Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/Vol10Issue4abst197P.pdf.

A comparison of novel N^G, N^G-disubstituted arginines on recombinant dimethylarginine dimethylaminohydrolase-1 activity and nitrite production in RAW264.7 macrophages.

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Endogenously occurring asymmetric dimethylarginine (ADMA) concentrations have been widely documented to increase in a range of cardiovascular conditions. One mechanism by which ADMA may act is through inhibition of nitric oxide synthases. Dimethylarginine dimethylaminohydrolases (DDAH) metabolise ADMA to form citrulline *in vivo*[1] and have been considered as targets for modulating ADMA levels[2].

Previously[1,2] we found that arginine analogues with an N^G-methoxyethyl substituent gave optimum inhibition with no effect on other arginine-processing enzymes[2, 4]. Closely related N^G,N^G-disubstituted arginines, i.e. direct analogues of ADMA, are also of interest for their potential as DDAH inhibitors, but few have been described in the literature. This study aims to evaluate the effects of N^G,N^G-disubstituted arginines on cell viability, inducible nitric oxide synthase activity and on DDAH1 activity to identify new lead compounds.

The desired analogues were not readily accessible by our previously published methods[2], therefore, the methodology of Katritzky's group for preparation of trisubstituted guanidines from di-(benzotriazol-1-yl)methanimine [5] was adopted to give a short series of acyclic and cyclic N^G, N^G-disubstituted arginine analogues, using secondary amines and ornithine.

RAW264.7 cells in 96 well plates ($1x10^4$ cells/ well) were stimulated with LPS (1µg/ml) in DMEM media (containing 10% FCS) and N^G,N^G-disubstituted arginines were added at 0.5-500 µM. Nitrite secreted was measured using Greiss assay[2] and cell viability was determined using MTT assay[2].

Human DDAH1 (QIAgenes construct, Qiagen) was expressed in *E. coli*BL21(DE3). DDAH1 was purified using His-IDA (MacheryNagel). DDAH1 activity was measured using colorometic citrulline assay based on Knipp[4]. Assays contained ADMA (10mM) in sodium phosphate (10 mM pH8.0); symmetric dimethylarginine (10mM), not a substrate for DDAH1, was used as blank. Experiments were carried out in duplicate, and repeated on at least 3 separate occasions, and analysed by two-way ANOVA.

N^G,N^G-disubstituted arginine analogues substituted with morpholinyl, piperidinyl, methoxyethyl/methyl and *N*-methylpiperazinyl groups did not significantly alter cell viability, the pyrrolidinyl substituted analogue at 500µM reduced cell viability to 39.2 ± 5.73 % (% of control, untreated cells where n=3, $p>0.001$). There was no significant effect of the morpholinyl and *N*-methylpiperazinyl analogues on nitrite production in the LPS stimulated RAW264.7 cells. At 500 µM the piperidinyl and the methoxyethyl/methyl analogues, respectively, reduced nitrite production in LPS stimulated RAW264.7 cells from 40.12 ± 1.27µM to 5.40±0.26 µM ($P<0.01$, n=4) and 15.92 ± 1.84 µM ($P<0.01$, n=4).

The *N*-methylpiperazinyl analogue (100µM) reduced DDAH1 activity to 26.99 ± 12.75% of control ($p=0.45$, n=3), the acyclic methoxyethyl/methyl analogue and the morpholinyl analogue (shown to be a weak inhibitor in a previous study by Kotthaus[6]) had a modest inhibitory effect on DDAH 1 activity.

In summary, we have found that the cyclic N^G, N^G-(*N*-methylpiperazinyl) arginine analogue specifically inhibits DDAH1, without effecting nitrite production or cell viability, and is a novel and interesting lead compound.

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