Communications to the Editor

Inhibitors of Efflux Pumps in *Pseudomonas aeruginosa* Potentiate the Activity of the Fluoroquinolone Antibacterial Levofloxacin

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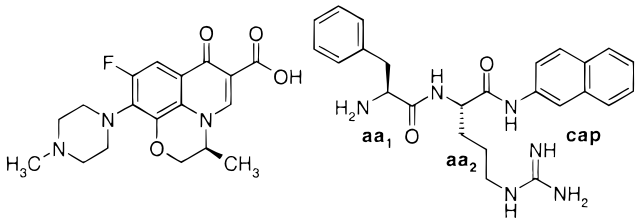
Received September 8, 1999

Introduction. The emergence of resistance to the major classes of antibacterial agents is recognized as a serious public health concern.[1] Drug inactivation, target modification, and alteration in target accessibility through drug efflux and decreased uptake are the three primary mechanisms that microorganisms employ in response to the pressures of antimicrobial therapy.[2] Drug inactivation and target modification are the two most studied and best characterized mechanisms of drug resistance;[3] however, active efflux of antimicrobial agents is increasingly being recognized as a major cause of antimicrobial resistance among a diverse set of antibacterial agents including β-lactams, macrolides, tetracyclines, and fluoroquinolones.[4] For example, of approximately 1000 clinical isolates of *Streptococcus pneumoniae* recently examined from the United Kingdom, 27% showed reduced susceptibility to the fluoroquinolones norfloxacin and ciprofloxacin, 45% of which were due to active efflux.[5] The inhibition of efflux transporters as a means of potentiating the activity of existing antimicrobial agents is an attractive avenue for drug discovery.[6] Several reports describing just such an approach have appeared wherein inhibitors of the drug-specific tetracycline efflux pump in *Escherichia coli* have been identified, although no in vivo efficacy data was presented.[7]

Recently, we embarked on a program to identify efflux pump inhibitors in *Pseudomonas aeruginosa* as a way of potentiating the activity of the fluoroquinolone antibacterial agent levofloxacin (Chart 1). *P. aeruginosa* is an opportunistic pathogen characterized by intrinsic resistance to a wide variety of antimicrobial agents. This property has been attributed both to the impermeability of the outer membrane as well as to the activity of several efflux systems,[8] four of which have been identified to date and which are encoded for by the structurally related *mexAB-OprM*, *mexCD-OprJ*, *mexEF-OprN*, and *mexXY* genes.[9] Results demonstrating that the inhibition of efflux pumps in *P. aeruginosa* could (i) overcome the intrinsic resistance in *P. aeruginosa* to fluoroquinolones such as levofloxacin, (ii) reduce the high-level acquired resistance to these agents, regardless of mutations in the target genes (*gyrA*, *parC*), and (iii) decrease the frequency of emergence of *P. aeruginosa* strains highly resistant to fluoroquinolones in clinical settings were an impetus for undertaking this project.[10]

Chart 1



Levofloxacin **1 (MC-207,110)**

From the outset, we pursued a strategy to identify a broad-spectrum agent that would simultaneously inhibit the MexAB-OprM, MexCD-OprJ, and MexEF-OprN pumps, an approach that was based on a variety of biological data. For instance, although MexAB-OprM is the only one of the four known multidrug-resistant pumps in *P. aeruginosa* expressed in the wild-type, there are reports of clinical isolates that are resistant to fluoroquinolones which demonstrate increased expression of any of the MexAB-OprM, MexCD-OprJ, or MexEF-OprN pumps.[11] In addition, we have shown that selective inhibition of the MexAB-OprM pump in strains that overexpress other efflux pumps has no effect on quinolone resistance.[10,12]

To identify potential broad-spectrum efflux pump inhibitors, we screened our synthetic compound and fermentation extract collection in the presence of levofloxacin using specifically engineered strains of *P. aeruginosa* that overexpressed each of the known pumps.[13] To quantify the activity of the inhibitors, we defined the term MPC_8 as the minimum concentration (μg/mL) of inhibitor required to decrease (potentiate) the MIC of levofloxacin 8-fold. Compounds with MPC_8s < 40 μg/mL were advanced for further profiling.

One compound identified from this screening effort was **1** (MC-207,110, Chart 1), a low molecular weight dipeptide amide. The compound had minimal intrinsic antibacterial activity (MIC > 512 μg/mL), potentiated the activity of levofloxacin 8-fold at 10 μg/mL, and was validated as a broad-spectrum efflux pump inhibitor in *P. aeruginosa* (Table 1). Followup studies were implemented to confirm that **1** was indeed blocking the efflux pumps.[14] Specifically, in a strain of *P. aeruginosa* lacking all three efflux pumps (PAM 1626), the MPC_8 of **1** was >40 μg/mL. The overall in vitro biological

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10.1021/jm9904598 CCC: $18.00
Published on Web 11/10/1999

Table 1. Activity of the Efflux Pump Inhibitors **1** (MC-207,110) and **13** Against PAM 1032, PAM 1033, and PAM 1034[a]

		MIC to levofloxacin (μg/mL) in the presence of efflux pump inhibitor							
		efflux pump inhibitor concentration (μg/mL)							
compd	strain[a]	0	1.25	2.5	5	10	20	40	MPC_8[b] (μg/mL)
1	PAM 1032	2	2	1	0.5	0.125	0.06	0.06	10
13		2	1	0.5	0.125	0.06	0.06	0.06	5
1	PAM 1033	4	4	2	0.5	0.125	0.125	0.125	5
13		4	2	0.5	0.125	0.06	0.06	0.06	2.5
1	PAM 1034	4	4	4	1	0.25	0.06	0.03	10
13		4	4	1	0.25	0.125	0.125	0.125	5

[a] PAM 1032, PAM 1033, and PAM 1034: laboratory strains of *P. aeruginosa* which overexpress the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps, respectively. [b] MPC_8: minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin 8-fold.

