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Putative Antibacterial Mechanisms for Angiotensin II Receptor Blockers

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ABSTRACT

Angiotensin II has profound actions in Th1 immune disease. It directly modulates Nuclear Factor-kappaB (NF-kappaB), an essential precursor to the generation of inflammatory cytokines and chemokines, including TNF-alpha. Corticosteroids exert their anti-inflammatory action by totally shutting down activation of NF-kappaB, while Angiotensin Receptor Blockers (ARBs) inhibit excessive NF-kappaB activation, allowing the phagocytes to respond to immune challenge in a less aggressive manner. The two anti-inflammatory mechanisms are fundamentally different. The new ARB, Olmesartan Medoxomil, has been identified as useful in treating Th1 inflammatory disease sarcoidosis, and the suggestion has been made that ARBs might also directly affect bacterial pathogens. Receptor proteins actively binding angiotensin II have been found on several species of bacteria. We found no match between the human angiotensin receptor and any similar protein in the 294 bacterial genomes currently sequenced, lending credence to the suggestion that bacteria may be using this host hormone in such a manner as to evade the host's immune system. Clearly, if an ARB was capable of actually inhibiting the supply of A-II, thus denying a microbe the ability to protect itself from destruction by phagocytosis, then that ARB could most definitely be classed as an 'antimicrobial'. The dosing of Olmesartan reported as most useful in immunomodulation, 40mg q8h, is well above the level needed to produce maximal hypotensive activity. It would thus seem likely that Olmesartan is acting on atypical receptors, perhaps directly upon pathogens, or upon some yet-to-be-defined human angiotensin-binding-protein(s).

Abbreviations:
IL-1beta, IL-6, IL-8 are Interleukins, inflammatory cytokines

CAM-1 is the 'intercellular adhesion molecule'
MCP-1 is 'monocyte chemoattractant protein 1'
TNF-alpha is 'Tumor Necrosis Factor alpha'
IFN-gamma is 'Interferon-gamma'
AT1, AY2 are Angiotensin Type 1 (and 2) receptors subtypes
CS-866 was the developmental name for Olmesartan (Benicar/Olmetec)
Th1, Th2 are two distinct immune responses, distinguished by their cytokine profile

Introduction

Angiotensin II (A-II) has profound actions in Th1 immune disease. Angiotensin II directly modulates the production of Nuclear Factor-kappaB (NF-kappaB) in the cytoplasm of mature phagocytes, causing nuclear release of messenger RNA (mRNA) to begin the transcription for many of the inflammatory chemokines and cytokines, including IL-1beta, IL-6, and IL-8, MCP-1, CAM-1, IFN-gamma and TNF-alpha [1,2,3,4,5,6,7,8]. The blockade of the A-II modulation pathway with Angiotensin II Type 1 Receptor Blockers (ARBs) reduces the generation of these cytokines, including TNF-alpha [5,6], and inhibits the inflammatory process [2,3,5,6].

In the light of new knowledge that Th1 inflammatory disease can be caused by bacterial pathogens [9,10], we need to determine whether ARBs might also be exerting any direct antibacterial action upon the pathogenic bacteria.

Angiotensin Receptor Blockade is Anti-Inflammatory

Marta Ruiz-Ortega has been researching the actions of angiotensin in immune diseases for some time. One of the first to identify A-II's direct stimulatory actions on NF-kappaB [1], Ruiz-Ortgea has since gone on to identify A-II as a key factor in inflammatory nephropathy, especially diabetic nephropathy [2,6], and to confirm the role of ARBs in controlling that inflammation.

Belline, et al, have also confirmed the observations of Iwai, et al, [7}, that phagocyctosis in a murine model of peritoneal inflammation is directly modulated by angiotensin [3]. Belline, et al, were further able to show that phagocytosis in murine peritoneal macrophages was attenuated by the ARB, Losartan, demonstrating that AT1 receptors were involved in "modifying the host resistance to infection".

