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# Study of 6-propyl-2-thiouracil as a radioprotector for the thyroid gland

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**Abstract:** The objective of the paper was to study the application of 6-propyl-2-thiouracil (PTU) as a radioprotector for the thyroid gland. Rat thyroid epithelial cells (FRTL-5) and human colon cancer cells (ARO81-1) were exposed to γ-irradiation with or without 1 mM PTU. Radiation response was analysed by clonogenic survival assay. Cyclic AMP levels were measured by Radioimmunoassay (RIA). The results showed that PTU increased the Surviving Cell Fraction (SF) at 2 Gy significantly ($p < 0.05$) in both cell lines. PTU increased extracellular levels of cAMP in all the treatments in a dose- and time-dependent manner for FRTL-5 cells. In ARO81-1 cells, a peak was observed at 24 hours in extracellular levels incubated with 1 mM PTU (36.97 ± 6.74 fmol/µg prot vs. control: 17.53 ± 3.9 fmol/µg prot, $p < 0.001$). Forskolin and dibutyril cAMP mimicked the effect of PTU on SF. Thus PTU appears to be a radioprotector for thyroid cells and could exert its effect through cAMP.

**Keywords:** thyroid; PTU; radiation; radioprotection; cyclic AMP.

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## 1 Introduction

Radiotherapy is a treatment modality used for the diagnosis and therapy of several malignancies. A major clinical concern is that the radiation dose that can be delivered to the patients is limited by the tolerance of normal tissue that also receives radiation doses similar to the tumour. Damage to normal tissue could be prevented by agents that preferentially protect it against the effects of ionising radiation, allowing also to deliver higher doses of radiation (Wang et al., 2002). These agents are known as radioprotectors. The mechanisms of action of radioprotectors are complex and generally include more than one mechanism (Prasad, 1995). During the past decades there has been a constant effort worldwide to find an agent to protect normal tissue during the application of radiation therapy (Maisin, 1998a; Nair et al., 2001).

Many studies have shown that the thyroid gland is very sensitive to ionising radiation (Ron et al., 1989; Thompson et al., 1994; Sarasin et al., 1999). The exposure to high external radiation doses produces changes in thyroid endocrine function and increases the risk of both benign and malignant thyroid nodules, especially when given during childhood or adolescence (Shalet et al., 1977; Schneider et al., 1993). It has also been demonstrated that the risk of hypothyroidism increases proportionally with the dose of radiation received (Constine et al., 1984; Hancock et al., 1995) while the risk of developing thyroid malignancies follows a linear dose response and decreases with higher doses of radiation (> 10 Gy) (Ron et al., 1995). The availability of a

radioprotective substance could be useful in patients who must be treated with irradiation, like those bearing Hodgkin's disease who have an elevated risk of thyroid cancer, Grave's disease and hypothyroidism as well after the treatment (Hancock et al., 1991). Antithyroid drugs have been used to treat hyperthyroidism since 1940. These drugs inhibit thyroid hormone synthesis therefore reducing their serum level. Among them methylthiouracil has been shown to protect thyroid gland from radiation injury in man and rat (Crooks et al., 1964; Crooks et al., 1965). This thioureylene drug, like 6-propyl-2-thiouracil (PTU) and thiourea, shares a simply chemical structure containing a sulphur atom like many radioprotective substances (Eldjarn and Pihl, 1958; Maisin and Doherty, 1960). These properties could be of utility for the application of these commonly used drugs as radioprotectors. On the other hand, derivatives molecules could be synthesised to be used as radioprotectors as well.

In the present study, we investigated the possible role of PTU as a radioprotector in the thyroid gland. To test the specificity of the compound, we also studied the effect in a human colon cancer cell.

