T24) or 44 (RT4) hours. At the end of the incubation steps, cells were collected and centrifuged at 800 rpm for 5 min. The supernatant was discarded, and 5 mL of ice-cold hypotonic solution (0.075 M KCL) was added. After cell fixation, the slides were stained with 5% Giemsa solution and analyzed under a light microscope at 1000× magnification. One thousand binucleated cells were analyzed in each slide. Doxorubicin was used as a positive control at a concentration of 0.4 μg/mL for 2 h, and all treatments were performed in triplicate.

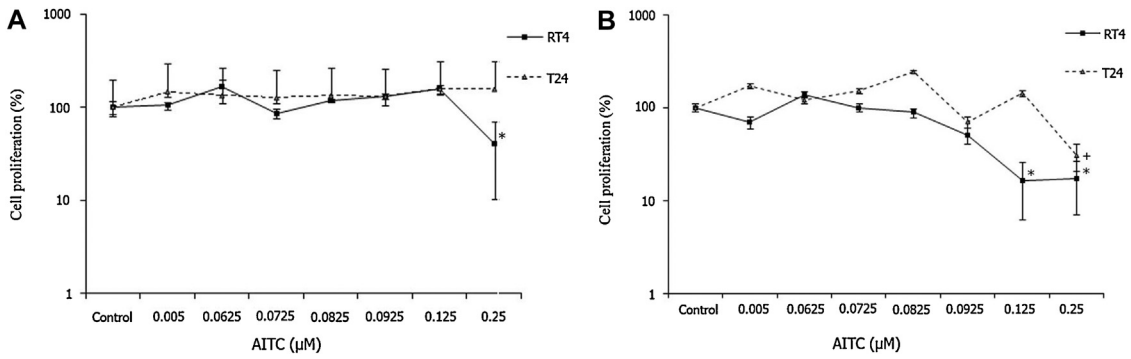

Fig. 1. Percentages of proliferation (logarithmic scale) in RT4 and T24 cell lines immediately (A) and three days (B) after treatment with allyl isothiocyanate (AITC). * $p < 0.05$ in relation to the AITC vehicle control (2% Tween 20%). Each point represents the mean value obtained from three independent experiments.

2.6. Cell cycle kinetics and apoptosis detection

For cell cycle kinetics, 2×10^5 cells were seeded into 12-well plates; 24 h later, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725 and 0.0825 μM for 3 h and then washed with Hank's solution. Fresh medium was added, and the cells were incubated again at 37 °C for 21 h. Afterwards, cells were detached using trypsin-EDTA, resuspended into fresh medium and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 200 μL of HFS (50 μg/mL propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100), placed on ice and protected from the light for at least 30 min. The percentage of cells in the G0/G1, S and G2/M phases were measured using GUAVA Cytosoft version 4.2.1 software. The cell cycle analyses were performed in triplicate.

A quantitative assessment for apoptosis was performed using a Guava Annexin reagent (Merck Millipore). Annexin V was used for detecting the externalization of phosphatidylserine to the cell surface and 7-AAD as an indicator of cell membrane structural integrity. Briefly, 2×10^5 cells were seeded into 12-well culture plates. After 24 h, the cells were treated with AITC at concentrations of 0.005, 0.0625, 0.0725 and 0.0825 μM for 3 h. Afterwards, cells were washed with Hank's solution, and fresh medium was added. Untreated cells and cells treated only with Tween 20 were cultured as controls. Cells were collected 21 and 45 h after incubation, resuspended into 100 μL Guava Nexin reagent for 20 min in the dark and immediately analyzed in the Guava easyCyte flow cytometer (Millipore) using the Guava System software. Data from 5000 cells were collected in each data file. Cellular status was defined as follows: unstained cells were classified as 'alive'; cells stained only by annexin V were classified as 'early apoptotic'; cells stained by both annexin V and 7-AAD were classified as 'late apoptotic'; and cells stained only by 7-AAD were classified as 'dead'.