The release of the inflammatory cytokines and chemokines in 'autoimmune' Th1 inflammation begins whenNF-kappaB is activated in the cytoplasm of monocytes, macrophages and dendritic cells (collectively, 'phagocytes'). NF-kappaB causes the phagocyte nucleus to emit a messenger RNA (mRNA) which contains the genetic code dictating transciption of the cytokines and chemokines in the cytoplasm [1,3,4,6].

Corticosteroids exert their anti-inflammatory action by totally inhibiting the activation of NF-kappaB [11] (in a dose-dependent manner).

Nevertheless, the two anti-inflammatory mechanisms are fundamentally different. Angiotensin II receptors on the phagocyte membrane are just one of the methods by which the immune system can be activated to deal with an invader. Besides the A-II receptor, there are Toll-like, and other receptors, each of which can activate NF-kappaB in order to help the phagocyte deal with the immune challenge. Angiotensin receptor blockade shuts down just one of the channels by which NF-kappaB can be activated to signal the inflammatory cascade. The phagocyte can still react to immune challenges sensed through the other receptors. Corticosteroids shut down NF-kappaB itself, and

totally suppress any attempt by the phagocyte to prevent the body from harm, whatever the source of immune challenge.

Unusual Clinical Observations

The authors have been observing beneficial effects of ARBs in a cohort of subjects recovering from Sarcoidosis. Sarcoidosis is a chronic inflammatory disease caused by an out-of-control Th1 immune response. These clinical observations seem to reveal a number of idiosynchrasies, perhaps indicating that the ARBs are having an effect on something other than just the Angiotensin II Type 1 Receptor (AGTR1).

Firstly, dosing requirements seem anomalous. Patients report that ARBs continued to give incremental symptomatic improvement at doses well above the levels giving maximal hypertensive activity. For example, Olmesartan (from Benicar/Olmetec) (CS-866) continues to incrementally suppress disease symptoms up to a dose of 40mg every 4 hours, with 40mg every 6-8 hours being the consensus optimum dosing. Yet this ARB achieves maximal hypotensive activity at a dose of 10-20mg qd [12]. Why would a dosage of ARB higher than that make any difference?

Secondly, different ARBs were not equally effective at suppression of disease symptoms. Olmesartan was reported as 'excellent', while Valsartan was a distant 'second best', Irbesartan 'useful'. Yet these ARBs all provide an 'insurmountable' blockade of AGTR1. How could one 'insurmountable' blockade differ from another 'insurmountable' blockade?

Finally, Valsartan did not seem to suppress 'sinus' symptoms. Even though it had adequate palliative effect on muscle pain, psychotic events [13] and dyspnea, it left some subjects with swollen and painful sinuses. After they changed to Olmesartan the 'sinus' problems disappeared. Since there is only one type of AGTR1 receptor in the body, how could any ARB not be active in the sinuses?

The *homo sapiens* Angiotensin II Type 1 Receptor

AGTR1 is a G-protein-coupled receptor (GPCR), a large family of cell-surface receptors which facilitate hormonal signalling through the phagocyte membrane [14]. Other GPCRs include the Toll-like Receptors, which also mediate the body's immune response [15,16]. An increased number of Angiotensin-II receptors are expressed in tissue inflamed by a Th1 disease compared with healthy tissue [17], due to up-regulation of AGTR1 receptors [18].

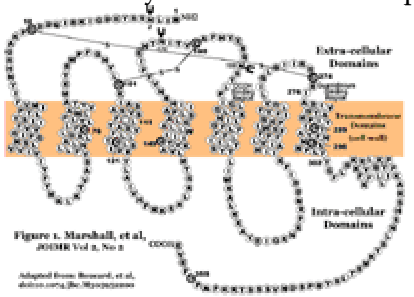

Figure 1. Schematic of the AGTR1 *homo sapiens* angiotensin type 1 receptor protein.

Figure 1 is a schematic diagram of AGTR1 (P30556) [14]. It consists of four extra-cellular domains, seven trans-membrane regions, and four intra-cellular domains. Signalling the presence of the extra-cellular homone Angiotensin-II seems to be accomplished by the structural changes which occur in transmembrane region 7 when Angiotensin II binds near the junctions of the extra-cellular domains and transmembrane regions 5 and 7. The

carboxyl terminus (O=C-OH) binds to the Lys-199 region of transmembrane 5, and the guanidinium group of Arg2 [$(NH_2)_2$-C-NH-C] binds to Asp-281 in transmembrane 7 [14,19].

