CLINICS

CLINICAL SCIENCE

Prima-1 induces apoptosis in bladder cancer cell lines by activating p53

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OBJECTIVES: Bladder cancer represents 3% of all carcinomas in the Brazilian population and ranks second in incidence among urological tumors, after prostate cancer. The loss of p53 function is the main genetic alteration related to the development of high-grade muscle-invasive disease. Prima-1 is a small molecule that restores tumor suppressor function to mutant p53 and induces cancer cell death in various cancer types. Our aim was to investigate the ability of Prima-1 to induce apoptosis after DNA damage in bladder cancer cell lines.

METHOD: The therapeutic effect of Prima-1 was studied in two bladder cancer cell lines: T24, which is characterized by a p53 mutation, and RT4, which is the wild-type for the p53 gene. Morphological features of apoptosis induced by p53, including mitochondrial membrane potential changes and the expression of thirteen genes involved in apoptosis, were assessed by microscopic observation and quantitative real-time PCR (qRT-PCR).

RESULTS: Prima-1 was able to reactivate p53 function in the T24 (p53 mt) bladder cancer cell line and promote apoptosis via the induction of Bax and Puma expression, activation of the caspase cascade and disruption of the mitochondrial membrane in a BAK-independent manner.

CONCLUSION: Prima-1 is able to restore the transcriptional activity of p53. Experimental studies in vivo may be conducted to test this molecule as a new therapeutic agent for urothelial carcinomas of the bladder, which characteristically harbor p53 mutations.

KEYWORDS: Bladder cancer; p53; Apoptosis; Prima-1.

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INTRODUCTION

Bladder cancer (BC) represents 3% of all carcinomas in the Brazilian male population and ranks second in incidence among urological tumors, after prostate cancer (1), with 73,310 new cases and 14,880 deaths estimated for 2012 in the USA (2).

Tobacco smoking, occupational exposure to aromatic amines, arsenic, radiation exposure from the radiation therapy of neighboring organs and the therapeutic use of alkylating agents are the main risk factors for the development of urothelial tumors (3).

A large number of genetic changes have been associated with the genesis and progression of BC, and many of them appear to abrogate the G1/S cell cycle checkpoint (4). In particular, p53 loss of function has been related to the development of high-grade muscle-invasive disease (5).

As a key gatekeeper of the G1/S checkpoint for cell-cycle progression, p53 is indispensable for maintaining genomic stability and keeping urothelial growth in check. Structural and functional p53 defects are found in over one-half of human urothelial carcinomas, contributing to genomic instability and consequently, numerous chromosomal aberrations. p53 mutations first affect one allele, followed by a second that may be a mutation, deletion or silencing by methylation of the additional wild-type allele, which disables the function of this tumor suppressor gene (6). Consistent with this action, the expression of p21 (WAF1), an important p53 downstream target, is downregulated in the majority of urothelial carcinomas that harbor p53 mutations (7). Several codons in the p53 gene appear to be preferentially mutated, including codons 280 and 285 (8). The specific mutation of these codons is rare in other epithelial tumors; thus, it has been suggested that they result from a urothelium-specific carcinogen or carcinogenic event.

Prima-1 (p53 reactivation and induction of massive apoptosis 1) is a low–molecular weight compound identified

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in a cell-based chemical library screen at the National Cancer Institute (National Institutes of Health, Bethesda, MD). This compound has been demonstrated to suppress the growth of cells that harbor mutant p53 by restoring the sequence-specific DNA-binding, wild-type conformation and transcriptional transactivation of mutant p53. Prima-1 has been shown to induce apoptosis in human tumor cells in a p53-dependent manner and suppress the growth of human tumor xenografts carrying mutant p53 (9).

In this study, we examined the effects of Prima-1 on the T24 and RT4 BC cell lines, which express a mutant and wild-type p53 gene, respectively, and analyzed whether this compound was able to induce p53 activity after UVC damage. To test this hypothesis, we evaluated whether exposing p53 wild-type (RT4) and p53 mutant (T24) cells to Prima-1 and ultraviolet radiation (UVC) alters the expression of p53 downstream genes that are related to apoptosis induction.