2.7. TP53 expression

Total RNA from the cultured T24 and RT4 cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using 6 μL of random hexamer primers (10×), 6 μL of reaction buffer (10×), 2.5 μL of dNTPs (25×) and 3 μL of MultiScribe (50 U/mL, High Capacity; Applied Biosystems). After incubation at 25 °C for 10 min and 37 °C for 2 h, the cDNA was stored at 4 °C and at −20 °C. Differential expression of *TP53* in the two cell lines was assayed using the TaqMan system (Applied Biosystems; Foster City, CA, USA). Each tube contained 2 μL of cDNA template, 5 μL of Master Mix TaqMan 2× (Applied Biosystems) and 0.5 μL of 20× primers/probe (Assays-on-Demand gene expression products; Applied Biosystems). β-Actin was used as a housekeeping gene. The reaction was performed using the following thermal cycler conditions: 94 °C for 10 min followed by 40 cycles at 94 °C for 30 s and 60 °C for 1 min. Fluorescence data were collected during each annealing/extension step. The reactions were performed using an Applied Biosystems 7500 FAST Real-Time PCR System and SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems). For every PCR sample, a negative (no template) control was processed as a routine control. Assays were performed in triplicate. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method [29].

2.8. Statistical analysis

Statistical analyses were performed using SAS software, v.9.2 (Statistical Analysis System, SAS Institute; Cary, NC, USA). For the cytotoxicity, comet and cell proliferation assays, data were analyzed using ANOVA and Tukey's test; for the cell proliferation assay (nonparametric distribution—T24 cells), the analysis was performed using a gamma distribution followed by a multiple comparisons test using the DIFF option of the GENMOD procedure; for clonogenic survival and apoptosis and cell cycle analyses, a Poisson distribution and a factorial analysis based on a binomial distribution were performed, respectively; for the micronucleus test, data were analyzed using the Poisson (RT4 cells) and binomial (T24 cells) distributions. Gene expression values were analyzed by one-way ANOVA followed by Tukey's test. The results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Cytotoxicity, cell proliferation and clonogenic survival assays

The data revealed a significant decrease of cell proliferation only in RT4 cells 3 h after treatment with 0.25 μM AITC (Fig. 1A). In contrast, significant decreases were detected for both RT4 (0.125 and 0.25 μM AITC) and T24 (0.25 μM) cells three days after treatments (Fig. 1B). No cytotoxicity was visualized 24 after treatments in either cell line (Fig. 2). The clonogenic survival assay revealed significant decreases in cell colonies (RT4 and T24) after AITC treatment at concentrations of 0.0625, 0.0725, 0.0825 and 0.0925 μM (Fig. 3).

3.2. Comet and micronucleus assays

All concentrations of AITC increased primary DNA damage in T24 cells; in RT4 cells, increases were observed for the three highest concentrations (0.0725, 0.0825 and 0.0925 μM) (Table 1). No significant differences were detected in the frequency of micronucleus between negative control and treated cells (Table 2).

Table 1
DNA damage (tail intensity) in bladder carcinoma cell-lines (RT4 and T24) treated with allyl isothiocyanate (AITC).

Cell line	Negative control[a]	Control Tween[b]	Positive control[c]	AITC (μM)				
				0.005	0.0625	0.0725	0.0825	0.0925
RT4	14.462 ± 1.024	12.055 ± 1.229	80.361 ± 6.127*	18.085 ± 3.118	19.871 ± 2.018	24.923 ± 6.330*	27.400 ± 3.264*	30.472 ± 3.820*
T24	5.807 ± 1.800	5.063 ± 0.218	89.430b ± 3.906*	14.807 ± 1.900*	13.903 ± 1.790*	15.767 ± 1.825*	13.933 ± 2.268*	15.557 ± 0.659*

[a] No treatment.
[b] Cells treated with 2% Tween 20 (vehicle control).
[c] Cells treated with methyl methane sulfonate (0.006 M).
* $p < 0.05$ compared to the Tween control.

Table 2
Frequencies of micronucleated (‰ MNC) RT4 and T24 cells after treatment with allyl isothiocyanate (AITC).

