Discovery of (2*S*,4*R*)-1-(2-Aminoacetyl)-4-benzamidopyrrolidine-2-carboxylic Acid Hydrochloride (GAP-134)[13], an Orally Active Small Molecule Gap-Junction Modifier for the Treatment of Atrial Fibrillation

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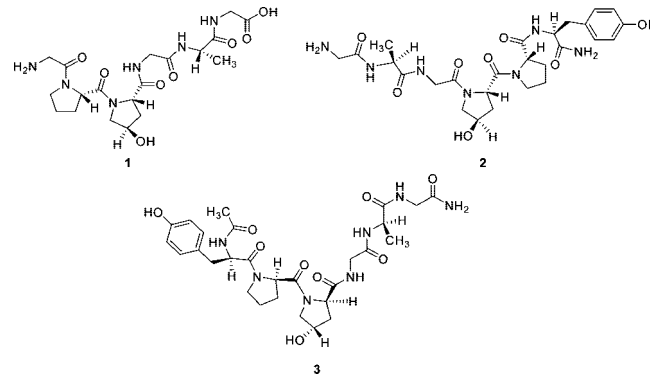
Abstract: Rotigaptide (**3**) is an antiarrhythmic peptide that improves cardiac conduction by modifying gap-junction communication. Small molecule gap-junction modifiers with improved physical properties were identified from a Zealand Pharma peptide library using pharmaceutical profiling, established SAR around **3**, and a putative pharmacophore model for rotigaptide. Activity of the compounds was confirmed in a mouse cardiac conduction block model of arrhythmia. Dipeptide **9f** (GAP-134) was identified as a potent, orally active gap-junction modifier for clinical development.

The isolation of a peptidic compound from bovine atria that restored rhythmicity in cultured cardiac myocytes led to the discovery of AAP[a] (**1**, Chart 1).[1,2] Subsequent mechanistic studies on **1** revealed that it lacked direct effects on classical ion channels, thereby fueling an interest in this new class of antiarrhythmic agents.[3] Dhein and co-workers reported on the synthesis and characterization of a series of AAP analogues, the most potent of which was AAP10 (**2**).[4] The underlying mechanism of action of **2** and its analogues reported in 1997 involved the re-establishment of gap-junction intercellular communication (GJIC) in adjacent cardiomyocytes,[5] thus reversing cardiac conduction slowing and electrical heterogeneity thought to be responsible for arrhythmias. Inherent enzymatic instability in first generation peptidic gap-junction modifiers **1** and **2** precluded their further development as useful antiarrhythmic agents. The discovery of rotigaptide (**3**) as a retroinverso analogue (reverse sequence of amino acids with concomitant replacement of L-amino acids with unnatural D configuration) of **2** represents a significant advance in the field and led to a second generation of enzymatically stable, efficacious gap-junction modifiers.[6] Compound **3** was evaluated clinically for the iv treatment of VF.[7] The goal of this study was to develop a third generation of small molecule gap-junction modifiers possessing the appropriate pharmaceutical properties required for oral administration to treat chronic conditions such as AF.

Chart 1. Antiarrhythmic Peptides AAP (**1**), AAP10 (**2**), and Rotigaptide (**3**)



The molecular target for this class of compounds remains elusive, although it has been reported that **2** and **3** may modulate the phosphorylation state of several serine residues near the C-terminus of connexin-43 (Cx43), the transmembrane spanning protein comprising the connexon hemichannel that ultimately forms the pore of the gap-junction.[7] Lacking knowledge of the molecular target precluded the use of a high-throughput screen to identify small molecule leads for optimization. An established in vivo mouse arrhythmia model involving gap-junction communication and uncoupling was utilized as the primary assay for activity.[8] In this model increased intracellular calcium concentration leads to uncoupling of gap-junctions and second degree cardiac conduction block. Compounds that maintain or re-establish GJIC produce a delay in the time required to induce cardiac conduction block.