MATERIALS AND METHODS

Cell Lines and Treatment

The human urothelial carcinoma T24 (p53 mt) cell line was kindly provided by Dr. Salvadori (Botucatu, Brazil). RT4, a primary urothelial carcinoma bladder cell line from a Caucasian (p53 wt) was purchased from EACC (Salisbury, UK). The cell lines were cultured in McCoy's medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Sigma, St.Louis, MO, USA) under an atmosphere of 5% CO_2 at 37℃. For the experiments, T24 and RT4 cells were seeded in 25 cm^2 tissue culture flasks and maintained at 37℃/5% CO_2 overnight to allow the cells to adhere. On the next day, the cells were exposed to 253.7 nm of UVC for 2 h to induce DNA damage. UVC radiation causes specific DNA lesions, and in addition to being a powerful tool for scientific studies, it is a methodology that is easily implemented in the laboratory because the majority of germicidal lamps emit UVC light (10). After exposure, the cells were treated with 50 μM Prima-1 (Sigma, St.Louis, MO, USA) for 1 and 192 h.

RNA Isolation and cDNA Synthesis

Following the above experiment, total RNA was isolated with the *mir*Vana™ Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. As a control, we used T24 and RT4 cell lines cultured in McCoy's medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) in a 5% CO_2 atmosphere at 37℃. RNA concentrations were determined by measuring the absorbance at 260/280 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were incubated at 25℃ for 10 min, followed by 37℃ for 120 min and 85℃ for 5 min. The cDNA was stored at −20℃ until use.

Quantitative Real-Time PCR (qRT-PCR) and Gene Expression

The expression level of 13 genes (Table 1) was analyzed by qRT-PCR using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Target sequences were amplified in a 10 μl reaction containing 5 μl of TaqMan Universal PCR Master Mix, 0.5 μl of a TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) (primers and probes, see Table I), 1 μl of cDNA and 3.5 μl of DNase-free water. The PCR cycling conditions were as follows: 2 min at 50℃, 10 min at 95℃ and 40 cycles of 15 seconds at 95℃ and 1 min at 60℃. A TaqMan B2M (Applied Biosystems, Foster City, CA, USA) (β2 microglobulin) Assay was used as an endogenous control.

We used the ΔΔCT method to calculate the relative expression of the 13 target genes using the following formula: ΔΔCT = (CT of the target gene in T24 and RT4 cells after 1, 18 and 192 h after exposure to 50 μM Prima-1 – the CT of the endogenous control under the same conditions) – (CT of the target gene in T24 and RT4 cells without Prima-1 treatment - CT of the endogenous control under the same conditions). The gene expression fold change was calculated as $2^{-\Delta\Delta CT}$.

Evaluation of the mitochondrial membrane potential by DePsipher

The DePsipher Kit (Trevigen, Gaithersburg, MD, USA) was used to measure the mitochondrial membrane potential under a fluorescence microscope (11,12). T24 and RT4 cells (2×10^5/ml) were seeded in a 24 well plate 24 h prior to the experiments. Then, the plate was exposed to 253.7 nm of UVC for 2 h for the induction of DNA damage. Prima-1 (50 μM) was added, and 18 h later, the cells were washed with PBS and detached from the cell culture wells with trypsin. As a control, we used T24 and RT4 cell lines exposed to UVC for 2 h in the absence of treatment. The

Table 1 - Assay identifiers (IDs) used for qRT-PCR.

