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# Modulation of cellular defense processes in human lymphocytes *in vitro* by a 1,4-dihydropyridine derivative

Nadezhda I. Ryabokon[a,b,*], Nataliya V. Nikitchenko[b], Olga V. Dalivelya[b], Rose I. Goncharova[b], Gunars Duburs[c], Maria Konopacka[a], Joanna Rzeszowska-Wolny[a,d]

[a] *Department of Experimental and Clinical Radiobiology, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Branch Gliwice, Wybrzeze Armii Krajowej 15, 44-101 Gliwice, Poland*
[b] *Laboratory of Genetic Safety, National Academy of Sciences of Belarus, Institute of Genetics and Cytology, Akademichnaya 27, 220072 Minsk, Belarus*
[c] *Laboratory of Membrane-Active Compounds and β-diketones, Latvian Institute of Organic Synthesis, Aizkaukles 21, LV-1006 Riga, Latvia*
[d] *Silesian University of Technology, Institute of Automation, Akademicka 16, 44-100 Gliwice, Poland*

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ABSTRACT

The aim of this pilot study was to assess whether a compound of the β-carbonyl-1,4-dihydropyridine series (AV-153 or sodium 3,5-bis-ethoxycarbonyl-2,6-dimethyl-1,4-dihydropyridine-4-carboxylate), which has high efficiency in stimulating DNA repair, can simultaneously modulate apoptosis in human cells. Peripheral blood lymphocytes of healthy donors were used in this study. DNA strand-break rejoining was assessed with the alkaline comet assay after a 3-h incubation of lymphocytes in the presence of a wide range of concentrations of AV-153 ($10^{-10}$–$10^{-5}$ M). Apoptotic and micronucleated (MN) cells were scored in phytohaemagglutinin-stimulated lymphocytes after a 72-h incubation with AV-153, using the standard cytokinesis-blocked micronucleus test. The study revealed dual effects of AV-153 on cellular defense systems against endogenously generated DNA damage: the compound *per se* simultaneously reduces DNA strand breaks and stimulates apoptosis, with a maximal efficiency of 76% and 42%, respectively; in contrast, after genotoxic stress (2 Gy of gamma-radiation) AV-153 reduces DNA strand breaks, the number of MN cells and apoptotic cells in a similar dose-dependent manner. A maximal efficiency of 67% was found for reduction of DNA strand breaks, while for MN cells and apoptotic cells the efficiencies were, respectively, 47% and 44%. While limited in number, these preliminary studies show the direct correlation between the efficiency of AV-153 in reduction of radiation-induced DNA breaks and MN cells on one side, and in reduction of apoptosis on the other. It suggests that the major target of the compound's action on genotoxic stress is DNA repair, followed by reduction of the number of damaged cells entering apoptosis.





## 1. Introduction

DNA repair and apoptosis represent two oppositely directed processes of the cellular defense system: while the DNA-repair machinery removes damage from DNA molecules and thus allows damaged cells to survive, apoptosis is a programmed cell death and eliminates cells with damaged DNA and thus protects cell populations from inheritance of defective genomes by subsequent generations. These processes compete not only for DNA as a substrate, but also for the energy in cells after the DNA-damaging impact of endogenous and exogenous factors (reviewed in [1]). Thus, during the induction and processing of base-excision repair (BER), poly(ADP-ribose) polymerase-1 (PARP-1) reduces $NAD^+$ and ATP levels dramatically in damaged cells (reviewed in [2,3]), but ATP is also strongly required for DNA ligation, a terminal stage in BER [4]. On the other hand, apoptosis needs $NAD^+$ for activation of apoptotic protease AP24 and ATP for numerous processes of caspase activation, nuclear changes, protein synthesis and disassembly of structural elements of damaged cells (for reviews see [1,5,6]). Besides, release of cytochrome *c* from mitochondria during initiation of apoptosis is associated with a drastic reduction of cellular ATP production (reviewed in [7]). Energy deficiency in these cells results in their death through ATP-independent necrosis (reviewed in [2,6]), i.e. an undesirable process because it can promote inflammation [8]. ATP concentrations at 25% of the basal level are considered to be the critical limit for apoptosis processing [9]. Thus the energetic state, as well as appropriate operation of the DNA repair and apoptosis pathways define the fate of genotoxically damaged cells.

