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Effects of an antimutagen of 1,4-dihydropyridine series on cell survival and DNA damage in L5178Y murine sublines

Olga Dalivelya, Natalya Savina, Tatyana Kuzhir, Iwona Buraczewska, Maria Wojewódzka, Irena Szumiel

Abstract In a series of studies it was shown that 1,4-dihydropyridine derivatives (1,4-DHP) show antimutagenic and anticlastogenic properties and accelerate repair of oxidant and ionising radiation generated DNA damage. Here, effects of one of 1,4-DHP compounds (sodium 3,5-bis-ethoxycarbonyl-2,6-dimethyl-1,4-dihydropyridine-4-carboxylate denoted as DHP) in X-irradiated L5178Y cells (murine lymphoma sublines, LY-R and LY-S) are reported. DHP treatment 1 h before, during and after X-irradiation gave a radioprotective effect in double strand break (DSB) repair competent LY-R cells: there was an increase in post-irradiation proliferation and cell viability as well as a slight acceleration of break rejoining as measured by the neutral comet assay. In the radiosensitive LY-S cells with impaired non-homologous end-joining system, the radioprotective effect was seen as enhanced growth and viability. There was, however, no effect on the DSB repair rate. Notably, there was no dependence of the biological effects on DHP concentration in the range of concentrations studied (1 nM – 100 μM), suggesting an all-or-none effect, as in cellular signaling induction observed in radioadaptation or bystander effect. We assume that DHP acts by decreasing fixation of radiation inflicted DNA damage, among others, by increasing the rate of DNA repair and enhancing the efficiency of checkpoint control. Direct confirmation of this assumption is necessary.

Key words 1,4-dihydropyridine • DNA repair • neutral comet assay • L5178Y cells • cytotoxicity • radioprotective effect

Introduction

DNA damage is a trigger of many pathological processes; therefore, knowledge of the molecular mechanisms and targets of antimutagens is important for understanding their etiology and pathogenesis. Antimutagenic effects may also be important from the point of view of normal tissue protection during cancer radiotherapy. We have earlier shown that the 1,4-dihydropyridine derivatives are efficient antimutagens and DNA repair modulators *in vivo* [3, 8, 9, 11, 12] and *in vitro* [15, 16]. In particular, alkaline single cell gel electrophoresis (comet) assays showed that DHP (sodium 3,5-bis-ethoxycarbonyl-2,6-dimethyl-1,4-dihydropyridine-4-carboxylate, previously designated as AV-153 [16]) reduced the number of endogenously generated DNA strand breaks in untreated human lymphocytes and human promyelocytic leukemia HL-60 cells [16]. In HL-60 and Raji (human B-lymphoblastic leukemia) cells exposed to 2 Gy of γ-radiation or 100 μM hydrogen peroxide there was a statistically significant increase in the single strand break rejoining rate [16]. Another compound of 1,4-DHP series, glutapyrone (disodium salt of 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)-glutaric acid), protected against ethyl methanesulfonate (EMS)-induced mutations in *Drosophila melanogaster* and chromosome breakage in mouse bone marrow [9, 11, 12].

O. Dalivelya, N. Savina, T. Kuzhir
Institute of Genetics and Cytology,
National Academy of Sciences of Belarus,
27 Akademicheskaya Str., Minsk 220072,
Republic of Belarus

I. Buraczewska, M. Wojewódzka, I. Szumiel✉
Department of Radiobiology and Health Protection,
Institute of Nuclear Chemistry and Technology,
16 Dorodna Str., 03-195 Warsaw, Poland,
Tel.: +48 22 504 1064, Fax: +48 22 811 15 32,
E-mail: izasz@orange.ichtj.waw.pl

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The purpose of the reported investigation has been to study the influence of an antimutagen of 1,4-dihydropyridine series on cell survival and X-ray-induced DNA damage in L5178Y murine sublines differing in radioresistance, repair of double strand breaks and metabolism of poly(ADP-ribose) (reviewed in [17, 18]). The present study is part of screening of 1,4-DHP derivatives synthesized in the Latvian Institute of Organic Synthesis.