Gene symbol	Gene name	Assays IDs
CASP2	caspase 2, apoptosis-related cysteine peptidase	Hs00234982_m1
CASP3	caspase 3, apoptosis-related cysteine peptidase	Hs00234385_m1
CASP7	caspase 7, apoptosis-related cysteine peptidase	Hs00169152_m1
CASP9	caspase 9, apoptosis-related cysteine peptidase	Hs00964603_m1
BBC3 (PUMA)	BCL2 binding component 3	Hs00248075_m1
BAK1	BCL2-antagonist/killer 1	Hs00940249_m1
BAX	BCL2-associated X protein	Hs00180269_m1
MDM2	Mdm2 p53 binding protein homolog	Hs01066930_m1
PMAIP1 (NOXA)	phorbol-12-myristate-13-acetate-induced protein 1	Hs00382168_m1
B2M	β2-microglobulin	Hs99999907_m1
CDKN1A (p21)	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Hs00355782_m1
TP53	tumor protein p53	Hs01034249_m1
APAF1	apoptotic peptidase activating factor 1	Hs00559441_m1
CYC1 (cytochrome-c)	cytochrome c-1	Hs00357717_m1

cells were incubated in the dark with 5 μg/ml DePsipher (5.5′.6.6′-tetrachloro-1.1′.3.3′-tetraethyl-benzimidazolyl carbocyanin iodide) solution for 30 min at 37℃, washed with reaction buffer with stabilizer, placed on a glass slide and covered with a glass coverslip. The stained cells were observed under an inverted fluorescence microscope using a fluorescein long-pass filter (fluorescein and rhodamine). DePsipher visualizes the potential-dependent accumulation in mitochondria, which is indicated by a fluorescence emission shift from red (590 nm) to green (530 nm).

Apoptosis analysis

Following the conditions previously reported, the morphological aspects of the cells were analyzed and photographed under a microscope.

Ethics

This project was submitted to the research ethics committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and approved at the August 5th, 2009 meeting (Protocol No. 0718/09).

RESULTS

Gene Expression Profile

The gene expression profiles after Prima-1 exposure to both cell lines are presented in Figure 1.

Prima-1 and Apoptosis

Pmaip 1 and Bbc3 are important genes induced by p53 that transcribe Noxa and Puma, respectively, and are essential for apoptosis induction. Noxa was found to be induced at 0 and 1 h in the T24 cells, while Noxa expression was detected at 1 and 18 h in the RT4 cells. Puma was not detected in RT4 cells but was overexpressed in T24 cells at 18 h.

Bak and Bax oligomerize and alter the permeability of mitochondrial membranes by facilitating the release of cytochrome c to activate caspases. In our model, only Bax was expressed by the T24 cells after 18 h, while Bak was not expressed. However, Bak was expressed in the p53 wt RT4 cells, while Bax was not expressed.

Our analysis revealed that caspase 9, Apaf-1 and cytochrome-c, which are involved in the activation of the caspase cascade, were progressively overexpressed in the RT4 cells; however, in the T24 cells, these genes were more expressed at 0 h and upregulated at 1 h; an even greater increase was observed at 18 h. Caspase 2 is considered an orphan caspase and thought to be an additional initiating caspase. We observed progressive caspase 2 overexpression in the T24 and RT4 cells. An analysis of the effector caspases 7 and 3 also demonstrated a progressive upregulation in both cell lines, with a peak at 18 h.

Prima-1 and p53 Activation

To demonstrate that the apoptosis induction was related to the reestablishment of p53 transcriptional activation, we evaluated the expression of MDM2 and p21, which are important p53-regulated genes. We found that p21 was expressed in RT4 cells at low levels compared with T24 cells after Prima-1 treatment, and the same pattern of expression was noted for MDM2.

An evaluation of p53 expression in cell lines exposed to UVC and Prima-1 revealed that while p53 was overexpressed immediately after exposure in the p53 wt RT4 cell line, the same expression level was detected after 18 h in the p53 mt T24 cell line.

Evaluation of the Mitochondrial Membrane Potential with DePsipher

In T24 cells treated with 50 μM Prima-1, there was a disruption in the $\Delta\Psi m$ that was similar to what was observed in RT4 cells exposed to UVC. This result was different from that found for T24 cells that did not exhibit a disruption in $\Delta\Psi m$ in the absence of Prima-1 (Figure 2).