* Corresponding author at: Laboratory of Genetic Safety, National Academy of Sciences of Belarus, Institute of Genetics and Cytology, Akademichnaya 27, 220072 Minsk, Belarus. Tel.: +375 17 294 91 79; fax: +375 17 284 19 17.
*E-mail address:* nrabakon@yahoo.com (N.I. Ryabokon).

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Induction and stimulation of apoptosis offer potential anticancer strategies by protecting against DNA damage and hence an increased synthesis of poly(ADP-ribose) which cause depletion of ATP required for apoptosis [10]. Further, new anticancer agents that induce apoptosis directly should be less mutagenic than existing drugs (reviewed in [11]). In our previous studies we have shown that AV-153 or sodium 3,5-bis-ethoxycarbonyl-2,6-dimethyl-1,4-dihydropyridine-4-carboxylate, a compound of the 1,4-dihydropyridine series, stimulates DNA break rejoining [12] through an increase of PARP activity induced by genotoxic stress in human cells [13]. We supposed that the observed high efficiency of AV-153 in reducing DNA damage could be followed by a reduction in the number of damaged cells entering into apoptosis. On the other hand, the compounds of this series, which have closely similar chemical structures and represent the β-carbonyl-1,4-dihydropyridine group, i.e. the analogues of the active center of the reduced form of $NAD^+$ or $NADP^+$ (NADH or NADPH), are known to have hydrogen- and electron-donating properties and to restore the pool of reduced $NAD^+$ and hence of ATP [14,15]. Thus, we also expected the stimulation of apoptosis due to energy regeneration in cell populations cultured in the presence of these compounds. The derivatives of 1,4-dihydropyridine have important pharmacological potential [15], and these various suggestions provided much of the impetus for this pilot study to examine if AV-153 modulates apoptosis in human cells, and if so, to define which of the two defense processes, DNA repair or apoptosis, is the preferred target for this modulation. In this manuscript we report for the first time that AV-153, a β-carbonyl-1,4-dihydropyridine derivative with a high antimutagenic [16,17] and genoprotective activity [12], protects human cells not only from DNA strand breaks and micronuclei, but also stimulates apoptosis *in vitro*. Nevertheless, at high levels of genotoxic stress this compound seems to have a higher efficiency in stimulating DNA repair, not the apoptosis pathway.

## 2. Materials and methods

### 2.1. Reagents

Sodium 3,5-bis-ethoxycarbonyl-2,6-dimethyl-1,4-dihydropyridine-4-carboxylate (AV-153) was synthesized in the Laboratory of Membrane-Active Compounds and β-diketones of the Latvian Institute of Organic Synthesis. Stock solutions were prepared in PBS and kept in the dark at 4 °C.

### 2.2. Lymphocyte isolation, treatment and incubation

Two similar groups of volunteer donors, non-smoking healthy females 25–43 years old were used here: group 1 (donors A, B and C) for study on the background levels of biological end-points and group 2 (donors B, D and E) for study on radiation-induced effects. One of the donors (donor B) was common for the two groups. Venous blood was taken from the donors before they had breakfast to diminish possible effects of diet. The blood was collected in heparinized tubes and cooled at 4 °C for about 30 min. Mononuclear lymphocytes were obtained on a histopaque-1077 (ICN) gradient. After isolation lymphocytes were washed twice in RPMI-1640 (Sigma–Aldrich) at 4 °C and seeded onto plates at a concentration of $5 \times 10^5$ $ml^{-1}$ in RPMI-1640 supplemented with 10% inactivated fetal bovine serum (FBS; Gibco), L-glutamine and 0.1% gentamicine. To assess cell viability, the standard Trypan-blue exclusion test was used according to the manufacturer's indications (Sigma–Aldrich).