Materials and methods

Cell culture

Two sublines of murine lymphoma L5178Y, LY-R and LY-S, were used, earlier characterized in detail [17, 19]. Cells of both sublines were maintained in logarithmic growth phase in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum at 37°C and 5% CO_2. For estimation of cell growth and viability, suspensions of cell density 25×10^3/ml (for treatment procedures without X-irradiation) and 100×10^3/ml (for irradiated cells) were used. The comet assay (single cell gel-electrophoresis) was carried out at cell density no less than 400×10^3/ml.

Treatment procedures

Exponentially growing cells were irradiated on 60 mm plastic Petri dishes with an X-ray machine at room temperature, with the use of an X-ray machine (ANDREX, Holger Andreasen, Denmark, 200 kVp, 5 mA, dose rate 1.2 Gy/min). Since the cell lines differ in radiosensitivity, approximately equitoxic doses of X-rays were used for irradiation: 1 Gy for LY-S and 2 Gy for LY-R cells. In cell growth and viability tests, X-irradiated cells were incubated at 37°C for 48 h. In the comet assay, cells of both sublines were irradiated with 10 Gy of X-rays at 0°C for estimation of initial DNA damage and at 37°C for repair analysis 15, 60 and 120 min after irradiation.

The 1,4-dihyropyridine derivative (DHP) at concentrations of 1 nM, 1 μM and 100 μM was added to the culture medium 1 h before irradiation and present there for the whole 48 h incubation in the growth and viability tests and from 0 to 120 min after irradiation in the comet assay. In micronuclei frequency determination, 1 h pretreatment before irradiation at concentrations of 1 nM or 100 μM DHP was carried out and DHP left to the end of post-irradiation incubation. (1 mM DHP was used to treat non-irradiated cells for comparison to control.)

Growth and viability tests

In growth tests, cell cultures, control and treated with DHP at concentrations indicated, were grown from the same cell density for 48 h. Relative cell numbers were determined (ratio of cell number in the treated culture to that in the control; direct count of morphologically normal cells), as well as viability by the dye exclusion test with trypan blue, as described below. Experiments were repeated at least 2 times.

Viability of cells subjected to X-irradiation and/or DHP treatment was estimated with the standard trypan blue test. For this purpose, irradiated or non-irradiated cells of both sublines were incubated for 48 h in the presence or absence of DHP, whereupon the dye was added at a final concentration of 0.2 mg/ml. The cells were scored using a Buerker hemocytometer.

Neutral comet assay

The modified method of neutral comet assay (single cell gel electrophoresis) [23] was used for estimation of DNA damage (predominantly DSB) and kinetics of their rejoining. The cell suspensions (4×10^5 cells/ml) were mixed with an equal volume of low melting-point agarose Type VII at a final concentration of 0.75%. Cell suspensions were cast on microscope slides precoated with 0.5% normal (regular) agarose Type IA. Samples were covered with cover slips and left on ice for solidification. Then, they were placed in a lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% *N*-lauroylsarcosine, pH 9.5) supplemented with 0.5% Triton X-100 and 10% DMSO. Lysis was carried out for 1–2 h at 4°C in the dark. After lysis, the slides were washed three times with an electrophoresis buffer (300 mM sodium acetate, 100 mM Tris-HCl, pH 8.3) and left in a fresh portion of the buffer for 1 h, then placed in a horizontal gel electrophoresis unit filled with a fresh electrophoresis buffer. The samples were electrophoresed for 1 h at 14 V (0.5 V/cm, 7–8 mA) at 8°C in the dark. After electrophoresis, the slides were rinsed with 0.4 M Tris (pH 7.5) and then stained with 1 μM 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, 50 μl per slide). For image analysis, pictures of 50 randomly selected comets per slide from two slides in three separate experiments were captured at 200× magnification using an epifluorescence microscope (Labophob-2, Nikon) equipped with a UV-1A filter block. Image analysis was carried out using the Comet v.3.1 (Kinetic Imaging Ltd., Liverpool, UK). The measure of damage was the tail moment (fraction of DNA in the tail times tail length). Data analysis was based on the mean population response or on the distributions of damage among cells. Statistical evaluation and plots were prepared with Microsoft Excel 2000 software.

