An *in vitro* based investigation into the cytotoxic effects of D-amino acids

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In the present study, cytotoxic effects of D-Ala, D-Pro and D-Lys are demonstrated. In an effort to study the possible mechanisms of the observed cytotoxicity, catalase activity, H_2O_2 generation, and apoptotic activity were measured in HeLa and MCF-7 cell lines. Although D-Lys is a poor substrate for DAO and therefore low H_2O_2 was detected, it was shown to provoke severe impairment of cellular integrity and survival. Interestingly, a very good substrate for DAO, such as D-Pro, did not substantially reduce cell viability. On the other hand, a moderate substrate for DAO, represented by D-Ala, was shown to moderately trigger toxicity in the tested cell lines. Although a correlation between the *in vitro* cytotoxicity of D-amino acids and the amount of H_2O_2 produced was absent, there was a good agreement between the ability of D-amino acids to trigger apoptosis and to provoke toxicity. Our results indicate that the toxicity of D-amino acids does not appear to be solely mediated by H_2O_2. Therefore, we hypothesize that other possible contributing apoptosis-mediated pathways might cause the observed toxicity.

Keywords: D-amino acids, toxicity, HeLa cells, MCF7 cells, catalase, apoptosis, oxidative stress

Numerous innovations in analytical methods have demonstrated the presence of D-amino acids in eukaryotes, including mammals (1–3). The most abundant D-amino acids in mammals are D-serine and D-aspartate (4). D-alanine is also present in mammals at moderate levels (4, 5). Recently, physiological roles of these D-amino acids in humans have been reported. For example, D-serine has been found to modulate the activity of *N*-methyl-D-aspartate (NMDA) receptor in the brain (6). It is postulated that D-serine binds to the coagonist binding site of the MDA receptor, which is involved in numerous physiological and pathological processes, including synaptic plasticity, learning, memory, neuronal cell migration, and neural diseases (7, 8). Furthermore, D-aspartate has

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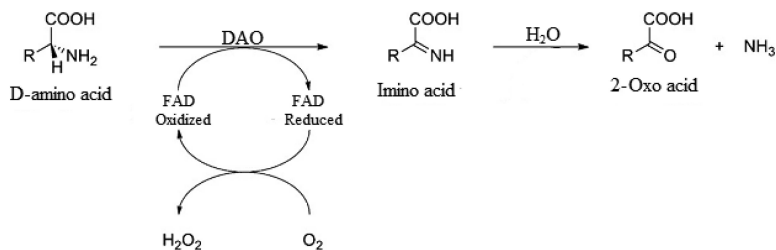* Correspondence; e-mail: S.Bardaweel@ju.edu.jo

Fig. 1. The D-amino-acid oxidase-catalyzed reaction. D-amino acid is oxidized to an imino acid with concurrent reduction of flavin adenine dinucleotide (FAD). The reduced FAD is subsequently oxidized while molecular oxygen is reduced to hydrogen peroxide. Consequently, the imino acid undergoes a non-enzymatic hydrolysis to the corresponding 2-oxo acid and ammonia.

been detected in nervous and endocrine tissues and is believed to be involved in the regulation of hormone release (9, 10), melatonin (11) and testosterone syntheses (12, 13). Additionally, the presence of free D-aspartate in the nuclei of mammalian tissues may suggest a role of D-aspartate in gene expression (14).

In mammals, D-amino acid oxidase (DAO) and D-aspartate oxidase (DAspO) catalyze the oxidative deamination of D-amino acids into the corresponding α-keto acids. D-amino acid oxidase was the first enzyme discovered in mammals (15). It has been found that DAO contains flavin adenine dinucleotide (FAD) as a prosthetic group and catalyzes the oxidative deamination of D-amino acids according to the reaction mechanism shown in Figure 1. During the initial step of catalysis, oxidation of D-amino acid into the imino acid and concomitant reduction of FAD occur. Subsequently, the reduced FAD is oxidized by oxygen to release hydrogen peroxide, and the imino acid is non-enzymatically hydrolyzed to produce the corresponding 2-oxo acid and ammonia (15, 16). DAO has a wide range of substrate specificity and metabolizes a large number of neutral and basic D-amino acids. Similarly, D-aspartate oxidase has FAD as a prosthetic group and catalyzes the oxidative deamination of acidic D-amino acids such as D-aspartate and D-glutamate, to produce the corresponding 2-oxo acid, hydrogen peroxide and ammonia (15, 16).