Cell line	Negative control[a]	Control Tween[b]	Positive control[c]	AITC (μM)				
				0.005	0.0625	0.0725	0.0825	0.0925
T24	0.08	0.05	0.38*	0.16	0.10	0.16	0.10	0.10
RT4	0	0	0.30*	0	0	0	0	0.03

[a] No treatment.
[b] Cells treated with 2% Tween 20 (vehicle control).
[c] Cells treated with doxorubicin (0.4 μg/mL). The values are expressed in ‰ MNC (frequency of micronucleated cells/1000 cells analyzed).
* $p < 0.05$ compared to the Tween control.

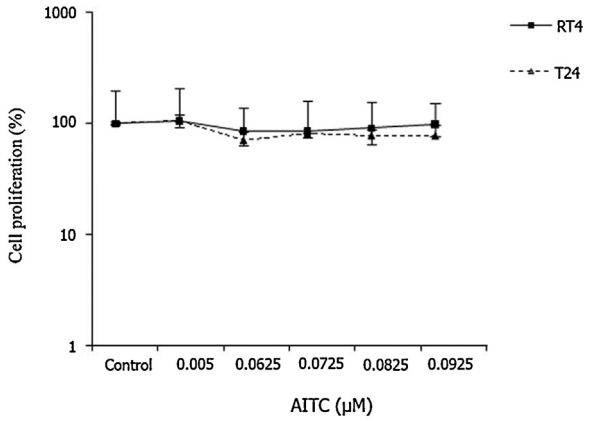

Fig. 2. Percentages of proliferation (logarithmic scale) in RT4 and T24 cell lines 24 h after treatment with allyl isothiocyanate (AITC). * $p < 0.05$ in relation to the AITC vehicle control (Tween 20%). Each point represents the mean value obtained from three independent experiments.

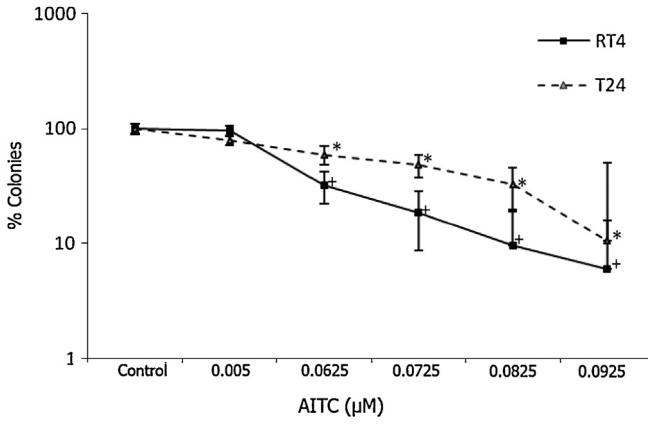

Fig. 3. Percentages of cell colonies (logarithmic scale) 10 (T24 cells) and 15 (RT4 cells) days after treatment with allyl isothiocyanate (AITC). $p < 0.05$ (*RT4; +T24). Each point represents the mean value obtained from three independent experiments.

3.3. Cell cycle analysis

Significant decreases in the numbers of RT4 cells in the S phase were detected after AITC treatment at 0.005, 0.0625, 0.0725 and 0.0825 μM. Moreover, a slight increase, but not significant, in G1 phase cells was also observed. For T24 cells, significant decreases ($p < 0.05$) in the numbers of cells in the G1 phase (0.0725 and 0.0825 μM) and the S phase (0.0625 and 0.0825 μM) accompanied by an increased number of cells in the G2 phase were detected, suggesting G2/M cell cycle arrest (Table 3).

3.4. Apoptosis

Increased necrosis (0.0625, 0.0725, 0.0825 and 0.0925 μM) and early apoptosis (0.0625, 0.0725, 0.0825 and 0.0925 μM) rates were observed in RT4 cells 48 h after treatment with AITC. For T24 cells, an increased necrosis rate was detected only 24 h after treatment with AITC at 0.0625 μM (Table 4).