Using this assay, scientists at Zealand Pharma generated a pharmacophore model for **3** based on the SAR of a hexapeptide library generated during the optimization campaign for **3**. The SAR highlights for **3** are illustrated in Figure 1. Truncation and amino acid substitution exercises on **3** showed that (i) the tandem

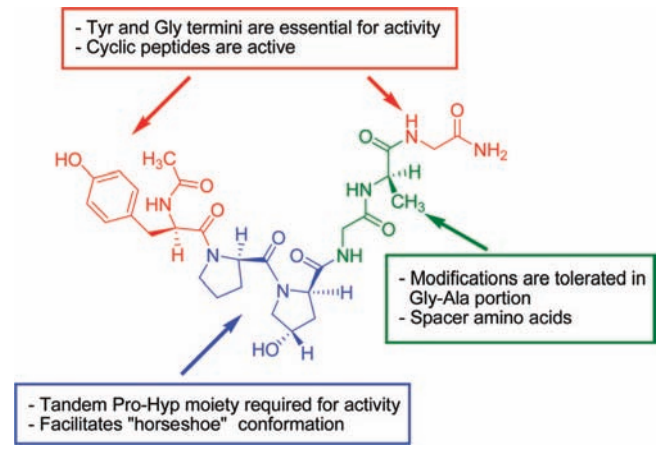


Figure 1. SAR trends derived from a hexapeptide library of rotigaptide analogues administered iv.

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[a] Abbreviations: AF, atrial fibrillation; VF, ventricular fibrillation; AAP, antiarrhythmic peptide; AV, atrioventricular; GJIC, gap-junction intercellular communication; Cx43, connexin-43.

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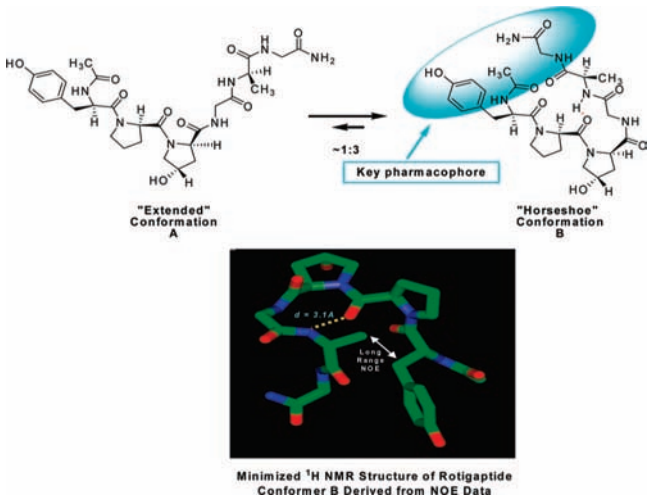


Figure 2. ^{1}H NMR based conformational analysis of **3** shows a 1:3 ratio of the extended (A) vs horseshoe (B) conformations.

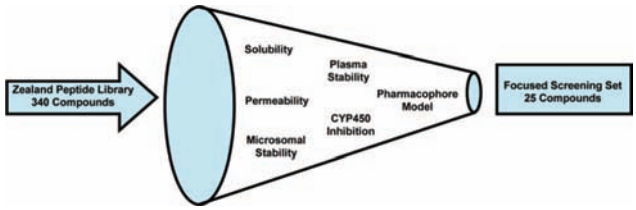


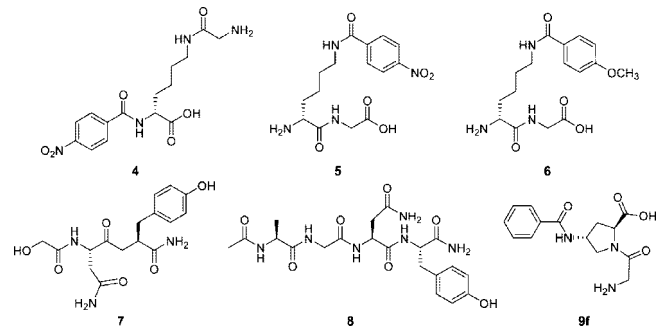
Figure 3. Triage of small peptide library to generate initial screening set for mouse $CaCl_2$ model.