A major characteristic of DAO and DAspO is their high specificity towards the D-isomers of amino acids; they are almost inactive towards the corresponding L-isomers. Broad substrate specificity of DAO is a plus when considering the overall concentration of the D-isomer of amino acids in food and drinks. The D-isomers of proline, methionine, isoleucine, alanine, valine and phenylalanine are good substrates for DAO (17). Nevertheless, D-ornithine, *cis*-4-hydroxy-D-proline, *N*-acetyl-D-Ala and D-Lys are oxidized at very low rates by DAO (17).

Although potential toxicity has been linked to D-amino acids, the fundamental mechanism by which toxicity occurs is not fully understood. In animal studies, administration of D-amino acids to rats and chicks resulted in growth inhibition. In addition, D-amino acids accumulation in certain tissues caused serious damage such as suppression of the synthesis of glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase (18). It has been suggested that the toxicity of D-amino

acids arises from the oxidative damage to cells by H_2O_2 formed upon the oxidation of D-amino acids (19). Supporting this suggestion, H_2O_2 appears to readily cross cellular membranes and cause oxidative damage to DNA, proteins and lipids (20–22). Furthermore, H_2O_2 has been found to induce apoptosis of tumor cells *in vitro via* activation of the Caspase cascade (23).

To assess the *in vitro* cytotoxicity of D-amino acids and to gain further insights into the molecular mechanisms that provoke the observed cytotoxicity, we investigated the cytototoxic effects and possible mechanisms of cytoxicity of three D-amino acids, D-alanine, D-proline and D-lysine, using several cell line models.

<div align="center">EXPERIMENTAL</div>

Mammalian cell lines and cell culture

The cell lines under investigation were human breast adenocarcinoma MCF7, MDA-MB-231 and MDA-MB-453 cell lines, the human ductal breast epithelial tumor cell line T47D, human pancreatic carcinoma MIA PaCa-2 cell line, T-cell leukemia HPB-MLT cell line, human Burkitt's lymphoma Raji cell line, EBV-negative Burkitt's lymphoma BJAB cell line, the human colon adenocarcinoma Caco-2 cell line, and human epithelial carcinoma HeLa cell line. Cell lines were cultured in high glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) containing 10 % heat inactivated fetal bovine serum (HI-FBS) (Invitrogen), 2 mmol L^{-1} L-glutamine, 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. Cell lines were maintained at 37 °C in a 5 % CO_2 atmosphere of 95 % humidity. The cells were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, optimal plating densities were determined.

Cell proliferation by MTS assay

MTS assays were carried out using the CellTiter 96 aqueous one solution cell proliferation assay (Promega Co., USA) according to the manufacturer's protocol. The yellow tetrazolium dye [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4--sulfophenyl)-2*H*-tetrazolium, inner salt] was reduced by metabolically active cells into the intracellular purple formazan product that is soluble in the tissue culture medium (24). The conversion of MTS into the aqueous soluble formazan product was accomplished by dehydrogenase enzymes found in metabolically active cells (24). The quantity of formazan product as measured by the amount of 490 nm absorbance was directly proportional to the number of living cells in the culture. To ensure exponential growth throughout the experimental period, as well as a linear relationship between absorbance at 490 nm and cell number when analyzed by the MTS assay, the following densities were used for each cell line: 1×10^4 cells per well for the MCF-7, MDA-MB-231, MDA--MB-453, T47D, MIA PaCa-2, Caco-2 and HeLa cell lines, and 2×10^4 cells per well for the HPB-MLT, BJAB and Raji cell lines.

