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# Identification and characterization of natural microbial products that alter the free D-aspartate content of mammalian cells

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ABSTRACT

Mammalian cells possess the molecular apparatus necessary to take up, degrade, synthesize, and release free D-aspartate, which plays an important role in physiological functions within the body. Here, biologically active microbial compounds and pre-existing drugs were screened for their ability to alter the intracellular D-aspartate level in mammalian cells, and several candidate compounds were identified. Detailed analytical studies suggested that two of these compounds, mithramycin A and geldanamycin, suppress the biosynthesis of D-aspartate in cells. Further studies suggested that these compounds act at distinct sites within the cell. These compounds may advance our current understanding of biosynthesis of D-aspartate in mammals, a whole picture of which remains to be disclosed.



Recent studies show that a variety of D-amino acids are present in mammalian cells and that they play important roles in physiological functions.[1–3] Among the free D-amino acids identified in mammals, D-aspartate (D-Asp) plays a crucial role in the central nervous, neuroendocrine, and endocrine systems. Several lines of evidence suggest that D-Asp plays an important role in regulating developmental processes, hormone secretion, and steroidogenesis.[4–7] Mammalian cells possess the molecular apparatus necessary to take up, degrade, synthesize, and release D-Asp; indeed, extracellular D-Asp is taken up by cells via L-glutamate (L-Glu) transporters. The affinity of L-Glu transporters for D-Asp is similar to that for L-Glu or L-Asp, and thus these transporters are involved in the uptake of these amino acids; however, their affinity for D-Glu is so low that it is essentially not transported at all.[8–10] Furthermore, D-Asp oxidase (DDO or DASPO; EC 1.4.3.1) is the only catabolic enzyme that stereospecifically acts on D-Asp in mammalian tissues. This enzyme is stereospecific for acidic D-amino acids and degrades not only D-Asp, but also D-Glu and *N*-methyl-D-Asp.[11–13] DDO is a flavin adenine dinucleotide-containing flavoprotein that catalyzes the oxidative deamination of D-amino acids to generate 2-oxo acids along with hydrogen peroxide and ammonia (in the case of *N*-methyl-D-Asp, methylamine is produced instead of ammonia).[14,15]

In contrast to the known functions of L-Glu transporters and DDO in the uptake and degradation, respectively, of D-Asp, little is known about the biosynthesis of D-Asp in mammals. Although D-Asp biosynthesis has been demonstrated in several cultured mammalian cells, such as rat pheochromocytoma PC-12 cells, rat pituitary tumor $GH_3$ cells, primary cultured rat embryonic neurons, MPT-1 cells (a subclone of the PC-12 cell line), and human cervical adenocarcinoma HeLa cells,[16–20] the enzyme responsible remains to be identified. In addition, the molecular component(s) that mediates the release of intracellular D-Asp also remains unknown. However, accumulating evidence suggests that different pathways in mammalian cells are responsible for releasing D-Asp. Indeed, two pathways responsible for the efflux of D-Asp of cytoplasmic origin have been identified; one through spontaneous and continuous release,[21,22] and the other through volume-sensitive organic anion channels.[22,23] An alternative pathway for the release of D-Asp via exocytotic discharge of vesicular D-Asp has also been proposed.[24,25]

*Abbreviations:* DDO, D-aspartate oxidase; HPLC, high-performance liquid chromatography; OPA, *o*-phthalaldehyde.

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D-Asp is present in specific cells within mammalian tissues, and the aforementioned mechanisms may be selectively employed by distinct cell types to regulate D-Asp homeostasis. Here, we screened 257 different compounds, including biologically active compounds of microbial origin and pre-existing drugs,[26] for their ability to alter the D-Asp content of mammalian cells.

Human embryonic kidney 293 cells are often used in cell biology research because they are easy to grow under standard conditions. Preliminary experiments detected D-Asp in 293 cells. Therefore, 293 cells were used for screening in the present study. To achieve this, the intracellular D- and L-Asp levels in cells treated with each target compound were measured by high-performance liquid chromatography (HPLC) using the *o*-phthalaldehyde (OPA)/*N*-acetyl-L-cysteine pre-column derivatization technique.[27,28] Example chromatograms are shown in Figure 1. The presence of D-Asp was confirmed by disappearance of the peak corresponding to D-Asp after a sample was treated with DDO (which was expressed in *Escherichia coli* and purified to near homogeneity, as described previously).[29,30] The D-Asp content of vehicle-treated cells was 21.8 ± 10.1 pmol/well, whereas the L-Asp content was 271.4 ± 86.5 pmol/well (mean ± standard deviation; $n = 10$). The percentage D-Asp with respect to total Asp (*D*% of Asp: D-Asp level/[D-Asp level + L-Asp level] × 100%) in vehicle-treated cells was 7.38 ± 1.90%. *D*% of Asp was the index against which all test compounds were screened. Mithramycin A, geldanamycin, and heptelidic acid were identified as candidates (Fig. 2) as the *D*% of Asp of cells treated with these compounds was approximately 9.33%, 15.8%, and 0.291%, respectively.