Designing and manufacturing ARBs is a multi-billion dollar business, and very few ARB molecular models have been deposited into the Protein Data Bank (PDB), or any other publicly available repositories. The model of olmesartan (shown in figure 2) was constructed from sources including its package insert and Sankyo Ltd brochures. A minimum energy conformation for the molecule (without regard for pH) was optimized using iterated Newtonian mechanics. This optimization was performed using Ghemical, a workstation-based molecular modeling package. Irbesartan was constructed similarly, while candesartan_cilexetil and valsartan were retrieved from the Department of Pharmaceutical Information Science, Tokyo University. Angiotensin II is available from the PDB, accession number 1N9V.

The carboxyl terminus of the insurmountable antagonists (including Candesartan, Irbesartan, Olmesartan and Valsartan) bind to the Lys-199 region of AGTR1. Transmembrane 7 is not affected at all. Despite this, the insurmountable antagonists effectively block the actions of angiotensin II (in man) by occluding this key region of the AGTR1 binding pocket.

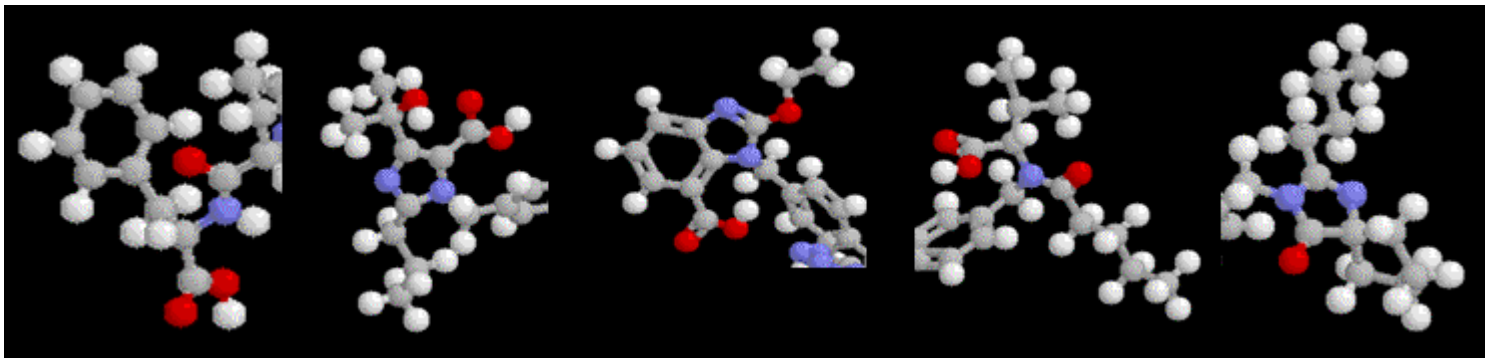

Figure 2. The carboxyl terminus of Angiotensin II and (resp.) Olmesartan, Candesartan, Valsartan and Irbesartan (note that Irbesartan has an O2, not a OCOH terminus).
[**Editor's Note: The authors supplied 3-D models of each of the ARBs.** JOIMR has created a special page where they
can be examined, interactively, in 3-Dimensions. Click here to access those models.
The images above have been supplied for those who cannot access the 3-D models]

The structure of the subject ARBs, and of Angiotensin II itself, are shown in figure 2 (angiotensin II is an octapeptide with the amino acid sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe).

There is significant variation between these ARB structures, and all are different from the carboxyl terminal region of A-II. Although they are extremely effective at binding the transmembrane-6 pocket of AGTR1 it is clear that small changes in the structure or conformation of the receptor might well render one, or more, of the antagonists ineffective. Perhaps if there were genetic mutations in the receptor, then those mutations might affect each ARB's ability to bind into the Lys-199 pocket. In fact, the AGTR1 gene does in fact have 5 reported mutations, but they are all in the transmembrane 7 region, and not adjacent to the Lys-199 pocket. Consequently, genetic mutations alone do not seem adequate to account for the differing ARB blockade effectiveness observed in the clinical studies. It must be inferred that we may be dealing with receptors other than the AGTR1...