Cells were gamma-irradiated in a $^{60}Co$ therapy source (Gammatron, Siemens, Germany) at a dose rate 0.8 Gy/min to a total dose of 2 Gy. For comet assays, irradiation was performed on ice to prevent DNA repair during this procedure. Control plates in all experiments were sham-irradiated under similar conditions. Equal aliquots of AV-153 at final concentrations of $10^{-10}$–$10^{-5}$ M were added to cell suspensions immediately after irradiation. After treatment cells were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.3. Assessment of DNA damage by alkaline single-cell gel electrophoresis (comet assay)

Cell samples were collected after 3 h of incubation with AV-153. In the case of irradiated cells, samples were also harvested just after irradiation in order to check the induction of DNA damage. DNA strand breaks were analyzed by comet assays as recommended in [18] and described previously [13,19]. After electrophoresis and fixation in 96% alcohol, slides were stained with ethidium bromide (2 μg/ml) and analyzed using a Leica DMRXA2 fluorescence microscope. All slides were coded before scoring. The level of DNA damage was assessed in arbitrary units (a.u.) according to Ref. [20].

### 2.4. Analysis of clastogenic effects by use of the micronucleus test

The conventional cytokinesis-blocked micronucleus (MN) assay was conducted according to the procedures of Fenech and Morley [21]. Briefly, 5 μg/ml lectin (phytohaemagglutinin, PHA, Sigma–Aldrich) was added to 5 ml lymphocyte culture at 0 min of incubation and 6 μg/ml cytochalasin B (Sigma–Aldrich) was added at 48 h of incubation after irradiation. Cells were harvested after 72 h incubation, gently washed in hypotonic solution, fixed in cold Carnoy's solution and dropped onto cold slides. After air-drying, slides were stained with May-Grünwald and Giemsa and analyzed at 600× magnification using an Olympus BX51 light microscope. One thousand binucleated (BN) cells were analyzed for the presence of micronuclei (MNi) in experiments with irradiated cells. When effects of AV-153 on endogenous rates of MNi were studied, 2000 BN cells were scored. The nuclear division index (NDI) was assessed by counting cells with 1, 2, 3 and more nuclei per 500 cells in total and using the following equation:

$$\text{NDI } (\%) = \frac{(N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)}{500},$$

where $N_1$–$N_4$ are the numbers of scored cells with 1–4 (or more) nuclei.

### 2.5. Assessment of apoptosis

Morphological analysis of apoptotic cells was carried out simultaneously with the cytokinesis-blocked micronucleus assay [21] in accordance with Ref. [22]. The conventional morphological criteria were used for recognition of apoptotic cells as described in Ref. [22]. Briefly, chromatin condensation and/or nuclear fragmentation with an intact cytoplasmic membrane served as main criteria. The total number of apoptotic cells per 1000–2000 analyzed BN cells was scored for each sample. Additionally, the results of analysis with May-Grünwald–Giemsa staining and optical microscopy were compared with results of fluorescence staining with ethidium bromide (2 μg/ml). Very close mean values of the results of these two analyses were obtained, as well as a statistically significant correlation between the results (Spearman, $n = 9$, $r = 0.70$, $P < 0.05$).

### 2.6. Statistical analyses

For each of the five donors, experiments were performed independently and repeated at least two times. The results were expressed as mean ± SD. The data were compared with the unpaired Student's *t*-test; Pearson correlation, Spearman rank-order analysis and regression analysis were also used from the STATISTICA software package (StatSoft, Tulsa, OK, USA).

## 3. Results

### 3.1. Effects of AV-153 on endogenous levels of DNA damage, MNi and apoptosis in human lymphocytes

As mentioned above, AV-153 stimulates DNA strand-break rejoining analyzed by comet assays. To determine whether AV-153 could affect the spontaneous incidence of apoptosis as well as chromosomal damage in parallel with its effect on DNA strand breakage, PHA-stimulated lymphocytes were incubated for 72 h with various concentrations of AV-153 from $10^{-10}$ to $10^{-5}$ M and the frequencies of apoptotic and micronucleated (MN) cells were studied. In parallel experiments, DNA strand breaks in non-stimulated lymphocytes incubated for 3 h in the presence of the same concentrations of AV-153 were analyzed with the comet assay. If AV-153 does not modulate apoptosis, it would be expected that it has no effect on the spontaneous frequency of apoptosis or the number of apoptotic cells following the reduction of DNA damage in the presence of AV-153. But as can be seen in Fig. 1, while the compound significantly reduced the spontaneous level of DNA strand breaks and induced a very slight decrease in MN cell frequency, it stimulated the appearance of apoptotic cells in a concentration-dependent manner. Maximal efficiency of AV-153 in reducing DNA damage (76%) was observed at a concentration of 1 nM, while maximal (42%) increase of apoptosis was at a concentration two orders of