## 2 Methods and materials

### *2.1 Cell lines and culture conditions*

Normal rat thyroid cells (FRTL-5) were cultured in Dulbecco's modified Eagle medium (DMEM/F12, 50:50 v/v) (GIBCO, Invitrogen Corporation, USA) supplemented with 5% Fetal Bovine Serum (FBS) (Natocor, Córdoba, Argentina), bovine thyrotropin (10 mU/ml), hydrocortisone (3.62 ng/ml), transferrin (5 μg/ml), insulin (10 mg/ml), somatostatine (10 ng/ml) and glycil-L-histidyl-L-lysine acetate (10 ng/ml) (Sigma, St. Louis, MO, USA). Human colon cancer cells ARO81-1 (Schweppe et al., 2008) were maintained in Roswell Park Memorial Institute 1640 medium (GIBCO) supplemented with 10% FBS. Both cell lines were kept at 37°C in 5% $CO_2$–95% air atmosphere in a humidified incubator.

### *2.2 Irradiation characteristics*

Cells were irradiated with a $^{60}$Co γ-ray source with a dose rate of 1 Gy per minute $\pm$ 5% (Oncological Institute "Ángel Roffo", Buenos Aires, Argentina) at different times in order to obtain radiation absorbed doses ranged between 1 Gy and 8 Gy. Corresponding controls were sham irradiated.

### *2.3 Clonogenic assay*

Exponentially growing cells were divided into the following groups: irradiated without the drug (a) and incubated with the drug before irradiation (b). Cells were seeded in T25 flasks 24 hours before the irradiation and incubated during that time with 1 mM PTU, 0.01 mM Forskolin (a diterpene that directly activates the membrane bound enzyme adenylate cyclase that calalyses the formation of cyclic AMP) or 1 mM dibutyril cAMP (dbcAMP) (Sigma). After being irradiated, cells were harvested, counted and seeded in 60 mm culture plates at different densities according to the radiation dose received. Cells were incubated for 9–14 days to allow colony formation. Cells were then fixed with 5%

glutaraldehyde and stained with 0.5% crystal violet. Colonies with more than 50 cells were counted. The surviving fraction of each radiation dose was normalised to that of the control to obtain a radiation survival curve. Curves were fitted according to the linear-quadratic model (surviving fraction = $\exp^{-\alpha(D)-\beta(D)2}$) using Origin 7.5 (OriginLab, USA). Surviving fraction at 2 Gy ($SF_2$) was calculated from each curve. Three independent experiments were performed; each point is the average of three plates.

### *2.4 Cyclic AMP measurement*

Cells were seeded in 24 well-cultured plates. Exponentially growing cells were incubated with the same medium containing different concentrations of PTU (0–1 mM), with or without the addition of the phosphodiesterase inhibitor 100 μM 1-methyl-3-isobutylxanthine (IBMX) for different lengths of time. After the treatment, culture medium was collected and frozen at –20°C for extracellular measurement. Cells were washed two times with ice-cold phosphate buffered saline and incubated with absolute ethanol at 4°C. After 30 minutes the supernatants were collected and stored at –20°C as well for intracellular measurement. Cyclic AMP concentration was determined by radioimmune assay according to the method described by Steiner et al. (1969) with some modifications (Del Punta et al., 1996). Cyclic AMP was iodinated with the limiting chloramine-T method, using TME-cAMP. The antibody was kindly provided by the NIH (National Hormone and Peptide Program, Dr A.F. Parlow). Protein content was determined by the Lowry method (Lowry et al., 1951). The results are expressed in fmol cAMP/μg protein. The results are the average of two independent experiments.

### *2.5 Statistical analysis*

All data are expressed as mean ± standard error (SEM). Differences between surviving fractions were calculated with Student's two tails *t*-test. Differences among experimental groups for cAMP levels were determined using one way ANOVA, followed by Tukey–Kramer multiple comparison test. Values were considered significant when $p < 0.05$.