Bacterial Receptors with affinity for Angiotensin II

Nickenig, et al, [20] were examining tissue cultured from a human skin biopsy specimen when they noticed something strange. Their tissue sample contained "angiotensin AT1 receptors and a putatively new angiotensin receptor activated by angiotensin(1-6), both coupled to signaling pathways involved in DNA synthesis". They noted the existence of this new receptor in their paper, but were at a loss to explain where it might have come from.

Earlier, in 1993, Whitebread, at al, had warned of "mollicute contamination" in rat tissue, documenting an atypical Angiotensin-II receptor site which disappeared after application of the 'antibiotic mixture BM-Cyclin' [21]. They had noted that it was not possible to study the rat A-II receptor in the presence of A-II receptors on contaminating mollicutes ('Mollicute' is a synonym for 'mycoplasma', tiny 'L-form' or 'Cell Wall Deficient' bacteria). The culprit bacterial species was identified as *Acholeplasma laidlawii*. Finally, they noted that the bacterial receptor could not be blocked with the ARB Losartan (Cozaar).

Bergwitz, et al,{22] accurately characterized an A-II receptor they found on *Mycoplasma hyorhinis*, and noted that the bacterium adheres tightly to the membrane of mammalian cells. They noted that the atypical A-II receptors might be due to host-parasite interactions, as the receptor's affinity for A-II was 7 times higher when the bacterium had infected a cell rather than when the bacterium was in isolated culture.They carefully confirmed that the bacterial A-II receptor was specific for A-II and Angiotensin I (A-I). The antibiotic bacitracin and aprotinin (a natural protease inhibitor) were both potent inhibitors of A-II binding to the receptor on M. hyorhinis. Neither bacitracin or aprotinin affected the sensitivity of the human A-II receptor, AGTR1.

Servant, et al, [23] isolated an atypical A-II receptor in rat pheochromocytoma cells. Bacitracin potently inhibited the actions of this atypical receptor, and enzyme immunoassay confirmed that the cells were contaminated with Mycoplasma hyorhynis. The A-II binding sites were eliminated by treating the tissue with 'BM-cycline'.

Using an A-II analog they estimated that the bacterial receptor was sized at 95kDa, significantly different in size from the mammlian AGTR1 receptor. Losartan was ineffective blocking Angiotensin-II from the bacterial receptor.

Smith [24] found that the bacterial receptor on M. hyorhinis has a marked affinity for A-I as well as A-II and that the affinities were related to PH. He therefore hypothesized that the microbe might be able to tell the PH of its environment by balancing the respective magnitudes of A-I and A-I binding to its receptor.

Bergwitz [22] concluded (in 1991) "**Although the biological significance of these binding sites is unknown, they .. may have implications for the involvement of mycoplasma in the pathogenesis of autoimmune disorders.**" It is hard to disagree.

From 1991 to 2004, and the Genome

The genomes of 294 bacterial species are now available in the NCBI Genebank, most of them completed. When one takes the gene for AGTR1 and runs a comparison between the human gene and all species in the Genebank (2,199,823 DNA sequences), it becomes obvious that only the mammalian species possess AGTR1 similar to *homo sapiens*. Takanayagi [25] observed "The deduced amino acid sequence of the human angiotensin II (Ang II) receptor was 95.3% and 94.2% identical to those of bovine and rat". As you traverse the mammalian phylogeny, the differences from AGTR1 of *homo sapiens* become more and more pronounced.

The genebank further confirms that there are no DNA sequences in any microbial species capable of transcribing a protein even vaguely similar to the human AGTR1.

We therefore took a small fragment of the transcribed AGTR1 gene, just the 30 amino acid sequence from position 181 to 210, covering the region where the key carboxyl terminus of A-II binds to the human AGTR1.