proline−hydroxy−proline motif was essential for activity, (ii) the glycine−alanine section showed a fair degree of tolerance for modification as long as proper spacing was maintained, and (iii) the N-terminal tyrosine and C-terminal glycine were essential for activity. Cyclization of the tyrosine and glycine using one of several spacer amino acids gave rise to several cyclic heptapeptides that were still able to prolong time to conduction block in the mouse $CaCl_2$ model. On the basis of these SAR data, a hypothesis was put forward that the terminal tyrosine and glycine units are required to be relatively close to each other in three-dimensional space, probably facilitated by the tandem proline units and one or several intramolecular hydrogen bonds formed in the hexapeptide backbone.

Further evidence to support this putative pharmacophore model was obtained from ^{1}H NMR conformational analysis (Figure 2) of **3**, which showed two major conformations, the "extended" and "horseshoe" conformations, in approximately a 1:3 ratio. A hydrogen bond between the proline carbonyl and the alanine NH stabilizes this conformation. On the basis of the long-range NOE observed between the tyrosine methylene protons and the alanine methyl group, the energy minimized computer-generated model of conformer B is shown in the lower part of Figure 2. This was consistent with a similar bias toward the horseshoe conformation reported for **2** by Dhein and co-workers.[9]

To generate a focused screening compound library for initial evaluation in the mouse $CaCl_2$-induced arrhythmia model, the Zealand Pharma small peptide library (∼340 compounds) was triaged using pharmaceutical profiling data, calculated properties, scaffold clustering, and adherence to the putative pharmacophore model based on the hexapeptide **3**. The 340 compounds were subjected (Figure 3) to pharmaceutical profiling assays including solubility at pH 7.4, PAMPA permeability at pH 7.4, rat microsomal stability, rat plasma stability, and CYP450 inhibition

Chart 2. Top Tier Hits from Focused Screen: Three Lys-Gly Analogues (**4**−**6**), Two Asn-Tyr Analogues (**7**, **8**), and Singleton Gly-Pro Analogue (**9f**)



(CYP3A4, CYP2D6, and CYP2C9) at 3 μM. Clustering of compounds allowed us to further reduce the screening set by selecting representatives from each structural class, with a bias toward smaller di- and tripeptides that adhered to the putative pharmacophore model.

In the initial screening iteration, the 25 representative small peptides were evaluated for their ability to prolong the time to cardiac conduction block in a mouse $CaCl_2$-induced arrhythmia model.[8,10] The compounds (in a volume of 100 μL/30 g mice) were administered iv (at doses of 10^{-12}, 10^{-11}, 10^{-10}, and 10^{-9} mol/kg) into the tail vein of anesthetized CD-1 mice equipped with ECG electrodes. Infusion of $CaCl_2$ solution (30 mg/mL, 0.2 (mL/min)/30 g mice) was initiated 3 min after drug or vehicle administration. The dose-dependent time lag (ΔT) to onset of AV block was measured for each compound and compared to vehicle effect and to the response seen with hexapeptide **3** as a positive control. A prolongation of the time to the onset of AV block was indicative of antiarrhythmic activity and suggestive of improved GJIC and conduction velocity.[8,10]

Six small peptides (Chart 2) whose potency and efficacy in the mouse $CaCl_2$ model were indistinguishable from hexapeptide **3** were ranked as top tier compounds for initial follow-up studies. The data for prolongation of the time to conduction block for **4**−**9f** and parent hexapeptide **3** are shown in Table 1. In a dose dependent manner, small peptides **4**−**9f** had a significant effect on ΔT values after iv administration, typically prolonging the time to AV block by ∼150 to over 200% versus control values. To further study the three scaffolds of hits identified in the initial screen, we selected **6** as a representative of the Lys-Gly scaffold, **7** as a representative of the Asn-Tyr scaffold, and Gly-Pro singleton **9f** for additional characterization. The graphical representation of data for **6**, **7**, **9f**, parent hexapeptide **3**, and vehicle control are shown in Figure 4.