Morphological analysis

After a 2 h of exposure to UVC radiation followed by 192 h of 50 μM Prima-1 treatment, we observed similar morphological changes in the T24 and RT4 cell lines (Figure 3).

DISCUSSION

In this study, we showed that Prima-1 induces apoptosis in a p53 mutant bladder cancer cell line by reestablishing p53 transcriptional activity.

Noxa was induced at 0 and 1 h in T24 cells, while in RT4 cells, Noxa expression was detected at 1 and 18 h. Puma was not detected in RT4 cells but was overexpressed in T24 cells at 18 h. Bak and Bax oligomerize, altering the permeability of the mitochondrial membrane by facilitating the release of cytochrome c and activating caspases. In our model, only Bax was expressed by T24 cells after 18 h, and Bak was not expressed at any time after treatment. In contrast, Bak was expressed by the p53 wt RT4 cells, while Bax was not expressed. A recent study showed that p53 participates in the intrinsic cell death pathway via the induction of Bax, Puma and Noxa (13). Thus, we can argue that in our model, in which T24 cells were exposed to UVC and Prima-1, intrinsic apoptosis pathway induction was promoted by the restoration of the p53 transcriptional function and the induction of Noxa, Puma and Bax expression.

Noxa and Puma are proteins transcribed by Pmaip 1 and Bbc3, respectively, which are important p53-induced genes that are considered essential mediators of the apoptotic arm of the p53 pathway (14,15).

In studies with knockout mice, some authors have demonstrated that Puma plays an important role in p53-mediated apoptosis in a wide range of cell types (15-17), while in this model of apoptosis, the role of Noxa is restricted to fibroblasts (18). The dramatic effect that the loss of Puma has on the sensitivity of different cell types in relation to p53-mediated cell death is particularly revealing (16,19), indicating that Puma is a crucial mediator of apoptosis in response to p53. Lomonosova et al. (20) demonstrated that other BH3 proteins along with p53, such as Puma, could overcome the dependence on Bax and Bak in mediating cell death. Thus, an explanation for our data may be that we observed the expression of Puma but not Bax and that the isolated expression of Bak, which was independent of Bax, is sufficient to induce apoptosis. In contrast, in the p53 wt RT4 cell line, we observed the occurrence of apoptosis independent of Puma expression. A difference in the described mechanism of apoptosis induction in this cell line has been described for cisplatin, which, along with the phosphorylation and stabilization of p53, activates the expression of Fas, Puma and caspase-10 (21).

A critical event for apoptosome assembly is the release of cytochrome c from the mitochondria, a step tightly regulated by members of the B cell lymphoma 2 (Bcl-2)