## 3 Results

### *3.1 Radioprotective effect of PTU*

Radioprotective effect of PTU was evaluated by the clonogenic survival assay after the exposure to a range of radiation doses. Exponentially growing cultures of both cell lines were pretreated with 1 mM PTU or the same volume of medium for 24 hours before the irradiation. The selected dose did not affect the proliferating rate (results not shown). As seen in Figure 1, FRTL-5 cells incubated with PTU showed an increase in post-irradiation survival with values of survival fraction at 2 Gy of 74.3 ± 14.1 compared to 53.3 ± 6.9 for control irradiated cells ($p < 0.05$). For ARO81-1 cells (Figure 2), the survival fraction at 2 Gy was also raised in those incubated with PTU, with values of 75 ± 8 compared to 56.9 ± 8 for control irradiated cells ($p < 0.05$).

**Figure 1** Cell survival of FRTL-5 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with PTU before the irradiation showed and increased in $SF_2$ from 75 ± 4.4 compared to 56.9 ± 4 for control irradiated cells ($p < 0.05$). The resulting points are the average of three independent experiments by triplicate ± SEM

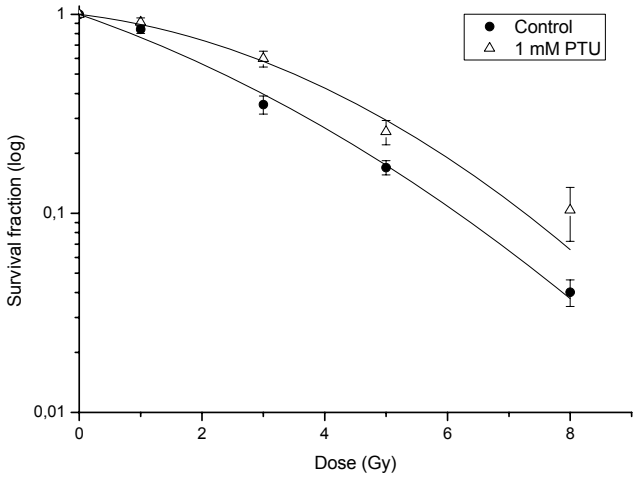
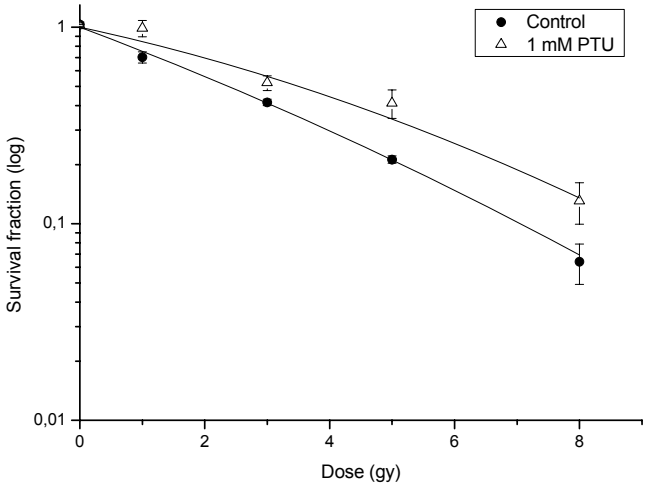



**Figure 2** Cell survival of ARO81-1 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with PTU before the irradiation showed and increased in $SF_2$ from 74.3 ± 7 compared to 53.3 ± 3.4 for control irradiated cells ($p < 0.05$). The resulting points are the average of three independent experiments by triplicate ± SEM



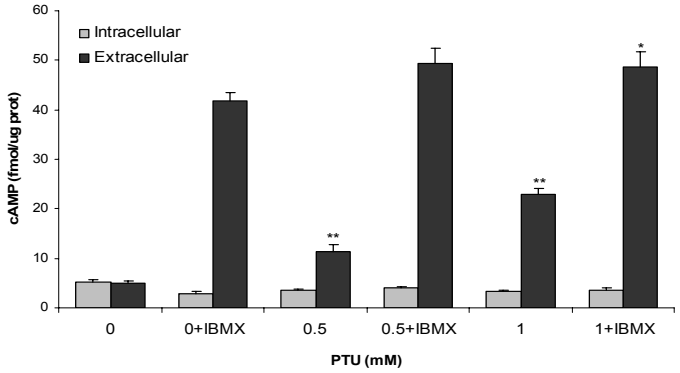

## *3.2 Effects of PTU in cellular cyclic AMP levels*

Exponentially growing cells of both cell lines were treated with different concentrations of PTU (0, 0.5 and 1 mM) for different lengths of time (5, 24, 48 and 72 hours). Cells were incubated with or without the addition of the phosphodiesterase inhibitor IBMX in