Compounds **6**, **7**, and **9f** were also evaluated in the rat atrial strip conduction velocity model.[11] In this model, metabolic stress (glucose and O_2-free media) induces conduction velocity slowing (measured with two microelectrodes) in rat atrial strips, presum-

Table 1. Effect of Tier 1 Compounds on Time to Cardiac Conduction Block in Mice Receiving an Intravenous Infusion of Calcium Chloride[a]

compd	10^{-12} mol/kg	10^{-11} mol/kg	10^{-10} mol/kg	10^{-9} mol/kg
4	192 ± 22	177 ± 12	151 ± 22	158 ± 9
5	124 ± 15	206 ± 19	197 ± 14	201 ± 30
6	220 ± 25*	133 ± 7	128 ± 11	164 ± 19*
7	188 ± 29*	232 ± 24*	203 ± 20*	187 ± 29*
8	228 ± 26	157 ± 8	205 ± 15	136 ± 11
9f	162 ± 9*	207 ± 21*	148 ± 20*	173 ± 11*
3	199 ± 30*	207 ± 26*	152 ± 10*	156 ± 15*

[a] Reported as % of control response, mean ± SEM; n = 7−11.

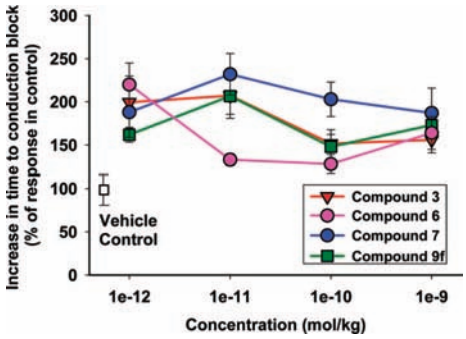


Figure 4. Mouse $CaCl_2$ data for **6**, **7**, **9f**, and hexapeptide **3** after iv administration.

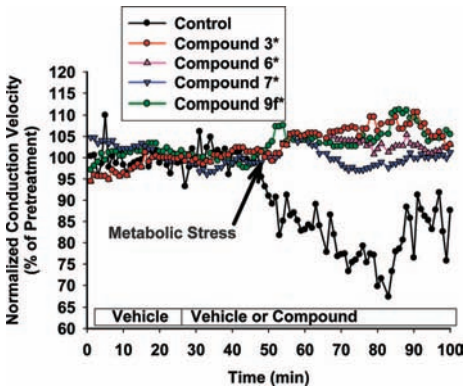


Figure 5. Compounds **6**, **7**, and **9f** prevent metabolic stress-induced conduction velocity slowing in rat atrial strips. Results are comparable to those seen with hexapeptide **3**.

ably due to the electrical heterogeneity that follows after the uncoupling of gap-junctions under these conditions. A compound that re-establishes GJIC would prevent stress-induced conduction velocity slowing in this model. At 10 nM, **6**, **7**, and **9f** prevented (Figure 5) the conduction velocity slowing seen in the control tissue after induction of metabolic stress. At this concentration, the compounds did not affect atrial contractility. The effects were identical to those observed with hexapeptide **3**.

Since the oral bioavailability of **3** in multiple species was negligible (unpublished data), a key focus of the follow-on campaign was to identify equipotent compounds with enhanced PK properties that could have potential to treat chronic AF. Preliminary rat PK studies were performed on the three prototypes **6**, **7**, and **9f**, and the data are shown in Table 2. With an oral bioavailability of 9% in the rat, **9f** was selected for additional characterization and was found to exhibit 21% oral bioavailability in the dog (unpublished results; for additional data suggestive of oral bioavailability of **9f** in dog, see ref 12). Compound **9f** did not demonstrate permeability characteristics in pharmaceutical profiling, thereby suggesting uptake by active transport mechanisms.