Micronuclei frequency determination

Microscopic preparations for the cytokinesis block micronucleus technique were made according to Fenech and Morley [4]. Briefly, cell cultures were incubated for 1 h with 1 nM or 100 μM DHP, X-irradiated (1 Gy, LY-S; 2 Gy, LY-R cells) and incubated for 16 h with 6 μM/ml cytochalasin B (Sigma) added immediately after irradiation. For micronuclei scoring, cells were subjected to a hypotonic treatment with 144 mM KCl solution (5 min, room temperature) and fixed in a cold methanol-glacial acetic acid solution (3:1). Microscopic preparations were made by dropping cells on slides and staining with Giemsa. Non-irradiated

Table 1. Effects of DHP (1,4-dihydropyridine derivative) on cell growth and viability of LY sublines after X-irradiation with 2 Gy (LY-R) or 1 Gy (LY-S)

Treatment	Subline LY-R			Subline LY-S		
	Relative cell number	Dead cells [%]	t; P*	Relative cell number	Dead cells [%]	t; P*
Control (non-irradiated)	1	2.13 ± 0.20		1	1.13 ± 0.13	4.17; <0.001
DHP 1 nM	0.72	2.43 ± 0.23	1.00; >0.05	0.51	0.39 ± 0.19	3.22; <0.001
DHP 1 μM	0.71	1.82 ± 0.23	1.03; >0.05	0.50	0.36 ± 0.19	3.35; <0.001
DHP 100 μM	0.65	1.66 ± 0.24	1.52; >0.05	0.48	0.61 ± 0.20	2.17; <0.05
X-irradiated	1	5.30 ± 0.19		1	10.75 ± 0.13	23.70; <0.0001
DHP 1 nM	1.34	4.31 ± 0.17	3.96; <0.001	1.21	5.42 ± 0.12	29.61; <0.0001
DHP 1 μM	1.45	4.10 ± 0.16	4.14; <0.001	1.09	6.69 ± 0.13	22.56; <0.0001
DHP 100 μM	1.53	4.72 ± 0.16	2.00; <0.05	1.13	6.58 ± 0.13	23.17; <0.0001

* Student's test evaluation of the difference from control (non-irradiated) or radiation alone data; t value and probability (P). Mean results from 3 experiments.

cells were also treated in the same way with 1 μM DHP to compare with the non-treated control. Also in that case, DHP was left for the whole 16 h incubation time. The procedure was according to Fenech [4]. Two microscopic slides were prepared for each experimental point and 1000 or 2000 cells scored. The experiment was repeated 3 times.

Results

Effects of DHP on cell viability and proliferation

Cytotoxicity of DHP at the concentrations of 1 nM, 1 μM and 100 μM was estimated in the trypan blue test after 48 h cell incubation in the presence or in the absence of DHP. Effect on cell proliferation was estimated in a 48 h growth test on the basis of relative cell numbers, as defined in the "Materials and methods" section.

The results are presented in Table 1 (numerical data and statistical significance from Student's t test) and in Fig. 1. In non-irradiated cells, DHP reduced the relative cell number by 30% in LY-R cells and by 50% in LY-S cells. The effect was independent of DHP concentration. In both LY sublines there was a slight decrease in percentage of dead cells. Thus, altered viability level could not affect proliferation; therefore, the decrease in relative cell number apparently resulted from slowed down cell cycle progression.