order to avoid the degradation of cyclic AMP. Levels of intracellular and extracellular cyclic AMP were then measured. As seen in Figure 3A, after 24 hours of incubation with PTU, extracellular cAMP levels were significantly increased in FRTL-5 cells treated with PTU at both doses (*$p < 0.05$). Groups treated with PTU plus IBMX showed a significant increase with 1 mM (*$p < 0.05$ for 1 mM PTU + IBMX vs. 0 + IBMX). There was no detectable effect of the drug in intracellular cAMP levels in comparison to the control. In addition, we evaluated whether this effect was time dependent. No changes in intracellular levels during the three days of treatment were observed (Figure 3B). On the other hand, extracellular levels were increased in a time-dependent manner in the groups treated with 1 mM PTU + IBMX (***$p < 0.001$).

**Figure 3** (a) Intra and extracellular cAMP levels in FRTL-5 cells incubated with different doses of PTU during 24 hours. PTU increased extracellular levels of cAMP at both doses in a dose-dependent manner in the groups without the addition of IBMX (**$p < 0.01$ vs. control). There were no differences in intracellular cAMP levels between groups.
(b) Effect of 1 mM PTU in cAMP levels of actively proliferating FRTL-5 cells. Extracellular cAMP levels were raised by PTU in a time-dependent manner in those groups treated with PTU+IBMX (***$p < 0.001$) while intracellular levels remained constant in time. Data points are the mean of two independent experiments ± SEM

(a)

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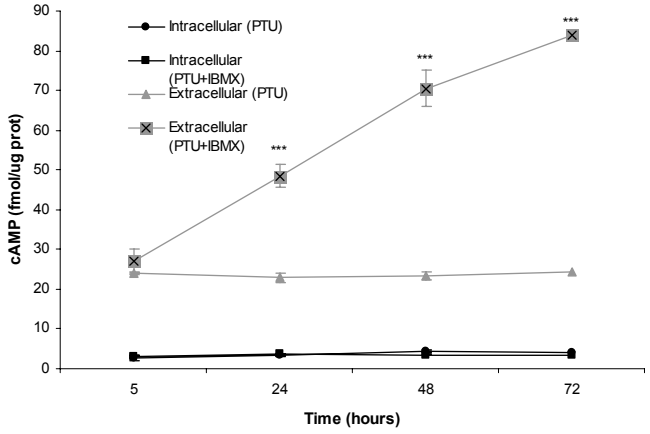
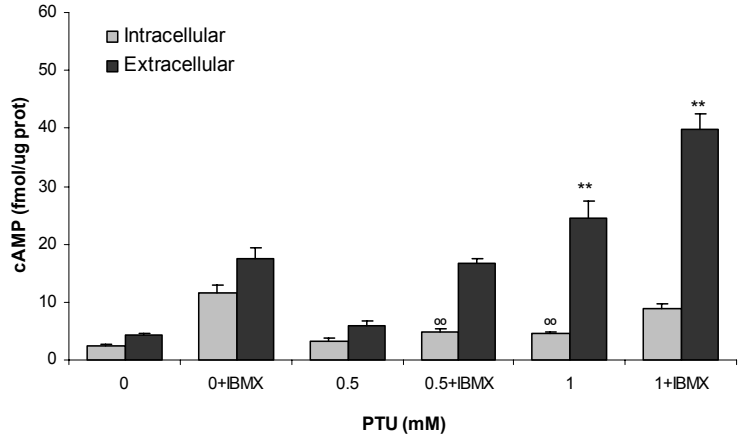