D-amino acids cytotoxicity in MCF-7, MDA-MB-231, MDA-MB-453, T47D, MIA PaCa-2, Caco-2, and HeLa cell lines

For the assay, cells were washed three times with phosphate buffered saline (PBS). PBS was then decanted and cells were detached with the non-enzymatic cell dissociation complex (Sigma Chemical Co., USA). Medium was added to a volume of 10 mL. The cell suspension was centrifuged at 1000 x g at 4 °C for 10 min and the pellet was re-suspended in 10 mL of medium to make a single cell suspension. Cells were counted using the trypan blue exclusion method and seeded into 96-well plates at the desired densities. A hundred µL of cell suspension was seeded and incubated per well to allow for cell attachment. After 24 h, the cells were treated with D-amino acids. Each D-amino acid solution, at a concentration of 1 mol L^{-1}, was prepared in BPS and passed through a 0.02-µm filter, then further diluted in the medium to produce the desired concentration. Cells were treated with various concentrations of each D-amino acid in four triplicates. Concentration ranges were 10–150 mmol L^{-1} D-Ala, 10–250 mmol L^{-1} D-Pro, and 1–60 mmol L^{-1} D-Lys Treated cells were incubated in a 37 °C 5 % CO_2 incubator for 24 or 48 h. At the end of the exposure time, MTS assays were carried out as previously described. The absorbance at 490 nm was read on a plate reader (Tecan Group Ltd., Switzerland). Control wells were prepared under the same experimental conditions but without the treatment with D-amino acids.

D-amino acids cytotoxicity in HPB-MLT, BJAB and Raji cell lines

Viable cells were counted and diluted with medium to give a final density of 2×10^4 cells per well. A hundred µL of cell suspension per well was seeded into 96-well plates. After 24 h, the cells were treated with D-amino acids. Cells were treated with various concentrations of each D-amino acid in four triplicates. The plates were incubated for 24 or 48 h at 37 °C 5 % CO_2. Cell viability was measured using the MTS assay as previously described. Control wells without the treatment with *D*-amino acid were prepared under the same experimental conditions.

Preparation of cell lysates

For each assay, HeLa and MCF-7 cells were grown in six-well plates. When the cells reached 80 % confluence, they were incubated with either 50 mmol L^{-1} D-Ala, 50 mmol L^{-1} D-Pro or 10 mmol L^{-1} D-Lys for 24 h at 37 °C in 95 % air, 5 % CO_2. At the end of the treatment, cells were harvested with the non-enzymatic cell dissociation complex before centrifuging at 1000 x g at 4 °C for 5 min. The pellets were re-suspended in 2 mL of solution consisting of 50 mmol L^{-1} phosphate buffer (pH 7.0), then lysed by several freezing-thawing cycles. The lysates were centrifuged at 3000 x g at 4 °C for 5 min, and the supernatants were frozen at –20 °C until used. Protein concentrations of the supernatants were determined by the Bio-Rad protein assay (Bio-Rad Inc., USA) using bovine serum albumin (BSA) as a standard.

Catalase assay

Catalase activity was determined as described previously by Aebi (25). The working solution was prepared by adding H_2O_2 to phosphate buffer. First, 50 mmol L^{-1} phosphate buffer (pH 7.0) and 30 mmol L^{-1} H_2O_2 solutions were prepared. Then, 0.3 mL of 30 mmol L^{-1} H_2O_2 solution was added into 100 mL of 50 mmol L^{-1} phosphate buffer (pH 7.0) to yield the working solution. Using phosphate buffer (50 mmol L^{-1}, pH 7.0) as a blank, absorbance of the working solution at 240 nm was measured and then adjusted to lie between 1.15 and 1.20 by diluting with buffer or adding more H_2O_2. When the enzyme assay was undertaken, 0.4 mL of the cell homogenate was added to 3.6 mL phosphate buffer (50 mmol L^{-1}, pH 7.0) to yield the assay solution. In the test cuvette, 1 mL of the working solution was added to the assay solution and the decrease of absorbance at 240 nm was recorded over a reaction course of 2 min. In a blank cuvette, 1 mL of phosphate buffer (50 mmol L^{-1}, pH 7.0) was added to 2 mL of the assay solution and the change in absorbance at 240 nm was recorded over a reaction course of 2 min.