In contrast, there was an increase in relative cell numbers in cell populations after exposure to X-rays (LY-R cells, 2 Gy; LY-S cells, 1 Gy) by 34–53% in LY-R cells and by about 14% in LY-S cells. In both LY sublines, this protective effect was reflected in viability increase, albeit to a different extent. The level of dead

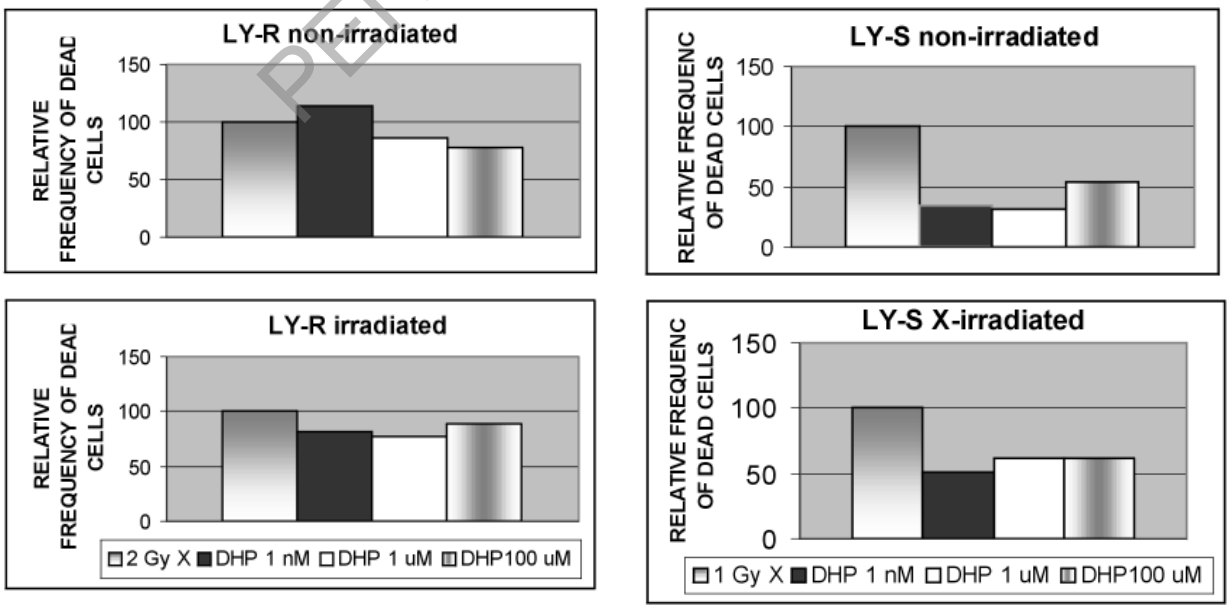

Fig. 1. Influence of DHP on dead cell percentage in LY-R and LY-S cells relative to the control level taken as 100% (non-irradiated or X-irradiated cell populations).

