D-amino Acids: Prospects for New Therapeutic Agents

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***Abstract*—*D*-amino acids are predominantly produced and utilized by bacteria. They are involved in the synthesis and cross-linking of peptidoglycan. Furthermore, oxidative catabolism of *D*-amino acids, via the *D*-amino acid dehydrogensae pathway, sustains energy production for cellular functions. Only a few decades ago, it was largely believed that free *D*-amino acids were restricted to bacteria. Often, *D*-amino acids were considered as the by-products of bacterial metabolism. Nevertheless, the occurrence of *D*-amino acids in mammals was recently confirmed by means of sensitive and advanced analytical methods. The physiological functions of *D*-amino acids in humans are still under investigation. The presence of astonishing amounts of certain *D*-amino acids in the human Central Nervous System suggests a vital role of *D*-amino acids in neuromodulation. Apparently, both prokaryotes and eukaryotes maintain tight regulation on the occurrence of *D*-amino acids in their systems through specific metabolic pathways. Previously, it had been reported that the accumulation of certain *D*-amino acids results in cellular toxicity. In this study, we investigate the potential of *D*-amino acids as prospective new therapeutic agents. Antibacterial, antifungal, and cytotoxic activities were evaluated against representative cellular models. Our findings indicate that, although *D*-amino acids are toxic on their own, their efficacy can be significantly improved by synergism with other therapeutic agents. The ability to use lower doses of both, the drug and *D*-amino acids, may be beneficial for the development of combinational remedies against resistant pathogens or cancerous cells.**

***Index Terms*—D-amino acids, antibacterial, antifungal, cytotoxicity**

I. Introduction

Amino acids are mainly found as the *L*-enantiomeric form in all kingdoms of life. However, significant amounts of *D*-amino acids are produced by bacteria; the major producer of *D*-amino acids in the ecosystem [1]. In bacteria, *D*-amino acids are involved in the synthesis and cross-linking of peptidoglycan [1]-[3]. Recently, it was shown that *D*-amino acids are released by diverse bacterial species in the stationary phase of growth and act as agents controlling cell wall assembly and modification [4]. Additionally, *D*-amino acids are used as an energy source via the metabolism by the *D*-amino acid dehydrogensae enzyme; a flavoenzyme that oxidatively transforms *D*-amino acids into their corresponding α-keto acids [5]-[7].

Recently, advanced analytical techniques have illustrated that the presence of *D*-amino acids is not limited to prokaryotes, as previously suggested. Indeed, significant quantities of *D*-amino acids were detected in mammals with the aid of sensitive analytical methods. Several reports verified the occurrence of some *D*-amino acids in the mammalian Central Nervous System (CNS) and peripheral tissues [8]-[12]. Of these *D*-amino acids, *D*-serine has been most broadly studied. It was found that *D*-serine plays a vital role as a neurotransmitter in the human CNS by binding to the N-methyl- *D*-aspartate receptor (NMDAr) [9]-[11]. *D*-serine has been shown to mediate numerous physiological and pathological processes, including NMDA receptor transmission, synaptic plasticity, and neurotoxicity [12]-[15].

Potential toxicity has been linked to *D*-amino acids in both prokaryotes and eukaryotes. Nonetheless, the underlying mechanism of toxicity is not fully understood. In bacteria, *D*-amino acids are likely to induce physiologically relevant alterations in the peptidoglycan structure [16]. Furthermore, a mixture of *D*-amino acids, produced in bacteria, prohibited biofilm formation and enhanced the disassembly of existing biofilms in some bacterial populations, suggesting a signaling role of *D*-amino acids in bacteria [17].

In animal studies, administration of *D*-amino acids to rats and chicks resulted in growth inhibition [18]. In addition, serious damages such as suppression of the synthesis of glutamate oxaloacetate transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase resulted from *D*-amino acids accumulation in animal tissues [18].

II. Results and Discussion

Previous work in our laboratory investigated the antibacterial activities of certain *D*-amino acids, including *D*-alanine, *D*-lysine, *D*-serine and *D*-proline against numerous pathogens "unpublished" [19]. Nonetheless, the potency of the examined *D*-amino acids was relatively low; as the minimum inhibitory concentration (MIC) values were in the millimolar ranges. Our results indicate that *D*-lysine, followed by *D*-alanine, exhibited the most potent antibacterial activities amongst the examined *D*-amino acids [19].

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The efficacy of several antimicrobial agents can be improved by synergism. Thus, to investigate possible synergism, and to enhance the antibacterial activity of *D*-amino acids, combinational regimens of *D*-alanine and *D*-lysine with antibiotic Cefuroxime-sodium were evaluated against numerous bacterial strains; including *Bacillus subtilis* ATCC 11562, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 29425, *Pseudomonas aeuriginosa* ATCC 11921, and *Xanthomonas vesicatoria* ATCC 11633. The evaluation of the *D*-amino acids' MICs was conducted according to the National Committee for Clinical Laboratory Standards (NCCLS), using the broth microdilution method [20]. To assess synergism, the antibacterial effects of several combinations between Cefuroxime-sodium with either *D*-alanine or *D*-lysine were evaluated by the chequerboard test, as previously described [21], [22].