H_2O_2 determination

H_2O_2 released by control cells or by cells (5×10^6 cells) treated with either 50 mmol L^{-1} D-Ala, 50 mmol L^{-1} D-Pro or 10 mmol L^{-1} D-Lys for 24 h was measured with an Amplex® Red hydrogen peroxide/peroxidase assay kit (Invitrogen) according to the manufacturer's protocol. The reaction mixture containing 50 µmol L^{-1} Amplex® Red reagent and 0.1 U mL^{-1} horseradish peroxidase in Krebs-Ringer phosphate was prepared and incubated at 37 °C for 10 min. After D-amino acid treatment for 6 h, 100 µL of the reaction mixture was added to each microplate well and incubated at 37 °C for 30 min before absorbance at ~560 nm was read on a plate reader. To correct for background absorbance, the value derived from the untreated control was subtracted for each point.

Cell death detection ELISA^{plus} assay

The cell death detection ELISA^{plus} assay (Roche, USA) was used to evaluate the apoptotic activity in the cells after incubation with either 50 mmol L^{-1} D-Ala, 50 mmol L^{-1} D-Pro or 10 mmol L^{-1} D-Lys for a period of 24 h. After treatment, the cells were lysed to release cytoplasmic histone associated-DNA-fragments that indicate that apoptosis activity. Absorbance was read at 405 nm. Higher absorbance correlated to increased apoptosis. Negative controls were obtained from untreated cells under the same experimental conditions.

RESULTS AND DISCUSSION

In vitro *cytotoxicity of D-amino acids*

Cytotoxic effects of D-Ala, D-Pro and D-Lys were examined *via* an *in vitro* system using several cancerous cell lines, as described in the Experimental section. Our choice of D-amino acids was based on their susceptibility to be metabolized by DAO. D-Pro

Table I. Percentage cell survival following exposure to D-amino acids

Cell line	D-amino acid		
	D-Pro[a]	D-Ala[a]	D-Lys[b]
HeLa	86 ± 2	63 ± 2	33 ± 1
MCF7	85 ± 3	78 ± 3	37 ± 2
T47D	83 ± 3	76 ± 3	28 ± 2
MD A-MB-231	86 ± 6	57 ± 4	29 ± 3
MDA-MB-453	82 ± 3	60 ± 3	26 ± 3
MI A PaCa-2	92 ± 4	86 ± 4	69 ± 3
HPB-MLT	95 ± 1	87 ± 1	77 ± 4
Raji	94 ± 2	88 ± 2	71 ± 3
BJAB	93 ± 2	87 ± 1	73 ± 6
Caco-2	89 ± 3	79 ± 3	41 ± 3

Mean value ± SD (n = 12).
[a] c = 50 mmol L^{-1}
[b] c = 10 mmol L^{-1}

represents an optimal substrate for DAO with a high turnover rate (17) while D-Ala represents a moderate substrate for DAO with an intermediate turnover rate (17). However, D-Lys represents a very poor substrate for DAO with a low turnover rate (17). According to our results, 10 mmol L^{-1} D-Lys and 50 mmol L^{-1} D-Ala exhibited remarkable cytotoxic effects when incubated for 24 h (Table I), or 48 h (data not shown), with the cell lines under investigation, whereas 50 mmol L^{-1} D-Pro exhibited a minimal cytotoxic ef-