P30556 DEFINITION Type-1 angiotensin II receptor, *homo sapiens*
181-210: afhyesqnst lpiglgltkn ilgflfpfli

This region is labelled the "OCOH Binding Region" in figure 1. Comparing this sequence against the entire translated genome database (tblastn) revealed a perfect match with homo sapiens, an almost perfect match with the genomes of beef cattle, pigs and rats, but zero correlation with any of the microbial genomes. Thus one can now say, definitively, that no microbial A-II receptor is identical with AGTR1, indeed, none are even structurally similar.

It can therefore be inferred that the microbial receptor has evolved to perform a totally different function from that of AGTR1 in man. Indeed, McLaren, et al, suggested that **the binding of host proteins by microorganisms may help them avoid recognition by the host immune system** [26].

Clearly, if an ARB was capable of actually inhibiting the supply of A-II to a microbe, thus denying it the ability to protect itself from destruction by phagocytosis, then that ARB could most definitely be classed as an 'antimicrobial'.

The Alternate Hypothesis - Unknown Human Genes sensitive to A-II

Several human genes have been reported to have angiotensin type II (AT2) receptor activity. The MAS Proto-oncogene, the angiotensin/vasopressin receptor (LOC171390), the melanocortin 2 receptor (adrenocorticotropic hormone)(MC2R), and AK007383, a mouse AT1 receptor-related protein, have all been reported to have receptor sites for A-II. With the exception of the latter, it is unlikely that any of the subject ARBs could be exerting a noticeable clinical effect on this predominantly AT2 receptor activity. Unlikley, but not impossible. Additionally, if angiotensin were binding to other, yet unidentified, receptors or proteins in *homo sapiens* then this would supply an alternate hypothesis - that the subject ARBs are affecting yet-to-be-determined metabolism in the human genome itself, and not in the bacterial genomes. More research needs to be done to totally preclude this possibility.

Some ARBs Cannot Blockade Microbial A-II Receptors

Losartan (DuP 753), the earliest of the ARBs, did not stop A-II from binding at the microbial angiotensin receptors [20,22, 23]. But Losartan was an earlier ARB, and binds competitively with angiotensin, not insurmountably like the more modern ARBs. Its structure is radically different. It cannot be inferred that just because Losartan failed to inhibit the bacteria from metabolizing angiotensin, that the modern ARBs would not do a better job. This is especially true at the higher doses which the clinical studies have indicated seem to be necessary.

Variations in the AT1 Receptor Structure

Fierens, et al, [27] explored the effect of variations in the structure of an angiotensin receptor, variations which the genebank confirms must exist between any bacterial angiotensin receptor and that of man. They susbtituted the Lysine near the carboxyl binding pocket near position 199 with Glutamine, and found that just this single substitution decreased the affinity 45-fold for candesartan (95% insurmountable), 10-fold for irbesartan (40% insurmountable) and 5-fold for losartan (surmountable). It is noteworthy that when this study was undertaken, olmesartan was not yet available.

Candesartan, irbesartan and losartan are all equally effective in the human AGTR1, yet offer widely different blockade effectiveness on atypical receptors. It could be expected that differences in activity would be also observable when blockading the (atypical) bacterial receptors.

Summary and Authors' Perspective

While there is little doubt that Angiotensin II contributes significantly to inflammatory disease, there have been surprisingly few studies performed to identify and confirm exactly how Angiotensin Receptor Blockade may ameliorate the damage and suffering from these diseases. The Angiotensin II receptor site(s) already identified on bacterial species must have some biological function. Clearly, if an ARB was capable of actually inhibiting the supply of A-II to a microbe, thus denying it the ability to protect itself from destruction by phagocytosis, then that ARB could most definitely be classed as an 'antimicrobial'. It was 1991 when Bergwitz [22] concluded "Although the biological significance of these binding sites is unknown, they .. may have implications for the involvement of mycoplasma in the pathogenesis of autoimmune disorders". It is evident that more needs to be done to identify exactly why bacteria metabolize angiotensin, whether they use fragments of this human hormone to enhance their ability to hide from the immune system [26], or whether they use them to directly pervert the very process of phagocytosis itself [28,29].