TABLE I. MIC OF *D*-AMINO ACIDS

	MIC (μg μL^{-1})			MIC (μg μL^{-1}) with Cefuroxime - sodium	
Strains/D-amino acid	*D*-ala	*D*-lys		*D*-ala	*D*-lys
B. subtilis	13±2	2±0.4		3±1	0.4±0.1
S. aureus	15±5	6±2		4±1	1±0.4
S. epidermidis	16±3	5±1		3±1	1±0.3
E. coli	24±4	11±3		6±2	2±0.5
P. aeuriginosa	26±5	13±2		7±2	2±0.8
X. vesicatoria	23±4	10±1		6±1	2±0.7

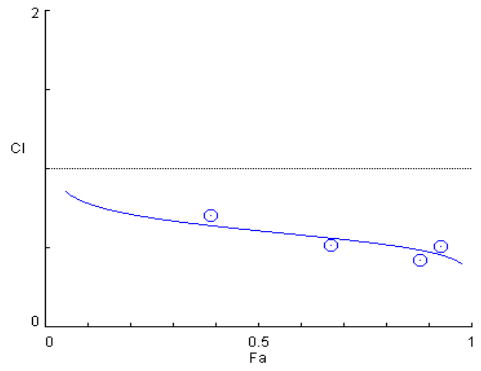

Figure 1. A representative Chou Talalay curve illustrating the synergy between *D*-lysine and Cefuroxime -sodium.

Our findings signify a dramatic reduction in the MIC values of *D*-alanine and *D*-lysine against the examined bacterial strains when combined with Cefuroxime-sodium (Table I). In addition, the results of pairing Cefuroxime-sodium with either *D*-alanine or *D*-lysine were evaluated for possible synergism through the utilization of the CompuSyn software program (version 3.0.1, ComboSyn, Paramus, NJ). Our data analysis confirmed the presence of synergism in all tested combinations (Fig. 1).

Furthermore, *D*-lysine and *D*-alanine were evaluated for their antifungal activities against *Candida albicans*; one of the microbial flora in humans that is responsible for several opportunistic infections [23]. Both *D*-amino acids demonstrated moderate antifungal activities against *Candida albicans*. The MIC values for *D*-alanine and *D*-lysine were 39 and 18μg μL^{-1}, respectively. Interestingly, upon the assessment of potential synergism between *D*-alanine or *D*-lysine and Amphotericin B, an approximately 6 fold reduction of the MICs was observed. Using the CompuSyn software program, synergism between either *D*-alanine or *D*-lysine with Amphotericin B was also confirmed (data not shown).

With the emergence of microbial resistance to several antibacterial and antifungal reagents, combinational remedies against microbial growth, at significantly modest concentrations of each individual compound, might aid treatment and prevention of microbial growth in several fields, such as agriculture, food-industry, surgical equipments and hospital surfaces. The natural occurrence of *D*-amino acids in the ecosystem, as well as the ease of their synthesis via the isomerization of their *L*-enantiomers, make them suitable targets for future development as antimicrobial agents.

In an attempt to study the effects of *D*-amino acids on eukaryotes, *in vitro* cytotoxicity of *D*-amino acids was evaluated and reported previously "in press" [24]. Additionally, the mechanism of the observed cytotoxicity was studied through measuring catalase activity, H_2O_2 generation, and apoptotic activity in HeLa and MCF-7 cell lines after *D*-amino acids treatment [24]. Our results indicate that the toxicity of *D*-amino acids does not appear to be solely mediated by H_2O_2, as previously suggested [24]. Apparently, other possible contributing apoptosis-mediated pathways might provoke the observed toxicity of *D*-amino acids.

According to our results, the cytotoxic effects of *D*-alanine and *D*-lysine were moderate when tested on eight different cancerous cell lines [24]. In an attempt to enhance the cytotoxic effects, and to be able to use considerably lower concentration of the chemotherapeutic agents, possible synergism between *D*-lysine and Doxorubicin on MCF-7 cell line was studied.

As shown in Fig. 2, the sensitivity of MCF-7 cells to Doxorubicin treatment was significantly enhanced ($P< 0.05$) when combined with 5 mM of D-lysine, suggesting a potential synergistic mode of action. Further work is undergoing to unravel the mechanism of the observed synergism and potentially enhance the sensitivity of cancerous cells to chemotherapeutic agents.

