

# Arylthioindoles: Promising compounds against cancer cell proliferation

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**Abstract.** Drugs that are able to modulate the microtubule dynamics either by inhibiting tubulin polymerization or by blocking microtubule disassembly are of great interest in anti-cancer therapy; a number of them are currently applied in clinical development. Tubulin polymerization inhibitors, including arylthioindoles, are characterized by the presence of an indole nucleus and have been obtained from natural sources or prepared by semi-synthesis. We characterized the effect of 5-bromo-3-[(3,4,5-trimethoxyphenyl)thio]-1H-indole (RS 2518) on the metabolism of human cell lines derived from solid tumors. We found that this new compound impairs cell adhesion, arrests the cells in the G<sub>2</sub>/M cell cycle phase and inhibits cell proliferation, thus leading to apoptosis. The described effects of RS 2518 on cancer cells have led to its selection as a lead compound for further studies. Some analogues have been developed and tested on a panel of cancer cell lines.

## Introduction

New anti-tumor agents are under investigation in both academic and industrial research laboratories worldwide. Drugs that modulate the microtubule assembly are of great interest in anti-cancer therapy, and a number of these drugs are currently employed in clinical development (1,2). Several tubulin polymerization inhibitors characterized by an indole nucleus have been obtained from natural sources or prepared by semi-synthesis. Arylthioindoles (ATIs) inhibit tubulin assembly by interacting with the colchicine site on  $\beta$ -tubulin close to its interface with  $\alpha$ -tubulin within the  $\alpha$ , $\beta$ -dimer (3). ATIs proved

to be effective inhibitors of tubulin polymerization and cancer cell growth, with activities comparable with those of colchicine and combretastatin A-4 (4,5).

In a recent study, we characterized the activity of the newly synthesized arylthioindole RS 2518 and reported its ability to bind  $\beta$ -tubulin and accumulate cells in the G<sub>2</sub>/M phase of the cell cycle, thus promoting the so-called mitotic catastrophe (6). This study aimed to define the destiny of tumor cells after incubation with a high concentration of RS 2518, i.e., 100  $\mu$ M vs. 10  $\mu$ M previously used (6).

## Materials and methods

**Cell cultures and treatments.** Human tumor cells (listed in Table I) and normal fibroblasts were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in DMEM supplemented with 10% FCS, 4 mM glutamine, 2 mM Na pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all reagents were from Celbio, Milan, Italy). Cells were trypsinized when subconfluent. Cell cultures were treated with the compound RS 2518 or its analogues (stock solution 10 mM in DMSO, kept at room temperature in the dark) at concentrations ranging from 0.5 to 100  $\mu$ M for 24 h, followed by either 24- or 48-h recovery. When the treatment caused the cells to detach, the attached and floating populations were analyzed separately. As a positive control of apoptosis, cells were treated with 100  $\mu$ M etoposide for 24 h.

**Cell morphology.** Cells grown in T75 flasks were observed under the microscope Olympus IX71 and photographed with a Nikon DS Chamber Head DS-5M (Enfield, CT, USA).

## Cell proliferation

**Cytotoxicity assay.** Cells were incubated with the drug, washed with PBS, trypsinized and pelleted. After the addition of 1 ml of 0.1 M NaOH, samples were vortexed and heated for 30 min at 50°C. Samples were then allowed to reach room temperature and kept at 4°C until the spectrophotometric analysis at 280 nm. Experiments in duplicate were repeated at least three times.

**MTT assay.** Cells were seeded in 96-multiwell plates at a density of 10<sup>3</sup>/100  $\mu$ l, while fibroblasts were seeded at a