Table II. IC$_{50}$ values of D-amino acids

Cell line	IC_{50} (mmol L^{-1})	
	D-Ala	D-Lys
HeLa	73 ± 6	18 ± 3
MCF7	73 ± 6	22 ± 4
T47D	74 ± 4	17 ± 5
MDA-MB-231	66 ± 7	15 ± 2
MDA-MB-453	52 ± 2	14 ± 3
MIA PaCa-2	75 ± 5	33 ± 6
HPB-MLT	102 ± 9	27 ± 5
Raji	85 ± 7	28 ± 5
BJAB	98 ± 5	29 ± 4
Caco-2	77 ± 7	23 ± 2

Mean value ± SD (n = 12).

fect under the same experimental conditions (Table I). Concentration-dependent cyto-toxicity was observed when D-Ala and D-Lys were incubated for 24 h with the cell lines under investigation and thus IC_{50} values were determined (Table II). To confirm that the toxicity observed with D-amino acids was specific to D-enantiomer and not to L-enan-tiomer, studies under conditions where cells were treated with various concentrations of L-Ala, L-Pro, or L-Lys were undertaken. Our results indicate that toxicity is only obser-ved for D-isomer but not L-isomer at concentrations up to 150 mmol L^{-1} of amino acids (data not shown).

Several potentially harmful byproducts of normal cellular metabolism directly in-fluence cellular functions and survival (26). Despite the fact that such harmful byprod-ucts are vital for signal transduction pathways (27) and reduction-oxidation status (26), overproduction of these highly reactive metabolites can initiate lethal chain reactions that ultimately lead to impairment of cellular integrity and survival (26). Although the toxicity of D-amino acids has been recognized, the reason for their toxicity has not been revealed yet. Several mechanisms were proposed to explain the toxicity associated with D-amino acids in eukaryotes. The most broadly recognized pathway of D-amino acids' toxicity in eukaryotes appears to be related to the oxidative damage to cells by H_2O_2 that occurs upon the oxidation of D-amino acids by DAO and/or DAspO (19). In an effort to study the mechanism of cytotoxicity, the catalase assay and direct H_2O_2 determination were undertaken, as described below.

Catalase activity

HeLa and MCF-7 cell lines were selected as representative cell lines to investigate the mechanisms of the observed cytotoxicity of D-amino acids. Around 5×10^6 HeLa cells and MCF-7 cells were incubated in 7 mL medium with either 50 mmol L^{-1} D-Ala, 50 mmol L^{-1} D-Pro, or 10 mmol L^{-1} D-Lys separately for 24 h. The cells were washed with PBS and collected for the catalase assay. After addition of the H_2O_2 working solution, the enzyme activity was determined by following the reduction of absorbance of cell ho-mogenate over two minutes. Comparing the specific activities (international unit/pro-tein concentration) of catalase, it is noticeable that catalase activities of HeLa cells and MCF-7 cells were significantly increased, about 10- and 8-fold, respectively, compared to
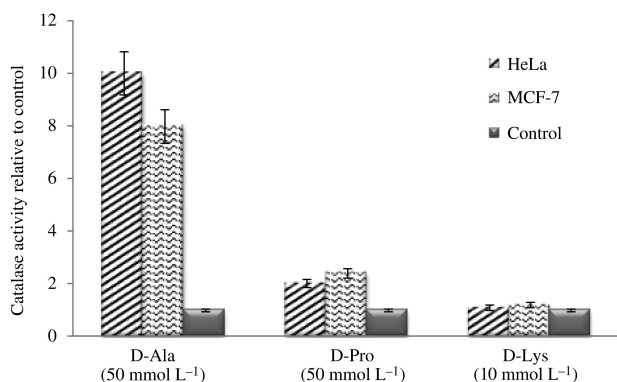

Fig. 2. Catalase activity following D-amino acid treatment for 24 h. Data represent the mean ± SD (n = 3) from blots.

the untreated control, in response to the addition of 50 mmol L^{-1} D-Ala (Fig. 2). Nevertheless, only a 2-fold increase of catalase activity in HeLa cells and a 2.4-fold increase in MCF-7 cells were observed when treated with 50 mmol L^{-1} D-Pro under the same experimental conditions. In contrast, compared to the untreated controls of each cell line, only a 10-15 % increase of catalase activity in both HeLa cells and MCF-7 cells was observed when treated with 10 mmol L^{-1} D-Lys.