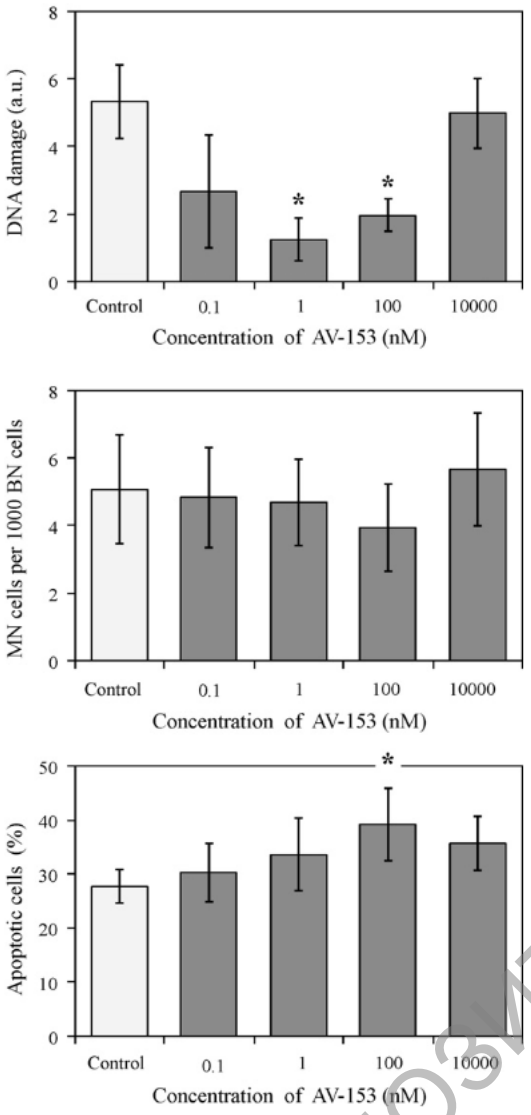


**Fig. 1.** Effect of different concentrations of AV-153 on the endogenous levels of DNA strand breaks, MN cells and apoptosis in human lymphocytes. Means and standard deviations are presented. *$P < 0.05$ when compared with the control level (Student's *t*-test).

magnitude higher. No evident effect of AV-153 on cell proliferation, assessed as NDI, was found (Table 1).

### 3.2. Effects of AV-153 on radiation-induced levels of the end-points studied

Further experiments with lymphocytes acutely irradiated with 2 Gy showed a significant increase in radiation-induced levels of all end-points studied (Fig. 2A). However, when cells were post-incubated in the presence of AV-153, a significant reduction of these end-points, including apoptosis, was observed (Fig. 2A). Thus in contrast to its effect on the endogenous rate of apoptosis, AV-153 affected apoptosis induced by genotoxic stress negatively. At the same time, no significant differences were found between the values of NDI in lymphocyte cultures after irradiation and incubation with AV-153 (Table 1).

Dose–response analysis of data on reduction of DNA damage, MN cells and apoptosis in the presence of AV-153 revealed that maximal efficiency of the compound was reached at nanomolar concentrations and its decrease at higher concentrations fitted a logarithmic function (Fig. 2B). The highest efficiency of AV-153 was found for reduction of DNA strand breaks, with a mean value for three donors of about 67%. The mean values of the efficiency in reducing MNi and apoptotic cells did not exceed 50% (Fig. 2B).

**Table 1**
Nuclear division index (NDI) in human lymphocytes after irradiation and culturing in the presence of AV-153.

| Cell treatment | NDI, mean ± SD |
|---|---|
| Control | 1.72 ± 0.14 |
| $+10^{-10}$ M AV-153 | 1.76 ± 0.09 |
| $+10^{-9}$ M AV-153 | 1.67 ± 0.11 |
| $+10^{-7}$ M AV-153 | 1.60 ± 0.12 |
| $+10^{-5}$ M AV-153 | 1.67 ± 0.05 |
| Control | 1.63 ± 0.08 |
| 2 Gy | 1.77 ± 0.10 |
| 2 Gy + $10^{-10}$ M AV-153 | 1.68 ± 0.02 |
| 2 Gy + $10^{-9}$ M AV-153 | 1.74 ± 0.11 |
| 2 Gy + $10^{-7}$ M AV-153 | 1.67 ± 0.01 |
| 2 Gy + $10^{-5}$ M AV-153 | 1.66 ± 0.05 |