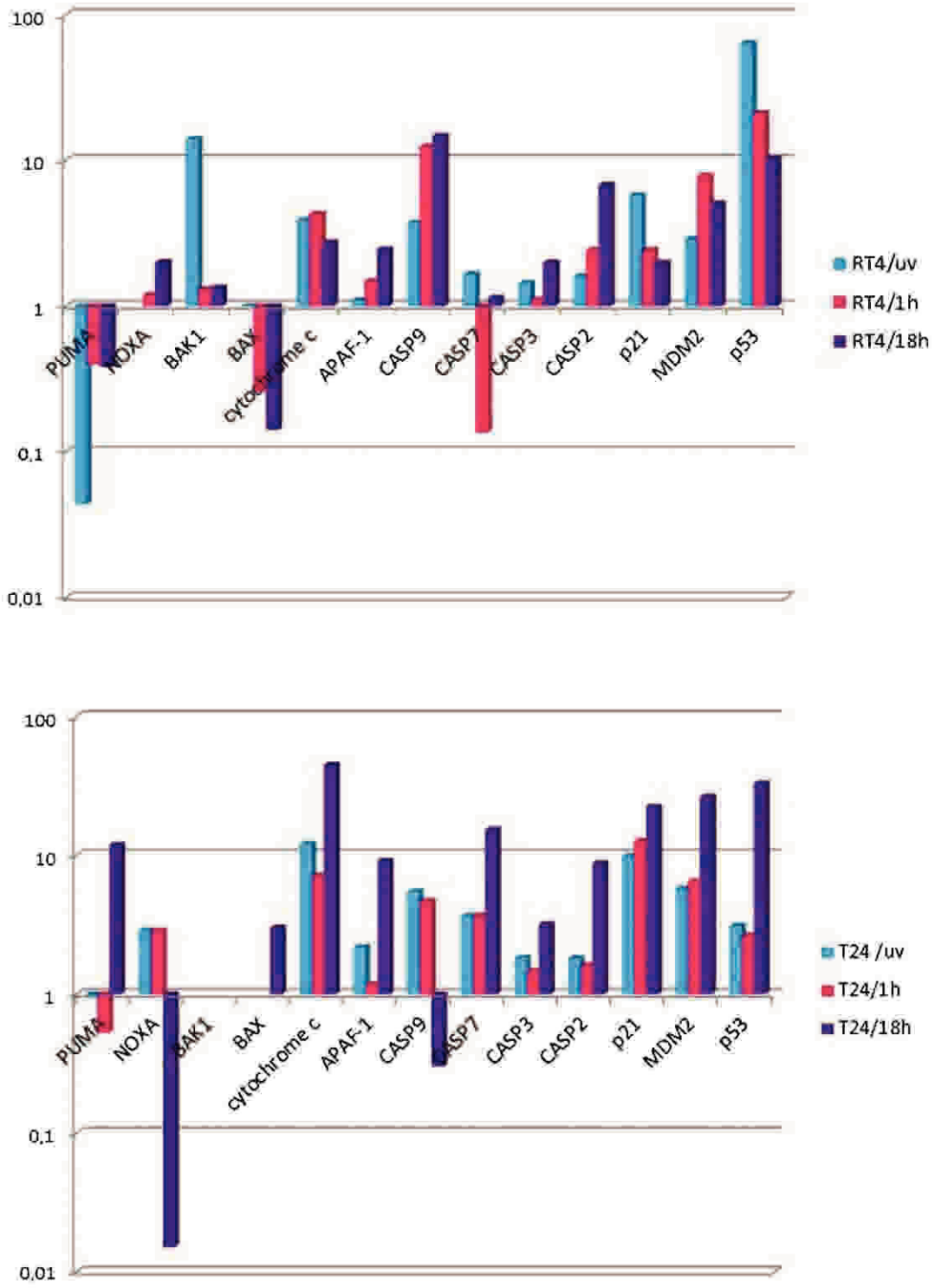

Figure 1 - Profile of MDM2, Puma, Bax, Noxa, Casp9, Casp7, Casp3, Casp2, p53, Apaf-1, p21 and cytochrome-c gene expression in T24 (p53 mt) and RT4 (p53 wt) cells after UVC radiation and treatment with Prima -1 for 1 and 18 h, compared with the control.

protein family (22). This family is comprised of three subfamilies, depending on the number of Bcl-2 homolog (BH) domains that they contain. The antiapoptotic subfamily consists of members containing BH4 domains, and Bcl-2 is the most well-known protein in this class. The two other subfamilies are proapoptotic in nature, either lacking the BH4 domain (Bax, Bak) or solely containing a BH3 domain (BH3-only). Once the BH3-only subfamily is activated, the inhibitory effects of the antiapoptotic Bcl-2 protein family are overcome, enabling the oligomerization of Bak-Bax within the mitochondrial outer membrane (MOM). Our results demonstrated differences in the involvement of Bak and Bax, and the former was shown to participate in apoptosis in RT4 cells and the latter in T24

