Synthesis and Pharmacology of a Noncompetitive Antagonist of Angiotensin-Induced Contractions of Vascular Smooth Muscle

[Sarcosyl]-[Cysteinyl (S-Methyl)]\textsuperscript{8}-Angiotensin II

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SUMMARY  The synthesis of an angiotensin II (A II) antagonist, sarcosyl-\textsuperscript{1}-cysteinyl(S-methyl)\textsuperscript{8}-angiotensin II \textsuperscript{[Sar\textsuperscript{1}-Cys(Me)\textsuperscript{8}-A II]}, showing partial organ selectivity and properties of a noncompetitive antagonist, is described. The compound was found to be an extremely potent antagonist on vascular smooth muscle both in vitro (pA\textsubscript{2} for rabbit aorta = 9.2) and in vivo on rat blood pressure (dose ratio of 10\textsuperscript{3} for ED\textsubscript{25} mm Hg during 1 \textmu g/kg per min infusion of antagonist). It was without effect on norepinephrine responses in both assay systems. In contrast, it was a considerably weaker antagonist on visceral smooth muscle (pA\textsubscript{2} for guinea pig ileum = 8.5; pA\textsubscript{2} for rat uterus = 7.9). Interestingly, in the vascular smooth muscle preparations, the compound also exhibited elements of a noncompetitive antagonist in that both the slope and maximum of the A II dose-response curves were reduced markedly. Qualitatively similar results were obtained with sarcosyl-alanyl\textsuperscript{8}-angiotensin II (Saralasin) on rabbit aorta. Moderate depression of maximum response was seen in guinea pig ileum but not in rat uterus. These effects on vascular smooth muscle were reversible in vitro but only partially reversible in vivo. 

Since the report of Marshall et al. (1970) that the reversed analogue, phenylalanyl\textsuperscript{4}-tyrosyl\textsuperscript{8}-angiotensin II, was a competitive inhibitor of angiotensin II (A II), a host of other analogues has been prepared which also inhibit the action of this hormone (see Peach, 1977; Regoli et al., 1974; Khoshla et al., 1974a). The structure-activity data generated to date for antagonists (as well as for agonists) have clearly indicated a pharmacologically heterogeneous population of receptors for A II in a variety of target cells. For example, an alkylating analogue of A II was shown to irreversibly antagonize the action of the parent hormone on guinea pig ileum but was without effect on isolated rat uterus or, in vivo, in the rat blood pressure assay (Paiva et al., 1972). In addition, des-Asp\textsuperscript{1}-Ile\textsuperscript{8}-angiotensin II (Ile\textsuperscript{2}-A III) is a more effective inhibitor of steroidogenesis induced by angiotensin III (A III, des-Asp\textsuperscript{1}-angiotensin II) than of an A II-induced effect (Sarstedt et al., 1975). Other antagonists have also been described which show some organ selectivity (Regoli et al., 1974). Many examples also exist in the agonist series (again, see Peach, 1977; Khoshla et al., 1974a, for discussion) including the postulated roles for angiotensin III (Goodfriend and Peach, 1975). It is a fact that this analogue is an extremely potent...
agonist on steroidogenesis in the adrenal cortex, although less effective on other angiotensin target tissues. In summary, there is different receptor specificity for A II in different target tissues—a property which should be exploitable in attempts to develop organ-selective agonists and antagonists.

Finally some antagonists have been described which show some characteristics of noncompetitive antagonists. As has been pointed out (Regoli et al., 1974), these invariably contain a sarcosine (Sar) residue in place of the aspartic acid in position 1.

Therefore, with the above in mind, we have prepared an analogue, sarcosyl-cysteiny/(S-methyl)-angiotensin II (Sar¹-Cys(Me)⁸-A II), in an attempt to generate a noncompetitive antagonist with enhanced potency and some degree of organ selectivity. The Sar residue was chosen since it is always present in analogues showing tendencies to non-competitive antagonism, and it seemed to produce more effective antagonists on vascular smooth muscle vs. visceral smooth muscle. The Cys(Me) residue was chosen in light of the report that the substitution of a threonine-O-methyl ether [Thr(Me)] residue at the carboxy terminus produced an analogue (Khoshla et al., 1977) showing enhanced antagonist activity vs. other aliphatic substitutions at this same position (Regoli et al., 1974). We reasoned that, if the nucleophilic character of the side chain oxygen was responsible for the enhanced potency, replacement of the oxygen with a more nucleophilic sulfur atom might further enhance potency. The results are reported here.