In order to better understand the relationship between the effects of AV-153 on DNA damage, MNi and apoptosis, we carried out correlation analysis of data on these end-points in cells after irradiation and post-incubation with AV-153. The usefulness of correlation analysis of MN cells, apoptosis and other end-points for assessment of possible processes taking place in human lymphocytes has been shown previously in other investigations [22,23]. Not surprisingly, the analysis revealed a strong correlation between the observed mean levels of unrepaired DNA strand breaks after 3 h incubation, and of MN cells (Pearson analysis, $r = 0.996$, $P < 0.001$; data not shown), because MNi can originate from the unrepaired breaks in DNA molecules. More importantly, the mean numbers of apoptotic cells that were reduced in the presence of AV-153 directly correlated with the reduced numbers of DNA strand breaks (Fig. 3A, Pearson analysis, $r = 0.961$, $P < 0.05$) and MN cells (Fig. 3B, Pearson analysis, $r = 0.989$, $P < 0.01$). These results suggest that in spite of its potency to stimulate apoptosis (data on endogenous levels), the main mechanism of AV-153 action in cells after genotoxic stress is stimulation of DNA repair, which is followed by a proportional decrease of damaged cells entering the apoptotic pathway.

## 4. Discussion

Here, we report the results of pilot investigations on modulation of defense systems in human cells, namely DNA repair and apoptosis, by AV-153, a compound of the 1,4-dihydropyridine series with high genoprotective activity, which has been shown previously to modulate DNA repair through regulation of PARP activity [13]. In this report, we demonstrate that in parallel with reducing DNA damage, AV-153 can also modulate apoptosis *in vitro*. It should be noted here that the wide range of AV-153 concentrations studied, from $10^{-10}$ to $10^{-5}$ M, were considerably lower than cytotoxic concentrations ($IC_{50}$ of AV-153 for human cells is between 10 and 15 mM) [12].

Cultures of human lymphocytes isolated from peripheral blood of five healthy donors were used in this study rather than human cell lines, in order to better reproduce the inter-individual variability in populations. The observed background, i.e. the endogenous levels of the end-points studied, were homogenous and close to published data that can be considered as the reference levels for the general population. Thus the usual endogenous level of DNA strand breaks in non-smoking healthy donors is about 15–30 a.u., according to [24,25], and an international collaborative study showed that the median MN cell frequency in non-exposed (i.e. normal) subjects is 6.5‰ [26], while the baseline apoptosis frequency in PHA-stimulated lymphocyte cultures after 72 h incubation is variable and can be about 1–15% [27,28].