In previous studies, we demonstrated that PC-12 cells biosynthesize D-Asp and release it from the cytoplasm via two pathways.[16,21–23] On the other hand, PC-12 cells do not express L-Glu transporters or DDO.[16,19] Therefore, it appears that PC-12 cells are a suitable model for investigating the role of mithramycin A, geldanamycin, and heptelidic acid in the molecular mechanisms that regulate D-Asp homeostasis in mammalian cells. We next measured the D- and L-Asp content of PC-12 cells treated with these compounds (and their levels in the culture media in which the cells were grown) by HPLC using the OPA/Boc-L-cysteine pre-column derivatization technique.[31] This technique replaced that used for screening because the D-Asp peak observed in the chromatogram derived from PC-12 cell culture medium (unlike that of 293 cells) was clearly separated from peaks representing impurities; therefore, the D-Asp content could be accurately measured. Briefly, cells ($1 \times 10^6$ cells) were seeded in 6-well plates and cultured in 2 mL of medium.[32] On the following day, the medium was replaced with 1.2 mL of fresh medium and the cells cultured for a further 24 h. The cells were then cultured for a further 24 h in the absence or presence of the test compounds prior to amino acid extraction (from cells and culture medium) and measurement of amino acid content, as described previously.[19] Example chromatograms are shown in Figure 3. On the basis of preliminary experiments, mithramycin A, geldanamycin, and heptelidic acid were used at concentrations equal to or lower than 0.2, 10, and 0.5 μM, respectively, to prevent any cytotoxicity. Interestingly, mithramycin A significantly reduced the D-Asp content of PC-12 cells (and culture medium) in a dose-dependent manner (Fig. 4A). Therefore, total D-Asp (D-Asp in the cells plus D-Asp in the medium) levels were significantly reduced after treatment with this compound. By contrast, mithramycin A had little effect on the L-Asp content of the cells and culture medium; therefore, the total L-Asp content was essentially unchanged (data not shown). On the other hand, geldanamycin also reduced the D-Asp content of PC-12 cells and culture medium, although with the exception of the D-Asp content in the culture medium after treatment with 1 μM geldanamycin, the differences were not significant (Fig. 4B). However, geldanamycin significantly reduced the total D-Asp content in a dose-dependent manner (Fig. 4B). By contrast, the L-Asp content of cells and culture medium, and hence the total L-Asp content, remained essentially unchanged (data not shown). Treatment of PC-12 cells with heptelidic acid had no significant effect on either the D- or L-Asp content of cells and culture media, or on the total D- and L-Asp content (data not shown). Taken together, these results suggest that mithramycin A and geldanamycin suppress the biosynthesis of D-Asp in mammalian cells.

In contrast to the effects of mithramycin A and geldanamycin on the intracellular D-Asp content of PC-12 cells, treatment of

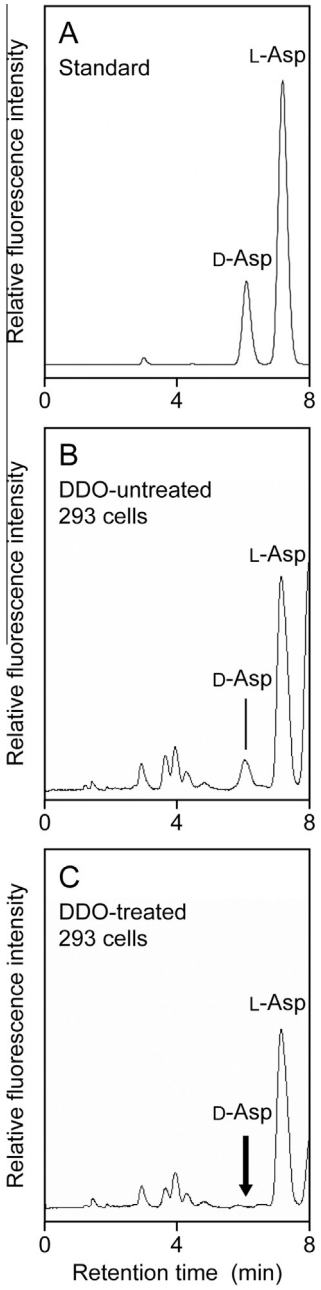


**Figure 1.** Typical examples showing the measurement of D- and L-Asp levels in 293 cells by HPLC using the OPA/*N*-acetyl-L-cysteine pre-column derivatization technique. (A) Chromatogram showing derivatives formed from standard D- and L-Asp and OPA/*N*-acetyl-L-cysteine. The D-Asp and L-Asp derivatives (20 and 60 pmol, respectively) were injected onto the HPLC column. (B and C) Chromatograms of samples prepared from 293 cells. A peak corresponding to D-Asp was detected (B). When the sample was treated with DDO, this peak disappeared almost completely (C), confirming that it represented D-Asp.