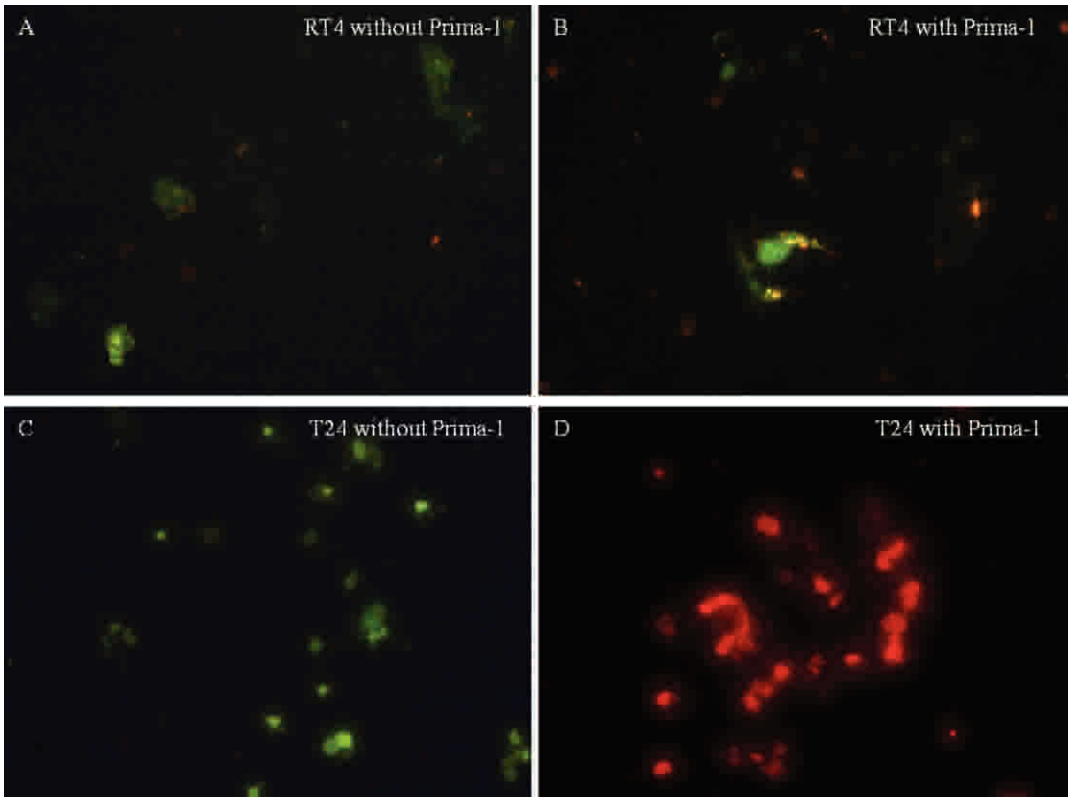

Figure 2 - ΔΨm evaluation by DePsipher **(A)** RT4 cells exposed to UVC in the absence of Prima-1. **(B)** RT4 cells exposed to UVC and treated with Prima-1 for 18 h. **(C)** T24 cells exposed to UVC in the absence of Prima-1. **(D)** T24 cells exposed to UVC and treated with Prima-1 for 18 h.

cells. Alternative routes reported by others help us understand these discrepant results. Lei et al. (23) showed that gossypol, a natural polyphenolic compound found in cotton seeds and used in infertility testing, is subsequently able to induce mitochondrial membrane rupture to release cytochrome c, and this effect is independent of Bax and Bak activation. Green et al. (24) postulated the existence of two alternative routes for cytochrome c release. The first is that the activation and oligomerization of Bax interacts with other mitochondrial membrane proteins, leading to release of cytochrome-c, and the second involves the opening of mitochondrial pores and a nonspecific release of cytochrome-c. In an experimental study with $bak^{-/-}/bax^{-/-}$ mice, Claveria et al. (25) found that the occurrence of programmed cell death was preserved.

In a study published by Katrhyn et al. concerning deconstructing p53 transcriptional networks in tumor suppression, the lack of Bax and Puma was not sufficient to diminish apoptosis in some cell types, which may explain the occurrence of apoptosis in the RT4 cell line even in their absence (13).