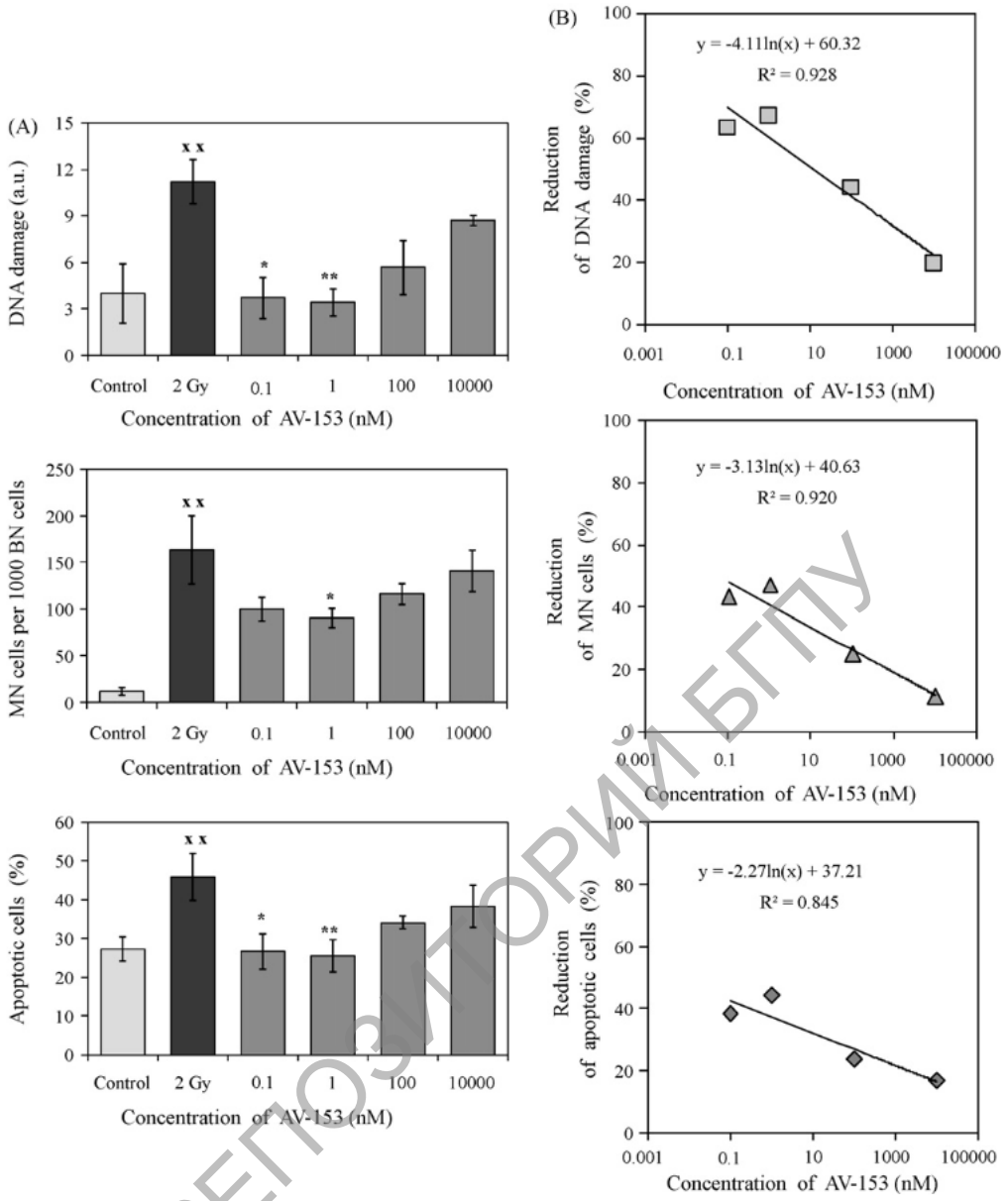




**Fig. 2.** (A) Effect of different concentrations of AV-153 on the radiation-induced levels of DNA strand breaks, MN cells and apoptosis in human lymphocytes; $^{xx}P<0.01$ when compared with the control level, $^{*}P<0.05$ and $^{**}P<0.01$ when compared with radiation-induced level (Student's *t*-test). (B) Concentration-dependent reduction of the endpoints (regression analysis).

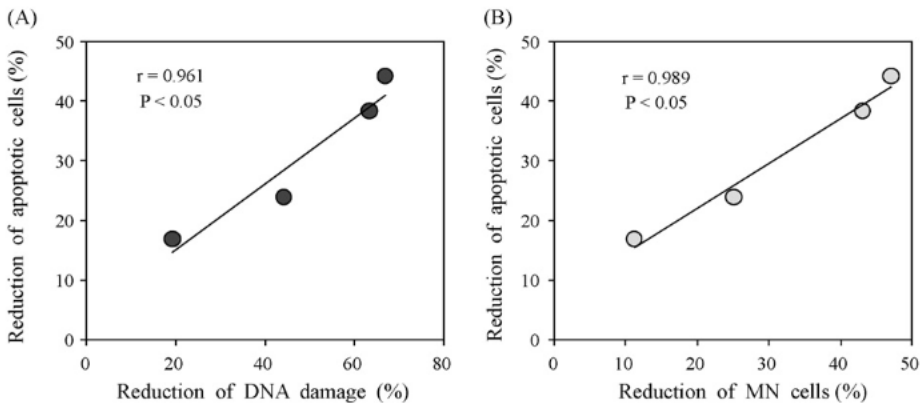


**Fig. 3.** Correlation between efficiency of AV-153 in reducing radiation-induced DNA strand breaks and apoptosis (A) and MN cells and apoptosis (B). Data are from Fig. 2B.