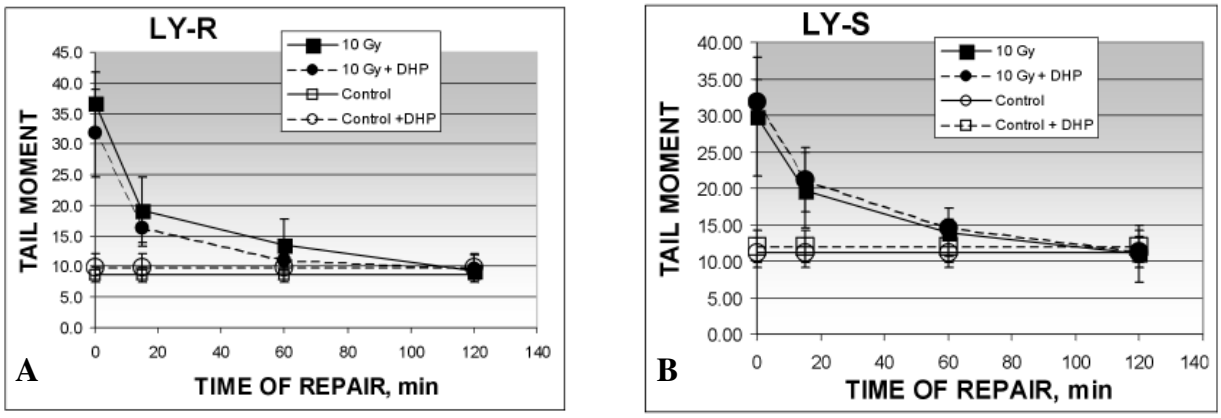

Fig. 2. Time course of rejoining of X-ray-induced DNA breaks in LY-R (A) and LY-S (B) cells untreated or treated with 1 nM or 100 μM DHP (present 1 h before irradiation and during the whole repair interval). The results for both concentrations were pooled because of lack of dependence on DHP concentration. The level of control and control + 1 μM DHP is indicated. The differences between tail moment values obtained for DHP treated and untreated X-irradiated cells are not statistically significant.

LY-R cells decreased by 10–20% only, that in LY-S subline was reduced by up to 60%. As in the case of unirradiated cells, there was no clear dose-dependent relationship in the latter case: the lowest dose (1 nM) was equally or even more effective than the higher doses of DHP.

Effects of DHP on repair of X-ray-induced DNA damage

Effect of DHP treatment in X-irradiated LY cells was also studied with the use of the neutral comet assay. To estimate the DHP influence on the endogenously generated DNA damage, the dose of 1 μM was chosen, whereas in experiments with irradiated cells, the effect of treatment with 1 nM and 100 μM DHP was analysed. The average tail moment value in LY-S cells (11.11 ± 0.37) exceeded that in LY-R cells (8.73 ± 0.1). DHP treatment did not affect much these values (LY-S, 11.99 ± 0.38; LY-R, 9.96 ± 0.32). It must be taken into consideration that tail moment value is a result not only of the endogenously generated ("spontaneous") DNA breakage level, but also of chromatin conformation.

Kinetics of DNA damage induced by X-rays in LY-R and LY-S cells treated or untreated with the 1,4-DHP derivative is presented in Fig. 2. In cells of both sublines, the main portion of induced DSBs was rejoined for the first hour of cell incubation, with about 60% of DNA damage being repaired during the first 15 min. In LY-R cells (Fig. 2A), DHP decreased the level of X-ray-induced DNA damage both immediately after irradiation and during a later incubation. It was interesting to note that the lowest and the highest doses of DHP were equally efficient against X-rays in this subline. When the tail moment value after X-irradiation alone was taken as 100%, then approximately 20% and more than 50% of X-ray-induced DNA damage was reduced at 0 min and 60 min, respectively, indicating an increased rate of DNA repair in the presence of DHP. It should be noted, however, that although a tendency to accelerated repair was reflected in these relative damage levels, the differences between absolute tail moment values were not statistically significant. Also, the residual damage level was identical in DHP-treated and untreated cells.

In LY-S cells both 1 nM and 100 μM DHP did not alter the rate of break rejoining both in absolute (Fig. 2B) and relative (not shown) tail moment values. The level of control and control + 1 μM DHP is indicated in the figure and it can be seen that there is no effect of DHP on tail moment in non-irradiated cells of both sublines.

Effects of DHP on micronuclei frequency

To check whether the effect of DHP treatment on DNA damage estimated with the comet assay is reflected in chromosomal damage, we determined the frequency of micronuclei in cells treated in a similar way as in the other tests, except that the endpoint was generation of micronuclei at 16 h after irradiation.