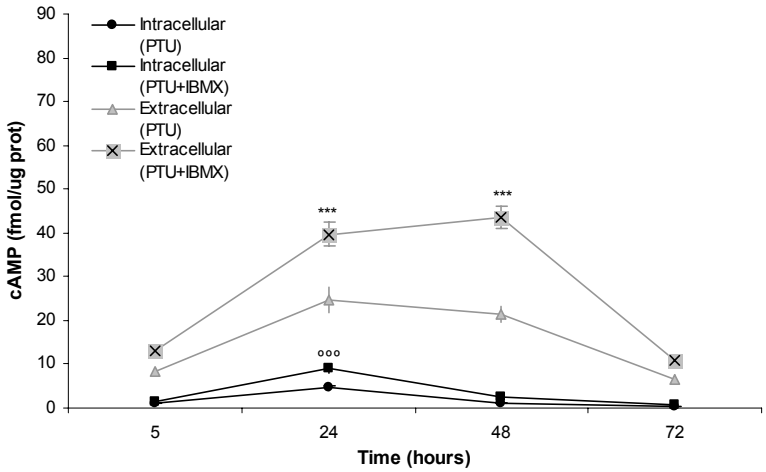
Intracellular cAMP levels in ARO81-1 cells were raised after 24 hours of pre-treatment with 1 mM PTU ($^{oo}p < 0.01$) (Figure 4A). Pre-treatment with PTU, with or without the addition of IBMX, elevated extracellular levels of cAMP significantly ($^{**}p < 0.01$) (Figure 4A). Figure 4B shows that 1 mM PTU induced a time-dependent increase of cAMP levels with a peak around 24 hours for total cAMP levels ($^{ooo}p < 0.001$ and $^{***}p < 0.001$ 1mM PTU + IBMX for intra and extracellular, respectively).

**Figure 4** (a) Intra and extracellular cAMP levels in ARO81-1 cells incubated with different doses of PTU during 24 hours. Intracellular levels were raised in the group treated with 1 mM PTU ($^{\circ\circ}p < 0.01$ vs. Control) while extracellular levels were raised too with 1 mM PTU, with or without the addition of IBMX ($^{**}p < 0.01$)
(b) Effect of 1 mM PTU in cAMP levels of actively proliferating ARO81-1 cells. PTU increased total cAMP levels with a peak at 24 hours ($^{\circ\circ\circ}p < 0.001$ and $^{***}p < 0.001$ for intra and extracellular levels, respectively). Data points are the mean of two independent experiments ± SEM

(a)




(b)



### *3.3 Modification of post-irradiation survival by cyclic AMP*

We found that pre-treatment of cells with PTU produced not only an increase in $SF_2$ in both cell lines after irradiation, but also an elevation in cAMP levels after 24 hours of incubation. To further investigate whether one mechanism of this radioprotective effect of PTU could be exerted by elevating cAMP levels at the moment of the irradiation, we incubated cells with agents that elevate cAMP levels. First, we incubated cells with 1 mM dbcAMP for a period of 24 hours. In FRTL-5 cells $SF_2$ was increased from 55.2 ± 10 to 65.1 ± 0.6 in treated cells ($p < 0.01$). In ARO81-1 cells dbcAMP increased $SF_2$ from 58 ± 2.5 to 67.8 ± 0.6 in treated cells. SF at higher doses used was not significantly changed in both cell lines. Forskolin increased $SF_2$ from 67.8 ± 5.1 to 83.3 ± 7.5 in FRTL-5 cells and from 55.8 ± 2.5 to 71.1 ± 5.5 in ARO81-1 cells. This effect was no statistically significant for 2 Gy, but we founded that for higher doses the increase was significant in both cell lines.