**Methods**

**Synthesis**

The tert-butoxycarbonyl-L-cysteine(S-methyl) [Boc-Cys(Me)-OH] was prepared in our laboratories from L-cysteine(S-methyl) using Boc azide according to the method of Anderson and McGregor (1967). The Boc-Cys(Me)-OH was crystallized as its dicyclohexylamine salt from ethyl acetate:petroleum ether:methanol (5:30:2) and was homogeneous as determined by elemental analysis (C,H,N) and thin layer chromatography (silica gel 60, Merck F-254) in three systems. The systems and RF’s were: Sys A, chloroform:methanol:acetic acid:water (4:1:20), RF = 0.63; Sys B, chloroform:methanol:acetic acid:water (60:30:4:1), RF = 0.73; Sys C, benzene:water:acetic acid (9:1:9), RF = 0.71. The amino acid eluted as a single symmetrical peak at 49 minutes using the standard 3-hour run conditions on a Beckman 119C amino acid analyzer.

Sar¹-Cys(Me)⁸-A II was prepared by solid-phase synthesis essentially as described by Stewart and Young (1969). The Boc-Cys(Me)-OH was attached to the resin using the cesium salt method of Gisin (1973). A substitution of 0.47 mm/g was achieved. The remaining residues (as their Boc derivatives) were sequentially added as previously described (Day and Freer, 1978). Side chain blocking func-

**Pharmacological Testing**

Isolated smooth muscles were mounted in 10-ml tissue baths (Metro Scientific), and the appropriate physiological salt solutions were gassed continually with an O₂/CO₂ mixture to give a pH of 7.4–7.6. Contractions were determined isotonically using a heart lever with gravity head and were recorded by kymography (Harvard Apparatus). Lever magnification was 6x. All experiments were carried out in duplicate on tissues from the same animal. When the antagonist was tested for its ability to modify responses to A II or norepinephrine (NE), it was preincubated for 10 minutes with one muscle and the dose-response curve compared with a previous control curve for the same preparation. The duplicate muscle was maintained in the control solution throughout the experiment and tested simultaneously to ensure that no major spontaneous changes in sensitivity occurred.

Specifically, rabbit aortic strips were prepared and cumulative dose-response curves generated as described by Puchgott and Bhardakom (1953). Rat uterus (estrogen-dominated) and guinea pig ileum assays were carried out as described previously (Freer, 1975).

Pressor assays were carried out in rats anesthetized with Dial/urethane as described by Chiue et al. (1979). Briefly, arterial blood pressure was recorded (Grass model 7 polygraph and Statham pressure transducer) via a cannula in the right carotid artery, and bolus injections of agonists were given intravenously via the jugular vein. Infusions of antagonist (18 µl/min in isotonic saline) were administered through a cannula in the right femoral vein.

Data for smooth muscle were normalized as percent maximum response ± SEM with the best agonist response of the control period being taken as 100%. Values of pA2 are as defined by Schild (1947).

All materials were of reagent grade quality. Asp¹-
Ile\(^5\)-A II was prepared in the authors' laboratory essentially as by Stewart and Young (1969) and Day and Freer (1978). Sar\(^1\)-Ala\(^8\)-A II was purchased from Peninsula Laboratories.

**Results**

As predicted, the Sar\(^1\)-Cys(Me)\(^5\)-A II was found to be a potent antagonist of A II-induced contractions in rabbit aortic strips (Fig. 1, upper), guinea pig ileum (Fig. 2), and estrus rat uterus (Fig. 3). The analogue was especially potent in the aortic strip preparation where it not only shifted the dose-response curve to the right but also reduced the slope and depressed the maximum response. The pA\(_2\) values were 9.2 and 8.5, respectively. It should be emphasized that the Sar\(^1\)-Cys(Me)\(^5\)-A II is 10-15 times more effective on the vascular smooth muscle of the rabbit aorta than on the visceral smooth muscle of the rat uterus.

When tested for its ability to block A II-induced responses in the rat pressor assay, the results were a similar, but not as dramatic, effect was seen with Sar\(^1\)-Ala\(^8\)-A II (Fig. 1, lower), although 10- to 15-fold higher concentrations (i.e., 25 and 50 ng/ml) were required.