Although X-irradiation was carried out with approximately equitoxic doses, the response of LY sublines considerably differed because of different duration of the G2 arrest: 4 h per Gy in LY-R cells and 11 h per Gy in LY-S cells as well as the apoptosis proneness [19]. This is reflected in the % of binucleated cells in the DHP-treated and X-irradiated cell populations (Fig. 3). A difference could be seen between LY sublines, in agreement with the radiation sensitivity, but DHP + X treatment did not alter the percentage of binucleated cells as compared with X alone. Thus, the progression through the cell cycle leading to successful mitosis did not seem to be affected by the treatment.

For micronuclei frequency, only preliminary data are available which, however, are consistent with the comet assay results. Also here, the effect did not depend on DHP concentration, so, the results for 1 nM and 100 μM were pooled. With micronuclei frequency after X-irradiation alone taken as 100%, the relative frequency (%) for DHP + X treated LY-R cells was 72.92 ± 1.77 (mean ± standard deviation) and this decrease was significant (chi square test, $\chi^2 = 8.7$); for LY-S cells the decrease was not significant (68.23 ± 10.42).

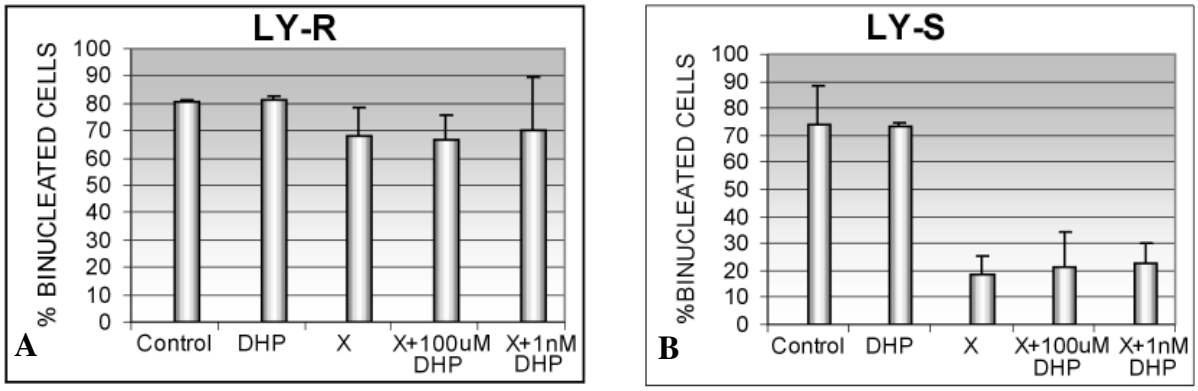

Fig. 3. Percentage of binucleated cells after 16 h incubation with cytochalasin B. (A) LY-R cells irradiated with 2 Gy X-rays without or with DHP treatment at the concentrations indicated (DHP present 1 h before irradiation and during the whole incubation interval). (B) LY-S cells irradiated with 1 Gy X-rays and treated as above. The differences between values obtained for DHP treated and untreated X-irradiated cells are not statistically significant.

Discussion

The present study is part of screening of DHP derivatives synthesized in the Latvian Institute of Organic Synthesis. So far, a pronounced antimutagenic and anticlastogenic activity was noted in studies on *Drosophila melanogaster*, murine tissues, human lymphocytes and a variety of human cancer cell lines [3, 8, 9, 11, 15, 16]. There were, however, few attempts at defining the mechanism of action of this group of compounds, except that accelerated DNA repair was seen, as estimated with the alkaline comet assay after treatment with hydrogen peroxide or methyl methane-sulfonate (MMS) as well as after γ-irradiation [16].

In studies with *Drosophila melanogaster*, the antimutagenic efficiency of 1,4-DHP derivatives was shown to depend on their electron-donating activity [8]; hence, they may act as antioxidants against spontaneous mutations. However, the protective effect against EMS supports the assumption that DHP derivatives also modulate DNA repair [11, 12, 15, 16], and indeed, accelerated rejoining of single strand breaks was observed [16]. Moreover, DHP considerably stimulated synthesis of poly(ADP-ribose) polymers and the increase correlated with an increase in DNA repair rate in hydrogen peroxide treated cells [15]. As discussed below, other possible targets of 1,4-DHP derivatives include progression through the cell cycle, DNA damage repair and fixation processes (notably, both dependent on poly(ADP-ribosylation)) and calcium ion homeostasis.