H_2O_2 determination

To support the H_2O_2-mediated toxicity pathway of D-amino acids and to establish a clear correlation between the cytotoxic effect of D-amino acids and their DAO turnover rate, we investigated the cytotoxic effects of various D-amino acids, including a good substrate for DAO with a high turnover rate (17) represented by D-Pro, a moderate substrate for DAO with an intermediate turnover rate (17) represented by D-Ala and a very modest substrate for DAO with a low turnover rate represented by D-Lys (17).

The amount of H_2O_2 released into the culture medium after a 6 h treatment was monitored in HeLa and MCF-7 cell lines. After the 50 mmol L^{-1} D-Ala treatment, H_2O_2 release increased the 13- and 10-fold compared to the untreated cells of HeLa and MCF-7 cells, respectively. Nevertheless, H_2O_2 release with 50 mmol L^{-1} D-Pro was enhanced only 2–3-fold relative to the untreated cells. Interestingly, H_2O_2 release with 10 mmol L^{-1} D-Lys was almost about the same as that of untreated cells (Fig. 3). Our results indicate that D-Lys, which is a poor substrate for DAO, and therefore low H_2O_2 is produced (Fig. 3), exhibited the highest toxicity amongst the tested D-amino acids (Tables I and II). On the other hand, D-Pro, which has a high DAO turnover rate, and thus high H_2O_2 level is expected to be produced, appears to exhibit minimal toxicity to most cell lines under investigation (Tables I and II).

In agreement with this, the catalase activity measured in HeLa and MCF-7 cell lines increased correspondingly to H_2O_2 produced after the D-amino acid treatment (Figs. 2 and 3). Catalase plays a central function in the antioxidant defense of the organism, as H_2O_2 is degraded to H_2O (28). As shown in Fig. 2, D-Ala treatment resulted in an 8–10
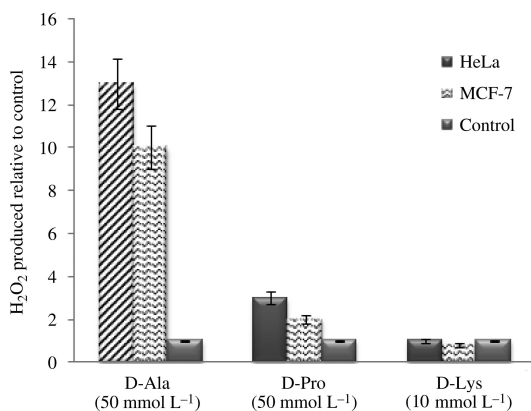

Fig. 3. H_2O_2 Accumulation in culture medium after D-amino acid treatment for 6 h. Data represent the mean ± SD (*n* = 3) from blots.

fold increase in catalase activity; nonetheless, significant accumulation of H_2O_2 occured (Fig. 3), which might outstrip the scavenging capacity of catalase and resulted in the observed toxicity. However, although the treatment with D-Lys altered neither the catalase activity nor the amount of H_2O_2 produced, it resulted in considerable toxicity, which was possibly not mediated by H_2O_2. Interestingly, a good substrate for DAO such as D-Pro showed only a 2–3 fold increase in catalase activity as well as in the amount of H_2O_2 released. As indicated by our results, D-Pro did not exhibit a noteworthy cytotoxicity compared to D-Ala or D-Lys.

Cell death detection ELISA[plus] assay

In an attempt to explore the possible mechanisms of cytotoxicity observed with D-Lys and D-Ala, we evaluated the apoptotic activities in HeLa and MCF-7 cells after treatment with D-amino acids. A cell death detection ELISA[plus] assay was used to verify whether the significant decrease in cell growth observed after the treatment with D-amino acids was the result of enhanced apoptosis in HeLa and MCF7 cell lines. Fig. 4 shows that maximum increase in apoptotic activity was observed after the treatment with 10 mmol L^{-1} D-Lys followed by 50 mmol L^{-1} D-Ala treatment in both cell lines. In contrast, D-Pro treatment did not appear to significantly increase the apoptotic activity in both cell lines. Interestingly, the apoptotic activities, measured by determination of DNA fragmentation, were in good agreement with the cytotoxic effects exhibited by D-Lys and D-Ala (Fig. 4).