**Figure 5** Cell survival of FRTL-5 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with 1 mM dbcAMP before the irradiation showed and increased in $SF_2$ from 58 ± 1.5 to 67.8 ± 0.4 in treated cells ($p < 0.01$). The resulting points are the average of three independent experiments by triplicate ± SEM

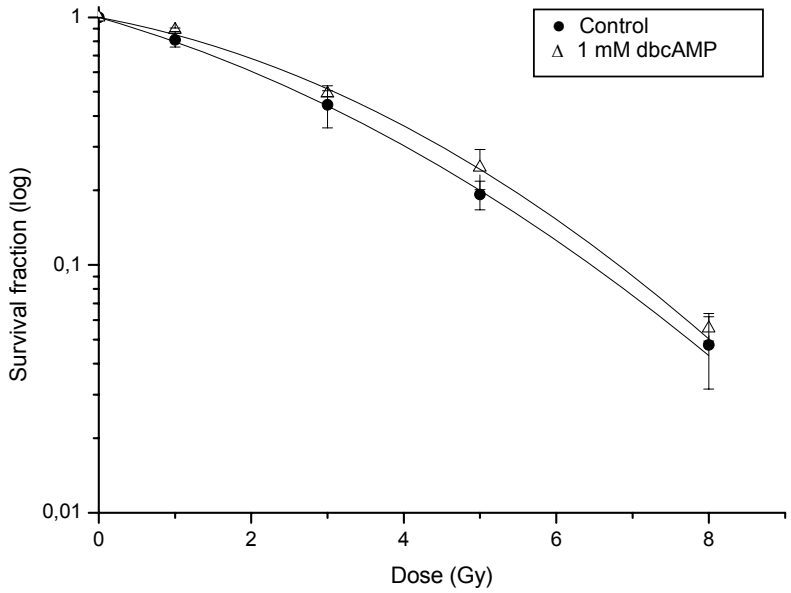


**Figure 6** Cell survival of ARO81-1 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with 1 mM dbcAMP before the irradiation showed and increased in $SF_2$ from 55.2 ± 5.8 to 65.1 ± 3.3 for control irradiated cells. The resulting points are the average of three independent experiments by triplicate ± SEM

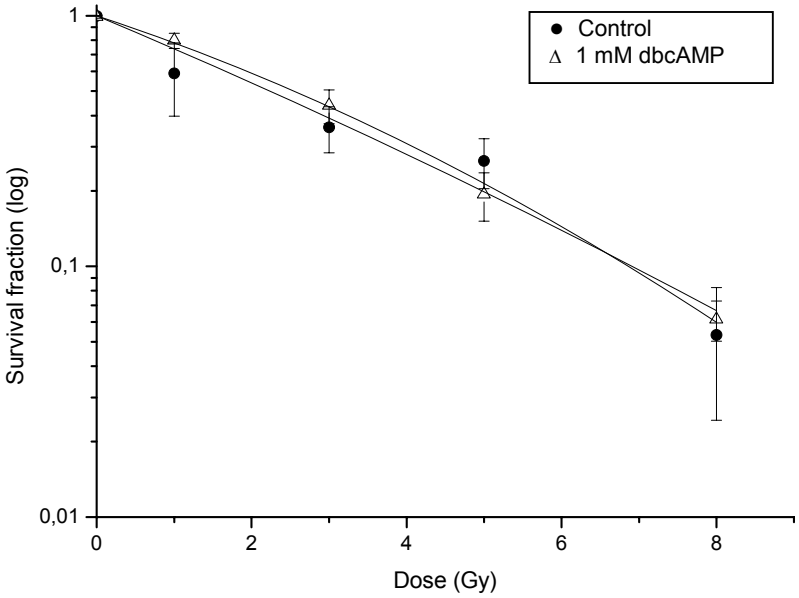



**Figure 7** Cell survival of FRTL-5 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with 10 μM Forskolin before the irradiation showed and increased in $SF_2$ from 55.6 ± 0.7 to 71.1 ± 3.9 in treated cells. The resulting points are the average of three independent experiments by triplicate ± SEM