Table 3
Cell cycle kinetics in bladder carcinoma cell lines (RT4 and T24) treated with allyl isothiocyanate (AITC).

		RT4	T24
G1 (%)	Control	40.90 ± 8.84	48.07 ± 1.25
	Tween 20	51.41 ± 12.30	49.38 ± 0.68
	0.005 μM	58.58 ± 6.91	42.29 ± 3.89
	0.0625 μM	59.00 ± 3.02	42.34 ± 5.95
	0.0725 μM	40.57 ± 3.09	30.47 ± 4.47*
	0.0825 μM	39.10 ± 2.37	14.94 ± 2.82*
S (%)	Control	22.75 ± 17.80	18.22 ± 1.23
	Tween 20	26.14 ± 25.75	22.70 ± 1.30
	0.005 μM	8.75 ± 1.72*	23.05 ± 1.73
	0.0625 μM	5.03 ± 0.82*	13.80 ± 1.44*
	0.0725 μM	8.05 ± 2.12*	16.89 ± 3.58
	0.0825 μM	6.24 ± 1.50*	5.72 ± 2.16*
G2 (%)	Control	24.94 ± 1.06	24.89 ± 1.97
	Tween 20	20.88 ± 7.48	21.14 ± 4.60
	0.005 μM	21.87 ± 2.09	27.96 ± 4.88
	0.0625 μM	17.28 ± 1.83	34.48 ± 5.81*
	0.0725 μM	21.89 ± 7.54	39.44 ± 13.49*
	0.0825 μM	18.64 ± 3.97	64.38 ± 0.70*

Percentage of cells in the G1, S and G2/M mitotic phases are expressed as the mean ± standard deviation from triplicate.
* $p < 0.05$ compared to the Tween control.

Table 4
Early apoptosis, late apoptosis and necrosis rates (%) in RT4 and T24 cells treated with allyl isothiocyanate (AITC).

	AITC	RT4		T24	
		24 h	48 h	24 h	48 h
Necrosis (%)	Control	11 ± 2	14 ± 1	2 ± 0	2 ± 1
	Tween	9 ± 1	24 ± 1	1 ± 0	3 ± 2
	0.005 μM	5 ± 1	24 ± 4	4 ± 1	2 ± 0
	0.0625 μM	11 ± 1	11 ± 3*	5 ± 0*	2 ± 0
	0.0725 μM	11 ± 2	6 ± 0,5*	4 ± 1	2 ± 0
	0.0825 μM	10 ± 1	8 ± 1*	4 ± 0	2 ± 0
	0.0925 μM	7 ± 1	6 ± 1*	4 ± 2	2 ± 2
Late apoptosis (%)	Control	22 ± 4	17 ± 2	8 ± 1	12 ± 3
	Tween	19 ± 4	9 ± 2	14 ± 1	12 ± 4
	0.005 μM	7 ± 1*	7 ± 2	15 ± 2	15 ± 1
	0.0625 μM	18 ± 1	18 ± 1*	29 ± 1*	18 ± 2
	0.0725 μM	34 ± 11*	35 ± 2*	20 ± 5*	26 ± 1*
	0.0825 μM	20 ± 0	15 ± 1*	19 ± 1	21 ± 8*
	0.0925 μM	18 ± 3	43 ± 0*	23 ± 2*	19 ± 2*
Early apoptosis (%)	Control	1 ± 0	5 ± 4	0 ± 0	5 ± 3
	Tween	6 ± 2	6 ± 1	1 ± 0	7 ± 1
	0.005 μM	3 ± 1	7 ± 3	1 ± 0	8 ± 2
	0.0625 μM	3 ± 0	10 ± 2*	1 ± 0	9 ± 1
	0.0725 μM	4 ± 0	13 ± 2*	1 ± 0	11 ± 2
	0.0825 μM	2 ± 0*	14 ± 2*	1 ± 0	10 ± 1
	0.0925 μM	3 ± 0	10 ± 3*	1 ± 0	12 ± 2

Percentage of cells in necrosis, late apoptosis and early apoptosis are expressed as mean ± standard deviation obtained from three independent experiments using the Annexin V assay (flow cytometry).
* $p < 0.05$ compared to the Tween control.