Compound **9f**was resynthesized according to the route depicted in Scheme 1.[13] (2*S*,4*R*)-1-*tert*-Butyl 2-methyl 4-aminopyrrolidine-1,2-dicarboxylate (**9a**) was benzoylated in the presence of $NaHCO_3$ to give **9b** which upon deprotection in HCl afforded aminoproline derivative **9c**. This compound was reacted with Boc-Gly-OH under standard peptide coupling protocol to give dipeptide **9d**. Saponification of the methyl ester followed by treatment with HCl to remove the Boc group afforded **9f** as a crystalline HCl salt. The sequence was highly efficient (overall yield = 56%), amenable for kilogram-scale, and was performed with no chromatography.

Table 2. Rat PK Parameters for **6**, **7**, and **9f** ($n = 3$)

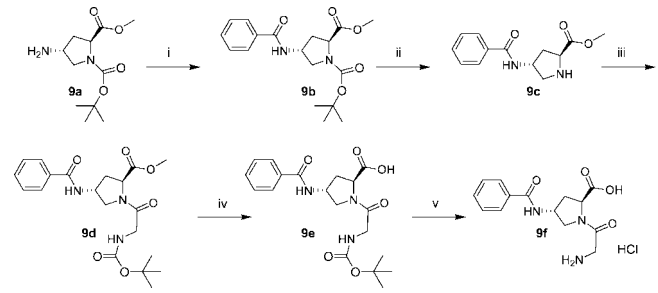
	6	**7**	**9f**
iv (0.5 mg/kg)			
Cl ((mL/min)/kg)	31 ± 12.3	55.4 ± 7.8	22 ± 4
V_{ss} (L/kg)	0.4 ± 0.4	0.9 ± 0.0	0.61 ± 0.28
$T_{1/2}$ (h)	0.3 ± 0.1	0.3 ± 0.0	0.8 ± 0.9
po (5 mg/kg)			
rat F (%)	2	0.7	9
$T_{1/2}$ (h)	1.5 ($N = 2$)	0.5 ($N = 2$)	1.9 ± 0.6
C_{max} (ng/mL)	28	31.5	105 ± 26
T_{max} (h)	0.75	0.4	0.5 ± 0
AUC (ng·h/mL)	71	36	360 ± 123

It has been shown that **3** attenuates calcein dye uptake and cell swelling by reducing permeabilization in cultured neonate rat ventricular myocytes.[14] Connexin hemichannels in the cell membrane are thought to regulate dye retention in this model, thus suggesting that gap-junctions and re-establishment of GJIC may play a cardioprotective role. Compound **9f** was evaluated for its ability to reduce dye uptake. Figure 6 shows that **9f** dose dependently reduces dye uptake in cultured C6 glioma cells in a manner similar to that of parent **3**.

Figure 7 illustrates an overlay (using MULTIFIT[15] routine within SYBYL) of the minimized structure of **9**[16] with the ^{1}H NMR derived horseshoe structure of **3**.[17−19] It is our working hypothesis that the benzoyl moiety of **9f** is mimicking the N-terminal tyrosine phenyl group of **3** while the glycine portion of **9f** might be providing a similar hydrogen bond donor−acceptor local environment as the C-terminal glycine carboxamide of **3**.

Finally, **9f** was evaluated for in vivo efficacy in the mouse $CaCl_2$ model after oral administration. For doses of 5−20 mg/kg po, **9f** significantly prolonged (Figure 8) the time to conduction block (~200% versus vehicle control; $n = 6-12$) in mice after the infusion of $CaCl_2$. Hexapeptide **3**, at 10 mg/kg, was inactive after oral administration, consistent with its poor oral bioavailability.

Scheme 1. Synthesis of **9f**[a]



[a] Reaction conditions: (i) PhCOCl, EtOAc, H_2O, $NaHCO_3$ (94%); (ii) HCl, Et_2O (99%); (iii) Boc-Gly-OH, $EtNCN(CH_2)_3NMe_2$·HCl, HOBT (74%); (iv) NaOH, MeOH (93%); (v) HCl (88%).