In contrast, the visceral smooth muscle preparations tested behaved somewhat differently. As can be seen, the guinea pig ileum (Fig. 2) did show some depression of maximum response (35% at 25 ng/ml antagonist), but in the rat uterus preparation, the Sar\(^1\)-Cys(Me)\(^5\)-A II behaved as a nearly classic competitive antagonist, although a slight (12%) but significant reduction in maximum response was noted (Fig. 3). The pA\(_2\) values were 8.5 and 7.9, respectively.
consistent with the in vitro data for vascular smooth muscle. As can be seen in Figure 4, there was a dose-dependent shift in the A II dose-response curve in the face of infusions of Sar 1-Cys(Me) 8-A II at 0.25, 1.0, and 5.0 μg/kg per min. Dose ratios at the 25 mm Hg response level were 12, 103, and 1380 times, respectively. Also, there was a dose-dependent decrease in the slope of the dose-response curve and, in the case of the highest infusion rate, there was a plateauing of the A II response. Remarkably, a bolus injection of 100 μg/kg of A II produced, on the average, only a 25 mm Hg increase in mean arterial pressure. No plateauing of responses was observed in the absence of antagonist. A dose of 250 μg/kg of A II produced essentially the same effect (i.e., a 28 mm Hg increase), indicating that during the infusion the blockade is only partially surmountable. It also should be noted that this compound exhibited transient pressor responses (10-15 mm Hg) when infused at 5.0 μg/kg per min.

To ensure that we were dealing with a specific effect on the angiotensin receptor, the Sar 1-Cys(Me) 8-A II was tested vs. NE on both rabbit aorta and in vivo in the rat pressor assay. The results in Figure 5 clearly show that, at doses which virtually abolish A II responses (i.e., 50 ng/ml, rabbit aorta and 5.0 μg/kg per min, rat pressor), there was absolutely no effect on NE responses. This included both slope and maximum responses.

The A II dose-response curves seen in the presence of Sar 1-Cys(Me) 8-A II are consistent with the possibility that this analogue exhibits some elements of a noncompetitive antagonist (i.e., decreased slope and depressed maximum response). To determine whether there might also be an irreversible component, recovery from the antagonist was studied on both aortic strip (Fig. 6) and rat pressor (Fig. 7) assays. As can be seen, there is full recovery 45 minutes after infusion (5.0 μg/kg per min) in the rat pressor assay. Surprisingly, however, the aortic strip showed only partial recovery 45 minutes after generating an A II dose-response curve in the presence of 50 ng/ml of Sar 1-Cys(Me) 8-A II. In fact, the A II dose-response curve after recovery was still 4-fold displaced from control, and the maximum response was only 68% of control. It
is worth noting that the tissues were washed repeatedly during recovery (a total of 20–25 bath volumes). Also, lest the presence of A II during the antagonist incubation should affect recovery, a series of experiments was carried out with no A II present. Under those circumstances, the recovery curve was identical to that shown in Figure 6.

Discussion

The compound described here, Sar1-Cys(Me)8-A II, does appear to be among the most potent A II antagonists reported to date. This is especially true for rabbit aortic smooth muscle for which a pA2 of approximately 9.2 was observed. Unfortunately, a direct comparison was not possible, but it appears that our Sar1-Cys(Me)8-A II analogue is somewhat more effective on rabbit aorta than the Sar1-Thr(Me)8-A II prepared by Khoshla et al. (1977). It should be noted that the cysteinyl analogue (i.e., Cys8-A II) has been prepared (Needleman et al., 1972). Unfortunately, it does not contain the sarcosyl residue and has not been tested on aortic strips. Therefore, it is not possible to compare it to our Sar1-Cys(Me)8 analogue. Although not strictly comparable, the in vivo pressor assays indicate that the Sar1-Thr(Me)8-A II is somewhat more effective, at least at low doses. Surprisingly, the Sar1-Cys(Me)8-A II analogue produced an insurmountable blockade of A II pressor responses (even bolus injections of A II of 250 μg/kg)—a property heretofore not reported for an A II antagonist. Both in vivo and in vitro, the antagonism was specific since responses to NE were unaffected even in the presence of large amounts of the analogue.

In line with our original rationale for the synthesis, we noted a decrease in activity when tested on nonvascular smooth muscle. It was clearly less active as an antagonist on the smooth muscle of the guinea pig ileum (pA2 = 8.5) and even less active on estrus rat uterus (pA2 = 7.9).