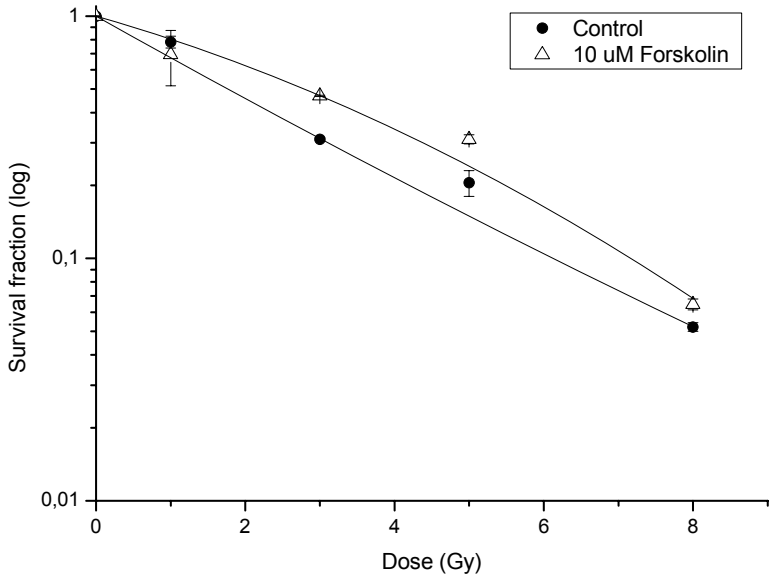
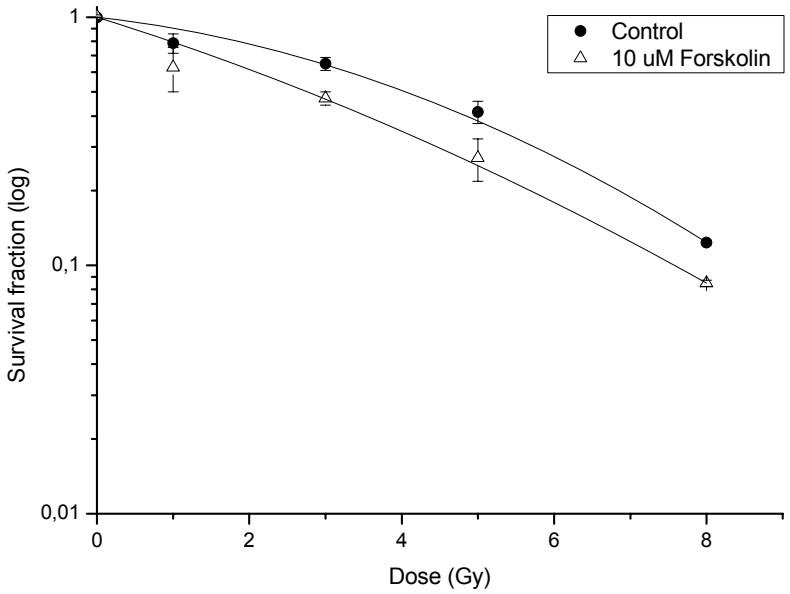


**Figure 8** Cell survival of ARO81-1 cells evaluated by the clonogenic survival assay after the exposure of different $^{60}$Co γ-ray doses. Cells incubated with 10 μM Forskolin before the irradiation showed and increased in $SF_2$ from 67.8 ± 3.6 to 83.3 ± 5.3 for control irradiated cells. The resulting points are the average of three independent experiments by triplicate ± SEM