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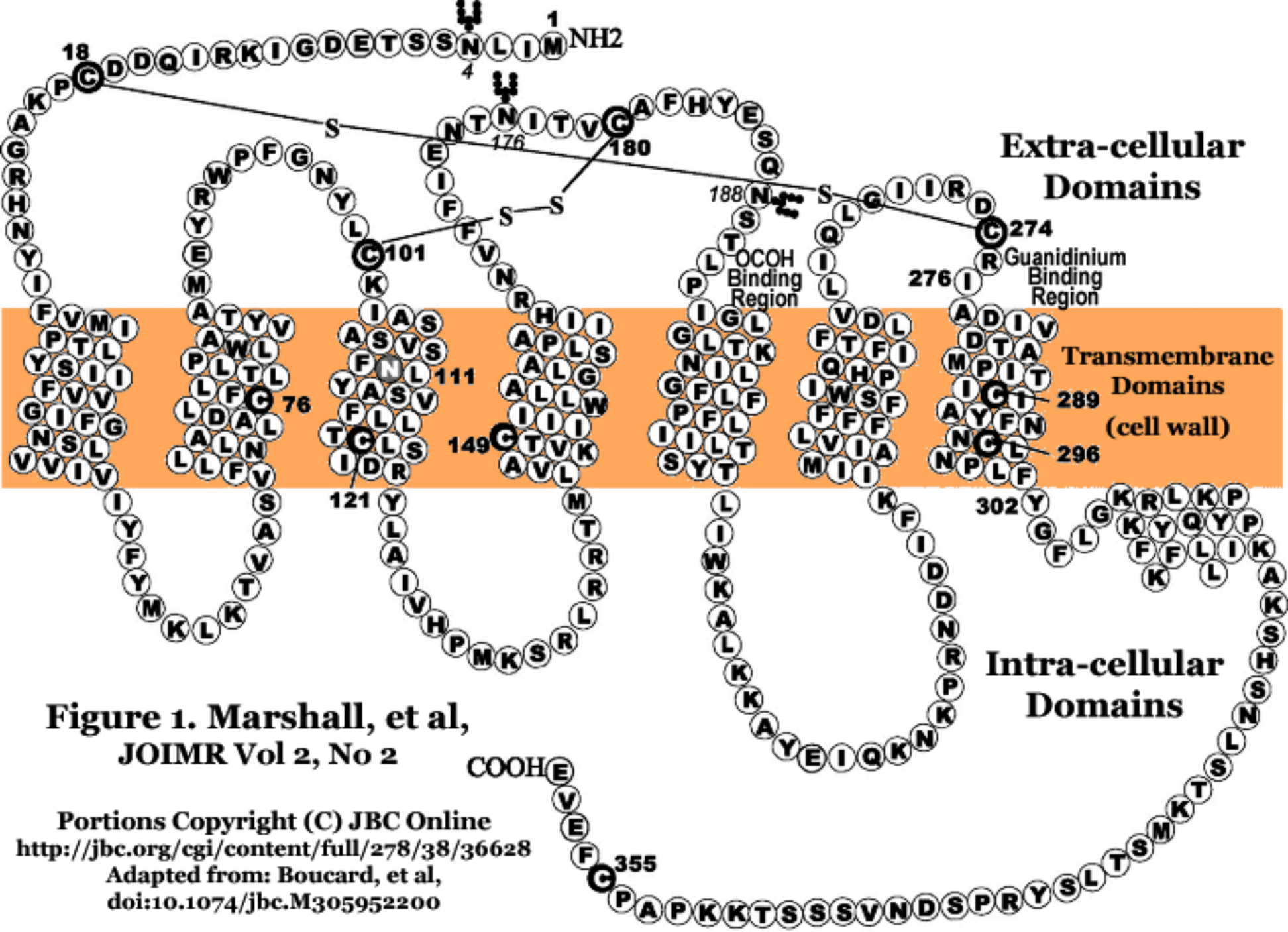

Figure 1. Marshall, et al,
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