In this study we found that a protective action of AV-153 in human cells can be observed not only at the level of DNA strand breaks analyzed with the alkaline comet assay, but also at the level of chromosomes since the MN test is commonly used for the assessment of chromosome fragments as well as chromosome loss and rearrangements [29]. Anticlastogenic effects of AV-153 have been previously found in polychromatic erythrocytes in the bone marrow of laboratory mice treated with the alkylating agent ethylmethanesulfonate [30], and in a parallel study similar properties were shown in the murine lymphoma cell subline LY-R after X-irradiation and incubation in the presence of nanomolar concentrations of the compound [31]. In this setting, it is important to underline the high efficiency of nanomolar concentrations of AV-153, as well as the logarithmic dose–response curves for DNA strand breaks, MN cells and apoptosis in the present study. Similar efficiencies and logarithmic dose–responses for DNA strand breaks arising spontaneously or after various genotoxic stresses have been previously described in human lymphocytes and in the cell lines HL-60 and Raji incubated with AV-153 [12].

Apoptosis or programmed cell death is a normal physiological process to protect tissues during development or after induction of cell damage through self-destruction and elimination of defective cells. The apoptosis assay gives information about damaged cells that were not detected as MN cells and it has been proposed to integrate the apoptosis assay into current DNA-damage assays [22]. Further, apoptosis in human lymphocytes is considered as a potential biological dosimeter thanks to its sensitivity and reproducibility in individual donors [32].

We found that AV-153 stimulates apoptosis in human lymphocytes (Fig. 1), but more interesting is that AV-153 had the inverse effect on radiation-induced levels of apoptosis (Fig. 2). These inverse effects in modulation of apoptosis are not the only ones observed for 1,4-dihydropyridines; there are many $Ca^{2+}$ channel antagonists or agonists for which a large body of evidence of modulation (stimulation or suppression) of apoptosis has been reported. More importantly, some atypical 1,4-dihydropyridines (glutapyrone and tautopyrone) which do not represent typical $Ca^{2+}$ channel blockers also demonstrate inverse effects in cardiac and brain tissues of mice *ex vivo*: they stimulate the increase of pro-apoptotic caspase-3 whereas they reduce its expression caused by azido-thymidine, a mitochondria-compromising anti-HIV drug [33].

More specifically, AV-153 showed a dual impact on cellular defense systems: it simultaneously reduced the endogenous levels of DNA damage and stimulated apoptosis (Fig. 1). Among other radioprotectors, some of the thiolamines protect cells against both radiation-induced DNA breaks and apoptosis, and their high efficiency in donating hydrogen atoms is considered as one of the mechanisms of impact on the cellular repair systems [34]. At the same time they act as inducers of apoptosis in unirradiated cells, perhaps due to their pro-oxidant properties [34]. In contrast to these thiolamines, AV-153 stimulated apoptosis in un-irradiated cells at low, non-genotoxic concentrations, at which it usually stimulates DNA repair. It should be mentioned here that the efficiency of 1,4-dihydropyridines in the repair of recessive mutations in *Drosophila melanogaster* strongly correlated with their electron-donating properties, and AV-153 was among the compounds with the highest value of electron-donating potency and the highest efficiency in repair [17].

In summary, the results of this pilot study suggest that at low (endogenous) levels of DNA damage and hence low energy consumption, AV-153 restores a gradually decreasing pool of ATP used in cells for various activities including defense systems, and thus acts as a simultaneous stimulator of DNA repair and apoptosis (Fig. 1). But, as mentioned above, during genotoxic stress the majority of cellular ATP is used for poly(ADP-ribose) synthesis during the first minutes of BER and, to a lesser degree, for later processing of stress-induced apoptosis. Taking this in mind, we suggest that during genotoxic stress DNA repair becomes the major target of AV-153 through recovery of ATP pools and hence stimulation of poly(ADP-ribose) synthesis, while apoptosis is not modulated directly so that highly damaged cells are not repaired in the presence of the compound. The level of radiation-induced apoptotic cells in our study directly followed the reduction of DNA damage in irradiated cells after incubation in the presence of different concentrations of AV-153 (Fig. 3). Further studies are required in order to understand the detailed mechanisms of the biological action of AV-153 and similar compounds with high genoprotective efficiency.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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