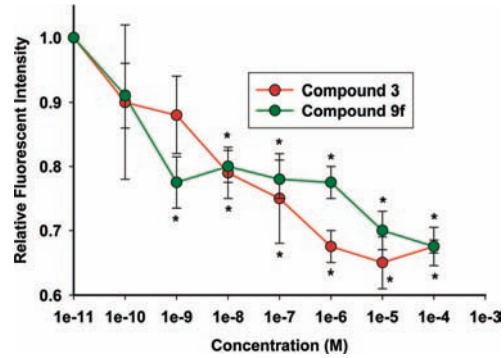


Figure 6. Dye uptake data for **3** and **9f** in C6 glioma cells.

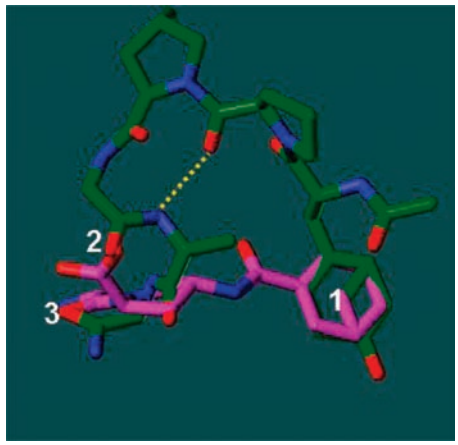


Figure 7. Pharmacophore overlay of **9f** (magenta) with the ^{1}H NMR derived putative bioactive conformer of **3** (green). Atoms used in MULTIFIT alignment are numbered: (1) centroids of benzoyl moiety of **9f** and *N*-tyrosinephenyl group of **3**; (2, 3) the glycine portion of **9f** with the C-terminal carboxamide of **3**.

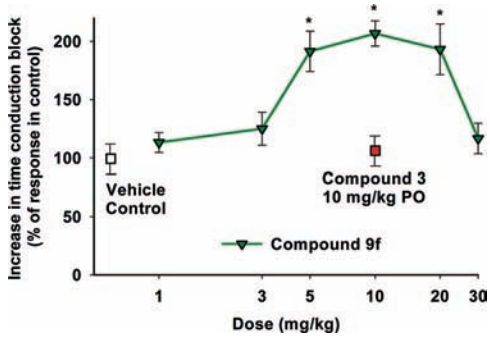


Figure 8. Mouse $CaCl_2$ data for **9f** and hexapeptide **3** after oral administration.

The bell shape dose response observed in this model has been observed previously with other antiarrhythmic compounds including verapamil and amiodarone and is likely model specific.[10]

In summary, we have reported our efforts to identify the first orally bioavailable small molecule gap-junction modifier. Screening of a focused subset of the Zealand Pharma small peptide library resulted in the identification and characterization of three small molecule chemotypes that displayed the same potency and efficacy as parent hexapeptide **3**. After demonstrating oral bioavailability in dog (21%), **9f** ((2*S*,4*R*)-1-(2-aminoacetyl)-4-benzamidopyrrolidine-2-carboxylic acid hydrochloride, GAP-134) was selected for additional studies and was shown to re-establish GJIC, prevent conduction velocity slowing in metabolically stressed atrial tissue, and reduce dye uptake and cell swelling in C6 glioma cells. The compound significantly prolonged the time to AV block in the mouse $CaCl_2$ arrhythmia model after oral administration. Compound **9f** was found to have a highly desirable in vitro and in vivo safety profile including (1) lack of direct effects on membrane ion currents, hERG inhibition, and CYP450 liabilities, (2) negative AMES result, (3) a benign NovaScreen profile, and (4) no effects on systemic hemodynamics.[20] On the basis of these and other data to be reported in due course, **9f** was selected as a development compound and is currently in phase 1 clinical trials for the treatment of AF.

Supporting Information Available: Procedures for the synthesis and characterization of **9f**, details of the in vitro and in vivo biological protocols, and details of the ^{1}H NMR and ^{13}C NMR conformational analysis and modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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