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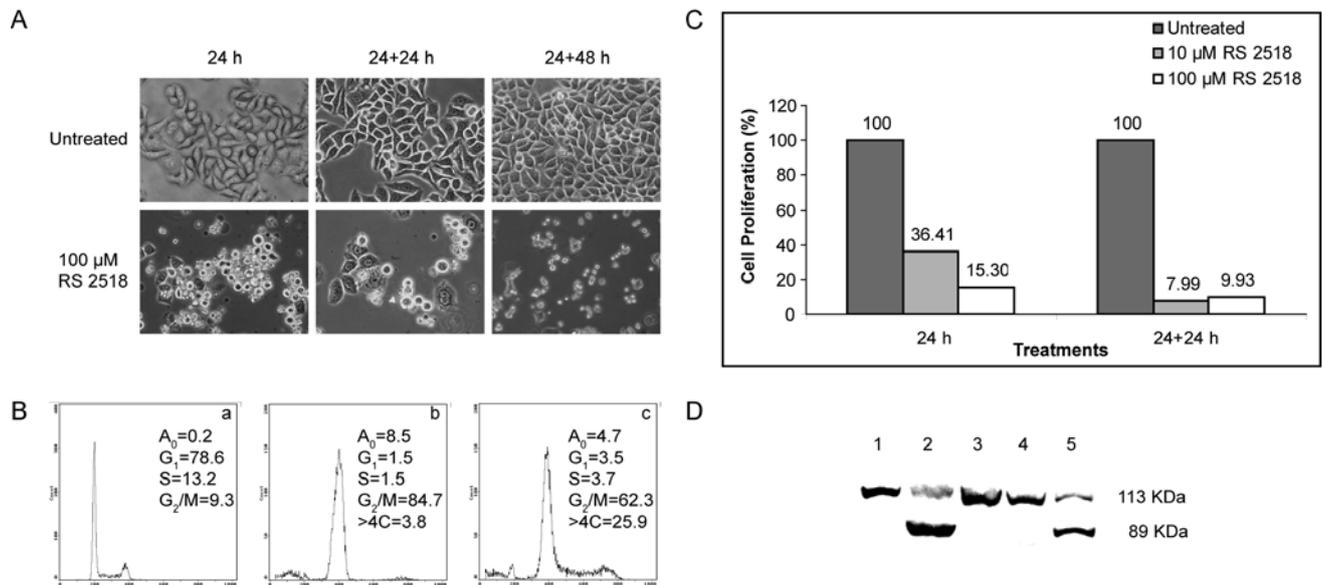


Figure 1. Biological effects of RS 2518 on HeLa cells. (A) Microscopic observation of HeLa cells untreated (upper panels) and treated (lower panels) with 100  $\mu$ M RS 2518 for 24 h, eventually grown for a further 24 or 48 h in drug-free medium. (B) Cell cycle analysis. Untreated samples (a) and cells treated for 24 h with 100  $\mu$ M RS 2518 (b) and further recovered for 24 h (c) were analyzed by flow cytometry. Cell distribution in the various cycle phases ( $G_1$ , S,  $G_2/M$ ) is reported. The  $A_0$  region defines cells with a DNA content  $<2C$ ; cells with DNA content  $>4C$  are considered as polyploids. A typical result out of three is shown. (C) Effect of RS 2518 on HeLa cell proliferation evaluated by cytotoxicity assay. Cells were treated for 24 h with 10  $\mu$ M (light grey columns) or 100  $\mu$ M RS 2518 (white columns) and further grown for 24 h in drug-free medium. Parallel, untreated samples (dark grey columns) were harvested after 24 or 48 h of growth. The proliferation rate of treated cells referred to the untreated samples. Data were expressed as the mean of three independent experiments. (D) Western blot analysis of PARP-1 proteolysis. Lane 3, untreated HeLa cells; lanes 1 and 4, attached fraction of cells treated with 100  $\mu$ M RS 2518 for 24 h (lane 1) and recovered for further 24 h (lane 4); lanes 2 and 5, floating population of cells treated with 100  $\mu$ M RS 2518 for 24 h (lane 2) and recovered for further 24 h (lane 5). Intact and proteolyzed PARP-1 have a MW of 113 and 89 kDa, respectively. Three independent experiments were carried out according to the procedure reported in Materials and methods.

Table I. Effect of RS 2518 on the proliferation of a panel of human tumor cell lines and normal fibroblasts.