III. MATERIALS AND METHODS

A. Chemicals

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA) and included: *D*-alanine,

D-lysine, *D*-serine, *D*-proline, Cefuroxime-sodium, Amphotericin B, and Doxorubicin. Bacteria were grown in Mueller-Hinton broth (MHB; Oxoid, Basingstoke, UK). *Candida albicans* was grown in sabouraud dextrose broth (Oxoid, Basingstoke, UK)

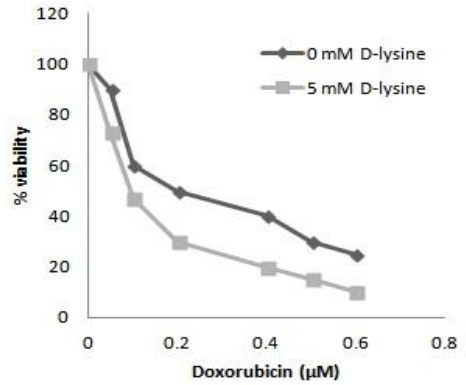

Figure 2. Percent viability of MCF-7 cells when treated with various concentrations of Doxorubicin in the presence or absence of *D*-lysine.

MCF7 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA), containing heat inactivated fetal bovine serum (HI-FBS) (Invitrogen), *L*-glutamine (Invitrogen), penicillin (Invitrogen) and streptomycin (Invitrogen).

B. Determination of Minimum Inhibitory Concentration

A stock solution of 1 mol L^{-1}, of each amino acid, was prepared in Phosphate Buffered Saline, PBS, and the pH was adjusted to 7.0 using HCl or NaOH. The stock solutions were filter-sterilized by passage through 0.45 μm membranes (Billerica, MA, USA) and serial diluted with the medium to the end point concentrations. MIC tests were conducted in 96 flat bottom microtiter plates (TPP, Switzerland). Each test well was filled with 100 μl nutrient broth. A sample (100 μl) of the stock solution was added to the first test well and mixed. A series of dilutions was then prepared across the plate. A 10 μl aliquot of the microorganism was used to inoculate each microtiter plate well to achieve a final inoculum size of 4×10^5 CFU/mL.

Wells with overnight culture, nutrient broth and bacterial inoculum but without amino acid treatment were assigned as positive growth controls, whereas negative controls were *D*-amino acid treated wells but without inoculums. All control wells were prepared and incubated under the same experimental conditions. Plates were incubated for 24 h at 37 ℃, with shaking. The wells were examined for microbial growth by naked eye before optical densities were measured at 600 nm (OD_{600}) using a Microplate Reader (Palo Alto, CA, USA). The minimum inhibitory concentration (MIC) value was described as the lowest *D*-amino acid concentration that inhibited $\geqslant$80% of microbial growth. Relative to the negative and positive controls, microbial growth in the test wells was detected as turbidit indicated by the optical density measured at 600 nm. MIC determination was carried out in triplicate (in same 96-well plate) and repeated three times for each microorganism.

C. Chequerboard Assay

In brief, serial 2-fold dilutions of the *D*-amino acid and the antibiotic were mixed in each well of a 96-well microtiter plates so that each row (and column) contained a fixed concentration of one agent and increasing concentrations of the second agent. Then, 10^5 CFU/mL of bacteria was approximately inoculated in each microtiter well, and the plates were incubated at 37 ℃ for 24 h with shaking. MICs were determined for the antibiotic at each *D*-amino acid concentration and for each *D*-amino acid at each antibiotic concentration. The combination inhibitory index (CI index) was calculated according to (1).

$$\text{CI index} = (\text{MIC of drug A in combination}/\ \text{MIC of drug A alone}) + (\text{MIC of drug B in combination}/\ \text{MIC of drug B alone}) \quad (1)$$

The interaction was described as synergistic when CI index was ≤ 1.0, additive if the CI index was =1.0, and antagonistic if the CI index was >1.0 [25], [26]. The Chou-Talalay Plot illustrates the result of the chequerboard assay and the CI values. The axis of the Chou-Talalay Plot represents the combination inhibitory index (CI) and the fraction affected (Fa) at each combination concentration.

D. Mammalian Cell Line and Cell Culture

The human breast adenocarcinoma MCF7 was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat inactivated fetal bovine serum (HI-FBS), 2 mmol L^{-1} *L*-glutamine, 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. Cells were maintained at 37 ℃ in a 5 % CO_2 atmosphere with 95 % humidity. The cells were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities were determined.

E. Cell Proliferation by MTT Assay

Cytotoxicity was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. For the assay, cells were washed three times with phosphate buffered saline (PBS) then PBS was decanted and cells were detached with the non-enzymatic cell dissociation complex (Sigma Chemical Co., USA). Cells were counted using trypan blue exclusion method and seeded into 96-well plates at the desired densities. Hundred μL of cell suspension was seeded and incubated per well to allow for cell attachment. After 24 h, the cells were treated with the *D*-amino acids or Doxorubicin. Cells were treated with different concentrations of each reagent in four triplicates. Treated cells were incubated in a 37 ℃ 5 % CO_2 incubator for 24 or 48 h. At the end of the exposure time, MTT assays were carried out. The absorbance at 490 nm was read on a plate reader (Tecan Group Ltd., Switzerland).

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