Caspase-9 is an initiator caspase and is thus involved in the initiation of the proteolytic cascade, which is associated with the intrinsic apoptosis pathway (26,27). Once activated, caspase-9 cleaves and activates the effector caspases 3 and 7, which are required for proteolysis and subsequent cell death (28). Our analysis revealed the effective initiators of apoptosis, i.e., caspase 9, apaf-1 and cytochrome-c, responsible for the activation of the caspase cascade. We demonstrated that there was an upregulation of these genes in the p53 wt RT4 cell line and the p53 mt Prima-1-treated T24 cells. There was a peak in the expression of caspase-9 in the T24 cell line after UVC radiation exposure, followed by a gradual decrease in its expression levels, while in the RT4 cells, the peak occurred later. Our analysis showed that the effectors caspases, caspase 7 and 3, progressively increased in cell lines, with a peak at 18 h for both. These data suggest that Prima 1 was able to effectively induce apoptosis in a p53 mutant bladder cancer cell line.

Caspase 2, which is considered an orphan caspase, is considered another initiating caspase. This protein is the most evolutionarily conserved of all caspases (29), and although there are numerous reports concerning the role of caspase-2 in apoptosis, there is much controversy regarding this protein, making it difficult to correctly place caspase-2 in the apoptotic cascade; hence, its role in apoptosis remains unclear, making it the orphan of the caspase family. Although caspase-2 shares some substrate specificity with executioner caspases (30,31), in many ways, it is considered an initiator caspase. Caspase-2 has been reported to cleave the pro-apoptotic Bcl2 family member Bid (32). This action is generally considered to be the mode through which caspase-2 initiates apoptosis. We also observed progressive caspase 2 overexpression in the T24 and RT4 cell lines, confirming the data published by Shen et al. (2008), who demonstrated that caspase 2 plays an important role in the intrinsic apoptosis pathway mediated by p53 promoting the translocation of Bax to the mitochondria, a key step for the release of cytochrome c (33).

The loss of mitochondrial membrane potential in the T24 strain occurred after 18 h of treatment with Prima-1,