Cell line	Origin (carcinoma)	1 $\mu$ M RS 2518 24 h	1 $\mu$ M RS 2518 24+24 h
HeLa	Uterine cervix	45.77 $\pm$ 0.007	33.12 $\pm$ 0.004
HCT116	Colon	67.97 $\pm$ 0.050	22.73 $\pm$ 0.022
SW613-B3	Colon	63.54 $\pm$ 0.016	63.64 $\pm$ 0.041
HT29	Colon	60.03 $\pm$ 0.016	32.53 $\pm$ 0.002
PC3	Prostate	53.39 $\pm$ 0.006	35.49 $\pm$ 0.027
MCF7	Breast	66.38 $\pm$ 0.008	76.89 $\pm$ 0.079
FO46	Normal fibroblasts	88.18 $\pm$ 0.010	111.72 $\pm$ 0.003

MTT assay was performed in quadruplicate; three independent experiments were carried out. Data are expressed as the percentage  $\pm$  SD compared to control cells considered as 100%.

Table II. Effect of RS 2518 analogues on cell proliferation.

Compound	HeLa	HCT116	Normal fibroblasts
RS 2439	57.07 $\pm$ 0.008	108.57 $\pm$ 0.011	89.11 $\pm$ 0.011
RS 2983	54.72 $\pm$ 0.004	70.72 $\pm$ 0.028	93.34 $\pm$ 0.007
RS 2999	51.67 $\pm$ 0.004	69.80 $\pm$ 0.009	87.31 $\pm$ 0.005
RS 3154	40.77 $\pm$ 0.006	52.32 $\pm$ 0.071	83.89 $\pm$ 0.005
RS 3162	52.37 $\pm$ 0.002	72.83 $\pm$ 0.062	94.26 $\pm$ 0.026
RS 3273	52.59 $\pm$ 0.001	81.93 $\pm$ 0.021	93.28 $\pm$ 0.004

MTT assay was performed in quadruplicate on cells treated with 0.5  $\mu$ M for 24 h. Three independent experiments were performed. Data are expressed as the percentage  $\pm$  SD compared to control cells considered as 100%.

density of  $1.5 \times 10^3 / 100 \mu$ l. Cells were treated 24 h later with different drug concentrations for 24 h followed by a 24- or 48-h recovery in a drug-free medium. At the end of the treatments, 20  $\mu$ l of CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT; Promega, Milan, Italy) were added to each well. Plates were then incubated for 4 h at 37°C in the dark and analyzed with a microplate reader (Gio De Vita, Roma, Italy) at 492 nm.

Experiments were performed in quadruplicate and repeated three times.

**Cell cycle.** To evaluate the distribution of a cellular population in the phases  $G_1$ , S and  $G_2/M$  of the cycle by flow cytometry, cells were seeded at a density of  $5 \times 10^5 / \text{ml}$  in 3 ml of complete medium in Petri dishes (6 cm in diameter). After 24 h, the medium was replaced with 1 ml of drug-containing medium. At the end of the 24-h treatment, cells were washed with PBS, trypsinized and recovered by centrifugation for 5 min

at 1500 rpm. The pellet was resuspended with 1 ml of cold 0.9% NaCl and 2 ml of cold 100% ethanol were added drop by drop (final concentration ~70%). The cellular suspension, kept at -20°C until the analysis, was stained with propidium iodide (30 µg/ml) for 30 min. RNA was digested with 2 mg/ml of RNase A for 30 min at room temperature. Finally, samples were analyzed with a Coulter Epics flow cytometer (Beckman Coulter, Milan, Italy) using the software XL. For each sample, at least 10,000 cells were analyzed. The fluorescence intensity was converted into histograms, and the percentage of cells in each phase of the cellular cycle was calculated with the XL2 software. Experiments were repeated at least three times.