Based on several pieces of evidence, it has recently been suggested that D-amino acids' toxicity is directly related to the formation of D-aminoacyl-tRNA. Under tight regulations of cellular protein syntheses and modifications, D-amino acids are usually not incorporated into protein structures. High substrate selectivity towards L-amino acids by aminoacyl-tRNA synthetases largely contributes to the preferential incorporation of L-amino acids in protein structures (29). However, it has been observed that *Escherichia coli* and *Bacillus subtilis* tyrosyl-tRNA synthetases can potentially catalyze the formation of D-tyrosyl-tRNA[Tyr] to the same extent as with L-enantiomer (30). Interestingly, extracts
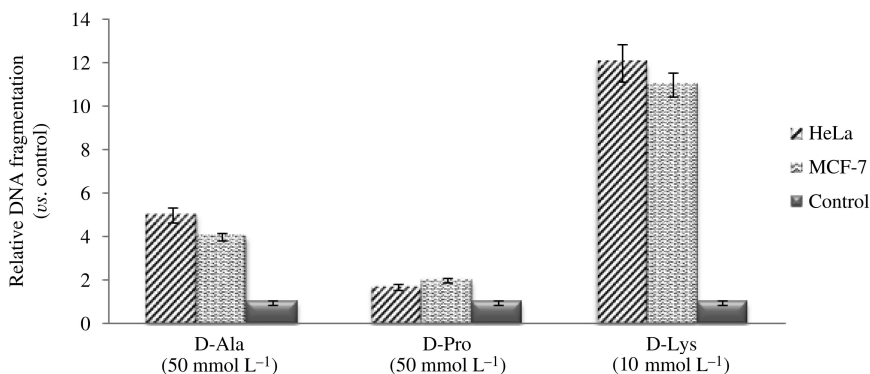

Fig. 4. Apoptotic effect after D-amino acid treatment for 24 h. Data represent the mean ± SD (n = 3) from blots.

of *E. coli*, yeast, rabbit reticulocytes, and rat liver were shown to have an enzyme activity capable of accelerating the hydrolysis of the ester linkage of D-Tyr-tRNA to release free tRNA and D-tyrosine (31).

The fate of D-aminoacyl-tRNA had never been studied previously. However, Dedkova *et al.* (32) demonstrated that D-methionine and D-phenylalanine charged on aminoacyl-tRNA were incorporated into proteins *in vitro*, suggesting that the toxicity of D-amino acids might arise from the mis-incorporation of D-amino acids in protein structures, resulting in the formation of functionless proteins.

Taken together, as demonstrated by our results, the toxicity of D-amino acids does not appear to be solely mediated by H_2O_2 as previously suggested. Using several measures, our results show that a correlation between the *in vitro* cytotoxicity of D-amino acids and the amount of H_2O_2 produced is absent, suggesting the presence of other possible contributing apoptosis-mediated pathways of toxicity. Ongoing studies that are attempting to unravel the correlation between the D-amino acids' toxicity and the formation of mis-folded proteins should shed light on other possible toxicity pathways.

CONCLUSIONS

In conclusion, in an attempt to study the possible mechanisms of the observed cytotoxicity of D-amino acids, we reported here the *in vitro* cytotoxic effects of D-amino acids with variable DAO turnover rates. Our results reveal that the oxidative damage which results from H_2O_2 production by DAO upon the oxidative deamination of D-amino acids, may not be the only mechanism contributing to toxicity. These findings may lend further support to the validity of the suggested hypothesis of D-amino acid mis-incorporation in protein structures, resulting in the formation of functionless proteins, hence, toxicity.

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