3.5. *TP53* expression

The data showed no significant differences for *TP53* mRNA expression after treatment with AITC in either cell line (Fig. 4).

4. Discussion

Epidemiological studies have shown that cruciferous vegetables may act on lung, breast, prostate, pancreas and bladder cancers, mainly because of their high concentrations of isothiocyanates (ITCs) [30]. Each vegetable may have one different ITC: for instance, phenethyl isothiocyanate (PEITC), derived from gluconasturtiin hydrolysis, is found in wasabi and watercress; benzyl isothiocyanate (BITC), derived from glucotropaeolin hydrolysis, is found in cabbage; and the allyl isothiocyanate (AITC), which is derived from sigrina hydrolysis, is found in mustard and horseradish [31]. ITCs may act by inhibiting cytochrome P450 isoforms, which

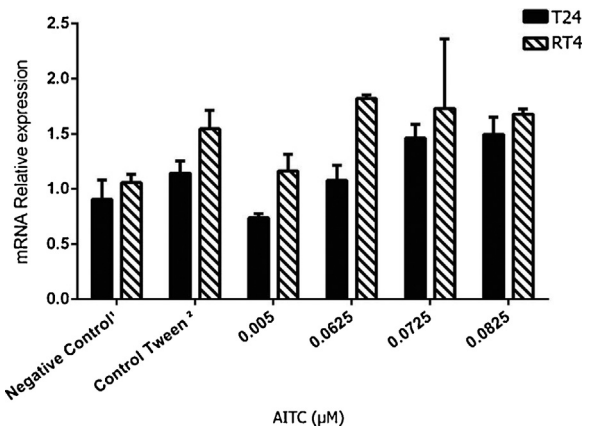

Fig. 4. Relative *TP53* mRNA levels in RT4 and T24 cell lines after treatment with allyl isothiocyanate (AITC). The relative expression values correspond to fold-change values. [1] No treatment; [2] cells treated with 2% Tween 20 (vehicle control); β-actin (endogenous RNA control).

modulate phase II enzymes and prevent DNA adducts caused by some carcinogens [30]. Mi [32] has observed significant inhibition of the proteasome activity in several cell lines, including cervical (HeLa), lung (A549), colon (HT29) and breast cancer (MCF-7) cells treated with BITC and PEITC. Additionally, this same author demonstrated that these compounds cause rapid accumulation of p53 and NF-kB (IkB) inhibition, apoptosis and G2/M phase arrest [32]. *In vitro* growth inhibition of UM-UC3 human bladder carcinoma cells was detected after treatment with AITC [33]. Furthermore, AITC is markedly less toxic to normal cells than to cancer cells, suggesting its selective activity [6,34]. The high bioavailability of AITC in urine has been suggested as an important feature for bladder cancer therapy [11]. In fact, some authors have described higher levels of AITC in urinary bladder tissues than in other organs after administration [33,35].

Therefore, based on the chemotherapeutic potential of AITC and considering the fact that chemotherapy exposure induces important adverse effects such as high systemic toxicity, lack of selectivity, and collaborate to resistance of these tumors after prolonged treatment, we tested the effect of this compound in wild-type and mutated *TP53* gene cells. The cellular response due to *TP53* mutations after different treatments is poorly understood, as the response depends on a complex signaling cascade. Hofseth et al. [36] reported that a mutant p53 impaired the DNA damage response and rendered the tumor cells more resistant to drug-induced apoptosis. In this way, it has been already shown that a functional *TP53*/p53 pathway improves gemcitabine cytotoxicity [37]. Moreover, a previous study from our group demonstrated higher apoptosis rates in wild-type cells compared to mutant cells for the *TP53* gene after cisplatin and gemcitabine treatments [24].