Aside from the potency of the compound, several other points are worth noting. First is the finding that this analogue exhibits properties of a noncompetitive antagonist at all doses tested. This depression of maximum response has been seen to varying degrees before (Regoli et al., 1974; Rioux et al., 1973; Khoshla et al., 1974b), particularly when using vascular smooth muscle. Paiva et al. (1977) have observed the same effect on uterine smooth muscle, although, with our analogue, the maximum response of uterus to A II was only slightly, although significantly, depressed (~12%).

Although the antagonist described here is clearly a potent compound, the nature of the inhibition is, we feel, of greater interest. Why, for example, should a peptide of this type exhibit noncompetitive antagonism? Since it has no reactive groups, affinity labeling is highly unlikely. It is not impossible, however, that the presence of the sulfur (with its unpaired electrons) in the side chain could coordinate an essential cation from the receptor. This principle has been used in the development of a potent inhibitor of angiotensin-converting enzyme (Cushman et al., 1977). A second explanation could be that the antagonist binds to the receptor to form, relative to A II itself, a slowly dissociating complex. Such a mechanism has been suggested to explain the phenomenon of tachyphylaxis to A II (Khairallah et al., 1966). This, we feel, is probably not the reason for the noncompetitive element of the antagonism. We say this since specific binding studies on both agonists and antagonists have not revealed any mass action data to support the existence of the very tight binding required to substantiate this kind of mechanism. For example, Sar1-Ala8-A II has been reported here to depress the maximum response of rabbit aorta to A II (Fig. 1), but it was indistinguishable from A II when measured in a radioreceptor assay (Devynck and Meyer, 1978). Similarly, Sar1-Leu8-A II depresses maximum response (Rioux et al., 1973) but has no greater affinity for receptors in aorta than does A II itself (Devynck and Meyer, 1978). On the other hand, there are pharmacological data to indicate that Sar1-A II, once bound, may dissociate more slowly than A II itself (Regoli et al., 1974).

Although we cannot definitely assign a mechanism for this noncompetitive antagonism, there are some data, admittedly indirect, which may help to explain the phenomenon. First is the fact that, in all instances, antagonists exhibiting the ability to depress the maximum response of rabbit aorta to A II have contained amino terminal modifications—primarily substitution of a sarcosyl residue for the aspartic acid. The additional observations (1) that Sar1-A II, in contrast to A II itself, produces tachyphylaxis of rabbit aorta (Moore and Khairallah, 1976), (2) that tachyphylaxis to A II may be correlated with interactions between agonist and an ex-
tracellular Ca\(^{2+}\) pool (Freer, 1975), and (3) that Sar\(^1\)-A II, again unlike A II, uses such an extracellular Ca\(^{2+}\) pool to mediate its contractile effects (Ackerly et al., 1977) are consistent with the idea that our antagonist may be operating, at least partially, through the same mechanisms that are responsible for tachyphylaxis in this preparation. Indeed, it would be interesting to know whether tachyphylaxis can occur in the absence of a stimulus effect. If so, these antagonists could be useful agents to study this common pharmacological phenomenon in the absence of agonist-induced perturbations of the test system.

It might be argued that the noncompetitive element of the inhibition might be due to the antagonist being, in fact, a weak partial agonist. We feel this is not the case since (1) no agonist activity was seen on aortic strips even at the highest bath concentration (50 ng/ml), (2) no agonist activity was observed in vivo except at the highest infusion rates (10–20 mm Hg increase at 5.0 μg/kg per min), and (3) Khoshsa et al. (1974b) have reported that thienylalanyl\(^8\)-A II is a partial agonist but shows only depressed maximums and decreased slopes in A II dose-response curves (aortic strips) after its own agonist activity is manifest. Although these data do not constitute a proof, they are supportive of our notion that this antagonist is not acting simply as a partial agonist.

A final point worthy of note is the greater selectivity (~10-fold) of our antagonist for vascular vs. visceral smooth muscle. It has been amply demonstrated (see Peach, 1977, for a recent review), and is confirmed again with this analogue, that the angiotensin receptor is in fact a pharmacologically heterogeneous population of receptors with distinctly different distributions in the various angiotensin target organs. The conclusion is inescapable that organ-selective antagonists will be forthcoming.

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