## 4 Discussion

Ionising radiation is commonly used in the diagnosis and treatment of cancer. The amount of radiation that can be delivered to the tumour to treat it efficiently is often limited by the toxicity to organs and surrounding tissues. After the Second War there were many attempts to develop chemical agents to protect humans against the military use of atomic weapons. The first to investigate the effect of cysteine in rats exposed to lethal doses of X-ray was Patt and collaborators, who founded that pre-treatment of rats reduced radiation induced lethality (Patt et al., 1949). After this, several drugs were tested as radioprotectors but their high toxicity excluded them from clinical use (Sweeney, 1979; Maisin, 1998b). Thyroid gland is very susceptible to ionising radiation. Thyroid function could be affected in patients treated with radiotherapy for head and neck cancer resulting in thyroid cell injury, immune-mediated damage and vascular injury (DeGroot, 1988; Hancock et al., 1995). The exposure to irradiation increases also the risk of developing radiation-induced thyroid cancer in these patients. Antithyroid drugs, such as methylthiouracil, have been tested as radioprotectors for the thyroid gland. Greig and Mclnnes (1966) showed that pre-treatment of rats with methylthiouracil before the irradiation decreased radiation-induced damage in the gland. In the present study, we studied the radioprotective role of the antithyroid drug 6-propyl-2-thiouracil in vitro. Pre-treatment of rat's normal thyroid cells with PTU before γ-irradiation resulted in a significant increased survival fraction compared to control irradiated cells, as evaluated

by clonogenic assay. One limiting step in the use of radioprotective agents for clinical radiotherapy is that an equivalent or even greater radioprotection of malignant cells may obstaculate any increase in therapeutic ratio (Andreassen et al., 2003). Therefore, we studied the effect of PTU in a human colon cancer cell line (ARO81-1). We founded that PTU also protected ARO81-1 cells. As seen in FRTL-5 cells, survival fraction was enhanced in treated cells. Antithyroid drug that contain the thioureylene grouping, among them PTU, are called thioureylene drugs (Taurog, 1976). This chemical structure that contains a sulphur atom is found in many radioprotective substances (Eldjarn and Pihl, 1958; Maisin and Doherty, 1960). The mechanism of action of these sulphhydryl compounds are very complex, and more than one mechanism is often involved (Prasad, 1995).

Adenosine 3′,5′-cyclic monophosphate (cAMP) is a cyclic nucleotide present in all mammalian cells. Many studies have shown that cAMP has many cellular functions and is involved in the regulation of many cellular processes like growth, differentiation and malignancy of some cell types (Prasad, 1975; Pastan et al., 1975). Factors like the rate of proliferation, the degree of differentiation and the point in the cell cycle during the irradiation exposure are influenced by levels of intracellular cAMP, which could modified the survival of irradiated cells (Lehnert, 1975). This possibility was first investigated by Prasad (1972) who founded that post-irradiation survival of CHO cells was increased after the incubation before the irradiation with agents that increased intracellular cAMP levels. Several investigators confirmed and extended the evidence of the radioprotective role of cAMP. For example, survival of Chinese V-7914-15 and thymocyte cells in culture after irradiation was elevated after the incubation with cyclic AMP stimulating agents (Ojeda et al., 1980). To see if a possible mechanism of action of PTU could be exerted by increasing cAMP levels before irradiation, we measured cAMP levels in both cell lines. We founded that cAMP levels were elevated in FRTL-5 cells incubated with different doses of PTU in a dose and time-dependent manner. This behaviour was also observed in ARO81-1 cells, with a peak in cAMP levels after 24 hours of incubation. Therefore, we incubated cells with dibutyryl cAMP or with Forskolin to elevate intracellular cAMP levels. Post-irradiation survival was modified by both drugs in both cell lines. The effect on survival fraction was greater with the addition of Forskolin in both cases. This difference could be related to the fact that dibutyryl cAMP enters to the cell more easily than cAMP (Posternak et al., 1962; Ryan and Durick, 1972), but before mimicking cAMP action it suffers one or two deacylation steps (Neelon and Birch, 1973). On the other hand, Forskolin activates directly adenylate ciclase to produce increasing intracellular cAMP levels. Therefore, it seems that a greater change in post-irradiation survival is obtained by elevating intracellular cAMP before irradiation.

In conclusion, the present study shows that PTU acts as a radioprotector in vitro through the elevation of intracellular cAMP levels.

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