Herein, we observed that AITC induced decreased cell proliferation three days after treatment at the highest concentrations and no cytotoxicity 24 h after treatment in both cell lines. These results provide evidence that the AITC concentrations used led to the loss of reproductive integrity, *i.e.*, decreased cell proliferation capacity, most likely because of sustained lethal damage. In contrast, some authors have reported cytotoxic effects of AITC (percentages of viable cells) in human brain malignant glioma cells (GBM 8401), though at concentrations higher that 1 μM [38]. It is important to remember that the clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony. This assay is widely used to examine the effects of agents with potential application in the clinic [39]. Therefore, the low survival observed in this assay could merely be the control of the lysis rates for cells that had already lost their reproductive potential at 24 h and/or three days [40]). Our present study also revealed increased amounts of primary DNA damage (comet assay) in both wild-type and mutant cells for the *TP53* gene treated with AITC. Interestingly, in mutated cells, even the two lowest concentrations of AITC were able to induce significant damage. Therefore, it seems that the *TP53* background must be considered with regard to the genotoxic potential of AITC. Nevertheless, although AITC was able to reach DNA, no mutagenic effect was detected by the micronucleus assay. These findings suggest that AITC, although genotoxic, is not aneugenic or clastogenic in the two cell lines. Similar results were previously reported by Lamy et al. [41] testing other ITCs. MTPITC, MTBITC and MTPeITC were genotoxic, but not mutagenic, in HepG2 cells. Additionally, it has been reported that AITC is unable to induce either chromosome aberrations or sister chromatid exchanges, even at highly cytotoxic doses [42]. The authors suggested that either the DNA was efficiently repaired or factors that trigger apoptosis might be active. Actually, several molecular events are involved in DNA repair.

Regarding the toxicogenomic effect of AITC, our results revealed no change in *TP53* gene expression in either cell line. Recently, it was demonstrated that PEITC might deplete mutant p53 without

causing changes in p53 mRNA expression [43]. One explanation for this finding was a possible mechanism involving protein modification *via* covalent binding to the ITC functional group, making the mutant p53 cells significantly more sensitive to PEITC-induced apoptosis than the wild-type cells. Similarly, we suggest that AITC can act at translational and/or post-translational levels, as we observed increased early apoptosis rates in the wild-type cells but not in mutant cells for the *TP53* gene, suggesting that AITC-induced DNA damage triggers apoptosis, most likely through p53 pathways. Kumar et al. [44] have discussed that decreased *BCL-2* and increased *BAX* expression, as well as CAD (caspase-activated DNase) activation by caspase-3, might also explain AITC induced-apoptosis.

When the effect of AITC on the cell cycle was analyzed, we detected activities dependent on the *TP53* status. While a decreased number of cells undergoing the S-phase was observed for the wild-type *TP53* cells, a significant decrease in the number of cells at G1 and S phases was found for the mutant cells, in parallel with an increased percentage of cells at G2/M phase (G2/M arrest). G2/M cell cycle arrest was also observed in human glioma cells after treatment with AITC [39]. Moreover, AITC is able to bind to cysteine residues and α- and β-tubulins in a bladder cancer cell line, promoting their degradation and ubiquitination and inducing cell cycle arrest in mitosis [12]. Studies using colon and prostate cancer cell lines treated with sulforaphane (which also belongs to the isothiocyanate family) demonstrated a G2/M cell cycle arrest, loss of *Bcl-2* gene expression and increased caspase activity [45,46].

In conclusion, AITC caused cell cycle arrest and increased apoptosis rates and genotoxicity dependent on the *TP53* status. However, we cannot rule out the possibility that these differences could reflect other intrinsic genetic alterations in the examined cell lines, which may also carry mutations in genes other than *TP53*. Therefore, further studies using other molecular targets need to be performed to better understand the mechanisms by which AITC may exert its antineoplastic properties against tumor cells.

Author contributions

All of the authors reviewed the manuscript. ALVS conducted all of the experiments, interpreted the data and wrote the manuscript. GNS suggested the experimental design, conducted the flow cytometry experiments and interpreted the data. EAC performed the flow cytometry experiments, micronucleus test and comet assay. DMFS was the advisor, contributed to the experimental design and data interpretation as well as critically read the manuscript.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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