**PARP-1 proteolysis.** Samples of  $2.5 \times 10^6$  cells (fresh or stored in liquid nitrogen) were resuspended with 100 µl of denaturing buffer (62.5 mM Tris/HCl pH 6.8, 4 M urea, 10% glycerol and 0.003% bromophenol blue), supplemented with β-mercaptoethanol (final concentration 4%). Cells were disrupted by sonication in ice (50 W twice for 20 sec). Extracts were then heated for 10 min at 65°C and run on 7.5% denaturing polyacrylamide gel. Protein transfer was performed at 200 mA for 3 h at 4°C and confirmed by staining the membrane with red ponceau (Sigma Aldrich). The membrane was saturated with PTN (PBS containing 10% newborn calf serum and 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with the PARP-1-specific MAb C2-10 (diluted 1:1000 in PTN; Alexis, Vinci Biochem, Italy). After 5 washings in PBS containing 0.1% Tween-20, the membrane was incubated for 30 min with the anti-mouse secondary antibody 77039, conjugated with horseradish peroxidase (1:10000 in PTN; Jackson Immunoresearch, Suffolk, UK) and then washed 5 times for 5 min in PBS. Visualization of the immunoreactive bands was obtained by a chemoluminescent substrate (SuperSignal West Pico from Pierce, Celbio). Three independent experiments were carried out.

## Results and Discussion

To define the destiny of cancer cells after incubation with a high concentration of RS 2518, i.e., 100 µM, we investigated the effect of the drug on cell metabolism. When treated with 100 µM RS 2518 for 24 h, HeLa cells showed a reduced adhesion property, resulting in cells floating in the culture medium (Fig. 1A). This effect was not reversed even after a further 24- or 48-h growth in a drug-free medium (Fig. 1A).

Given that ATIs interfere with tubulin metabolism, we analyzed cell cycle distribution by flow cytometry. In HeLa cells treated for 24 h with 100 µM RS 2518, we observed a net increase of cells accumulated in the G<sub>2</sub>/M phase (Fig. 1B), reaching 84.7% at the end of the treatment and 62.3% after a further 24-h growth in the absence of the drug. The apparently weaker effect observed after the recovery time may be explained by the appearance of a cell fraction with a DNA content of >4C, which corresponds to multinucleated cells; this population reached 25.9% and possibly originates from endoreduplication events occurring when cells are blocked in mitosis.

We investigated the effect of the compound RS 2518 on HeLa cell proliferation. Using the cytotoxicity assay, we found that RS 2518 considerably affected cell proliferation in a time-

and dose- dependent manner (Fig. 1C). Compared to control cells, samples treated for 24 h with 10 µM RS 2518 showed a residual proliferation of 36.41%, which further decreased after a 24-h recovery in a drug-free medium (7.99%). Treatment with the highest concentration (100 µM) affected cell proliferation even further (Fig. 1C). Notably, we found that the effects observed at the highest concentration include the activation of caspase-dependent apoptosis, as evidenced by PARP-1 proteolysis (Fig. 1D), a marker of this process (7). The apoptotic destiny of G<sub>2</sub>/M-blocked cells was already reported for other tubulin-interacting drugs (8), e.g., paclitaxel (9) and platycodin D (10). The ability to induce apoptosis is extremely important, given that cancer cells are usually refractory to undergo apoptosis when treated with chemotherapeutic agents (11).

We then extended the analysis of the effect of RS 2518 to a panel of cancer cell lines (Table I) and to normal human fibroblasts FO46. The data reported in Table I refer to a 24-h treatment with 1 µM RS 2518 and revealed that the compound affected the metabolism of the cancer cell lines involved (estimated IC<sub>50</sub> ~0.5 µM), while it did not interfere with normal fibroblast proliferation.

Based on these promising data, we evaluated the effect of six analogues of RS 2518 (namely RS 2439, 2983, 2999, 3154, 3162 and 3273) on the cancer cell lines HeLa and HCT116. The results obtained (Table II) showed that HeLa and HCT116 cell proliferation was affected by a 24-h treatment with 0.5 µM of each compound, with the exception of RS 2439 on HCT116 cells. Of note is that human normal fibroblasts were insensitive to the drugs.

Taken together, these data demonstrate for the first time that the ATI RS 2518 is able to affect tumor cell proliferation by promoting a significant accumulation of cells in G<sub>2</sub>/M, leading to apoptosis. RS 2518 was thus selected as a lead compound for further investigation as a potential anti-tumor drug. Recent data showed that ATIs used in this study are efficient in inhibiting the proliferation of several tumor cell lines (12).

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