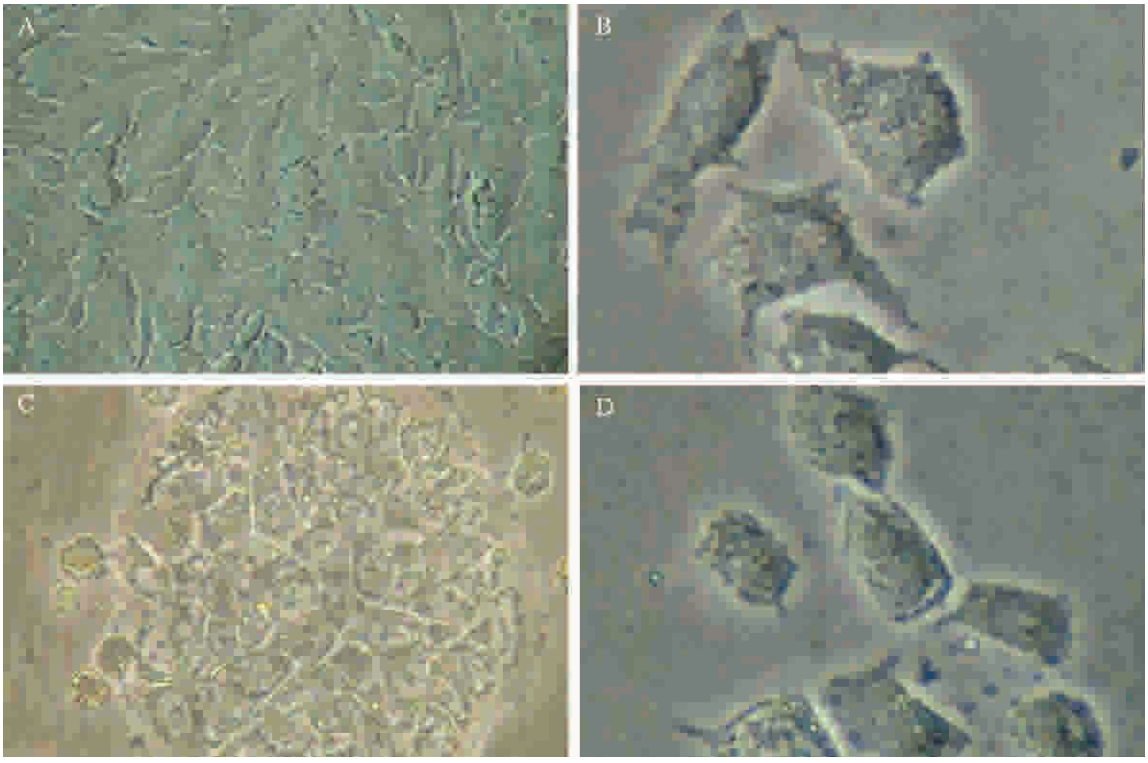

Figure 3 - Microscopic images of T24 cells prior to UVC exposure **(A)** and 192 h after exposure and treatment with Prima-1 **(B)** Microscopic images of RT4 cells prior to UVC exposure **(C)** and 192 h after exposure and treatment with Prima-1 **(D). A** and **C** (100 x)**. B** and **D (**400 x**).**

demonstrating that this mechanism is a direct target of this compound and confirming the findings of Shen et al. (33).

The morphological features observed in the T24 and RT4 cell lines after 24 h of treatment with Prima-1 demonstrates morphological changes, which became more pronounced with increasing treatment time.

To demonstrate that apoptosis induction was related to the reestablishment of p53 transcriptional activation, we evaluated the expression of other important genes regulated by p53: MDM2 and p21. We found that p21 was expressed at lower levels in RT4 compared with T24 cells upon Prima-1 treatment, and the same was observed for MDM2. These results suggest that Prima-1 was able to restore p53 function because after treating the T24 cells (p53 mt), we observed an increase in the expression of p21 and MDM2, which are important genes activated by p53. The gradual increase in the expression of p21 after UVC radiation exposure and subsequent Prima-1 treatment is further evidence for the restoration of p53 because the activation of p53 induces the expression of many proteins, including p21 (34). Messina et al. studied the effect of Prima-1 in thyroid cancer cells and also demonstrated an increase in p21 expression following treatment with this compound (35).

In evaluating the p53 expression in cell lines exposed to UVC and Prima-1, we found that p53 was overexpressed immediately after treatment in the p53 wt RT4 cell line, and the same level of expression was detected after 18 h in p53 mt T24 cell line. However, the p53 expression level in T24 cells after cell damage was significantly lower than that found in RT4 cells. Our findings also revealed that Prima-1 acts to increase the p53 mRNA levels in p53 wt cell lines. The mechanism involved in this phenomenon has not been previously reported and requires additional study.

There are no data in the English literature demonstrating Prima-1 activity in bladder cancer cell lines. p53 mutations are highly associated with high-grade invasive urothelial carcinomas, and it is well known that there is a lack of suitable therapeutic options for these cases (36). The possibility of restoring p53 activity may be of great interest, and the possibility of using it intravesically is tempting. We have previously reported a preliminary study evaluating Prima-1 as a potential treatment in an *in vivo* model of orthotopic urothelial carcinoma. Although this treatment was not effective, there were no side effects, and more studies are necessary to consider this compound as an option in clinical practice (37).

In conclusion, we demonstrated that Prima-1 is able to reactivate p53 function in p53-mutated bladder cancer cell line by promoting apoptosis, inducing the expression of Bax and Puma, activating the caspase cascade and disrupting the mitochondrial membrane independent of Bak in the T24 cell line (p53 mt).

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■ AUTHOR CONTRIBUTIONS

Piantino CB was responsible for the development and execution of the study. Reis ST was responsible for the real-time experiments. Viana NI and Morais DR cooperated in the maintenance of cell cultures. Silva IA

collaborated in the preparation of solutions and dilutions. Antunes AA, Dip Junior N and Srougi M collaborated in project development. Leite KRM generally supervised the project.

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