The observations on LY cells, reported above, indicate that the effect on DSB repair may be important in the radioprotective activity, since – as summarised in Table 2 – this effect was weaker in LY-S cells that are DSB repair-defective (reviewed in [20]). Nevertheless, some protection was observed even in these cells, indicated by determinations of relative cell number and viability tests. It seems of importance that DHP considerably slowed down proliferation of non-irradiated cells. Moreover, this effect was independent of DHP concentration (see Fig. 1). In another study, DHP treatment did not alter the distribution in cell cycle phases [16], thus, apparently not causing any arrest at a specific checkpoint; neither did it exert any lethal effect, as judged from the viability tests. Hence, it was plausible to assume that DHP facilitated or enhanced this aspect of the cellular response to DNA damage that consisted in arrest in cell cycle progression. The arrest depends on a complicated signaling system (review in [13]) and exerts a protective effect by favoring repair and preventing damage fixation. Nevertheless, the percent-age of binucleated cells (Fig. 3) did not reflect any change in the cycling compartment of the cell populations under examination. This aspect of DHP action awaits further investigation with the use of other incubation intervals for cytochalasin B treatment, as well as the use of flow cytometry to assess cell cycle progression.

It is tempting to assume that 1,4-DHP derivatives, which belong to well known voltage-dependent Ca^{2+} channel blockers act through modulation of calcium ion transport. There are some older positive reports, e.g. diltiazem, a benzothiazepine calcium channel blocker, alone or in combination with 1,4-DHP blockers, protected against bone marrow damage (cytogenetic damage, cell death) and mortality in whole body irradiated mice [5–7]. Nevertheless, survival studies with human cell lines (HeLa and MDA-MB-231) involving blocking of the radiation-induced increase in cytoplasmic calcium concentration with another member of 1,4-DHP family, nifedipine, did not reveal a

Table 2. Summary of DHP effects in LY cells, X-irradiated with approximately equitoxic doses (LY-R, 2 Gy; LY-S, 1 Gy)

Feature examined after DHP pre-treatment and X-irradiation	LY-R	LY-S
DNA breakage rejoining	Slightly increased	Unchanged
Micronuclei frequency	Decreased by 27%	Unchanged
Early lethal effect (48 h growth)	Decreased by 43%	Decreased by 14%
Viability (48 h after irradiation)	Enhanced by 20%	Enhanced by 60%

connection between the radiation effects on cellular Ca^{2+} homeostasis and cell survival [21]. Other more recent data indicate that calcium is required for ionising radiation-induced cell cycle regulation and protein kinase C activation, but the increase in cytoplasmic calcium concentration is not a universal cellular response to irradiation [10]. Finally, glutapyrone, unlike classical 1,4-DHPs, lacks calcium antagonistic or agonistic properties, whereas it has an antimutagenic and antineoplastic activity [9, 11, 12, 22].

The lack of dependence of the biological effects on DHP concentration in the range of concentrations studied (1 nM – 100 μM), suggests an all-or-none effect, as in cellular signaling induction observed in radioadaptation [2, 18] or bystander effect (reviews in [1, 14, 17]. It should be added that radioadaptation has recently been interpreted as a result of decrease in damage fixation rather than repair stimulation [18]. Although at present only an indirect support for such assumption is available, it may be speculated that in the case of DHP the antimutagenic effect is based on the same principle. Furthermore, damage fixation is poly(ADP-ribose) polymerase-dependent and its activity is modulated by DHP [15]. The reported results must be treated as preliminary, but they warrant further investigations concerning the mechanisms of action of DHP in cells with damaged DNA.

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