Table 2. Intrinsic Antibacterial Activity and Activity of Efflux Pump Inhibitors Against PAM 1032[a]

compd	MIC[b] (μg/mL)	MPC_8[c] (μg/mL)	VOL_{SYN}[d]
1	> 512	10	1962
2	> 512	10	1390
3	512	5	1760
4	> 512	> 40	463
5	256	5	2825
6	512	5	2343
7	> 512	40	486
8	> 512	> 40	183
9	128	2.5	2612
10	256	2.5	2223
11	> 512	> 40	906
12	> 512	10	2056
13	> 512	5	2586

[a] PAM 1032: laboratory strain of *P. aeruginosa* which overexpresses the MexAB-OprM efflux pump. [b] MIC: minimum concentration (μg/mL) of efflux pump inhibitor required to inhibit the growth of PAM 1032. [c] MPC_8: minimum concentration (μg/mL) of efflux pump inhibitor required to reduce (potentiate) the MIC of levofloxacin. [d] VOL_{SYN}: volume of synergy (see ref 15).

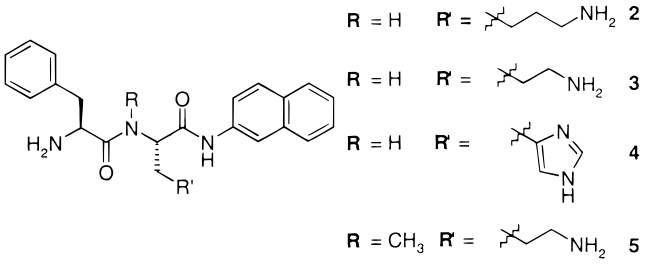
profile and structural simplicity of **1** were major factors in choosing this agent as the lead compound in our program.

We began an extensive medicinal chemistry program to optimize the biological and physicochemical properties of this lead compound. Herein we describe a portion of our efforts in this area and disclose the first known class of broad-spectrum efflux pump inhibitors for *P. aeruginosa*. Additionally, using a prototypical compound from this study, we provide evidence of efficacy in an in vivo model of infection.

Synthesis and Biological Evaluation. Standard peptide coupling techniques were used in the preparation of the target dipeptide amides. The final products were purified by reverse-phase MPLC and tested as their bis-TFA salts. The structural identity of each compound was confirmed by ^{1}H NMR and HRMS, and the purity of the compounds was established by two diverse HPLC systems.

Compounds from the chemistry effort were tested using a modification of the screening assay, and the results are displayed in Tables 1 and 2. Interactions between the inhibitors and levofloxacin were quantified by the determination of the MPC_8 and the use of MacSynergy II 3-D analysis.[15] This method is suitable in determining the extent of synergism (or additivity) of activity between two drugs. To establish selectivity, a collection of randomly chosen compounds was examined for their ability to inhibit P-glycoprotein (P-gp), a mammalian efflux pump that is associated with multidrug resistance. None of the compounds tested inhibited P-gp at concentrations where they inhibited the bacterial efflux pumps (data not shown).

Chart 2



Results and Discussion. A strain (PAM 1032) of *P. aeruginosa* overexpressing *mexAB-OprM* was used as a marker of biological activity. As expected, the activity of the compounds tested in this strain mirrored the results obtained in the wild-type strain (PAM 1020) because PAM 1020 already has a basal expression of *mexAB-OprM* and does not express *mexCD-OprJ* or *mexEF-OprN*. In general, the extent of potentiation, or synergy, between the inhibitor and levofloxacin increased as the MPC_8 decreased.

We initially examined the structure−activity relationships (SAR) of the middle amino acid (denoted as "aa_2", Chart 2). A brief study showed that a basic amino acid was a prerequisite for activity and that although substitution with L-lysine gave a compound (**2**) that was as potent as the lead, introduction of the unnatural amino acid L-ornithine (L-Orn) gave **3**, which was 2-fold more potent than **1** (Table 2). Results showing that the *N*-δ-methyl-L-Orn and the *N*,*N*′-δ-dimethyl-L-Orn analogues were inactive (data not shown), coupled with the lack of activity of the histidine derivative **4**, demonstrated the importance of a basic, primary amino group for biological activity. The ornithine moiety provided synthetic simplicity, and it became the standard middle residue in successive compounds.

Although compounds **2** and **3** were effective as potentiators of levofloxacin activity in vitro, they were not stable upon incubation with mouse, rat, or human serum at 37 °C. Followup HPLC studies demonstrated that cleavage of the peptide linkage between "aa_1" and "aa_2" occurred following the administration of mouse serum, results which were understandable given that the compounds contained natural (L) amino acids. To circumvent this problem, we prepared the *N*-methyl derivative **5** that was both stable to serum proteases and as potent as compound **3** (Table 2).

While studying the stability of compounds **2** and **3**, we continued, in parallel, our SAR study by examining

Chart 3

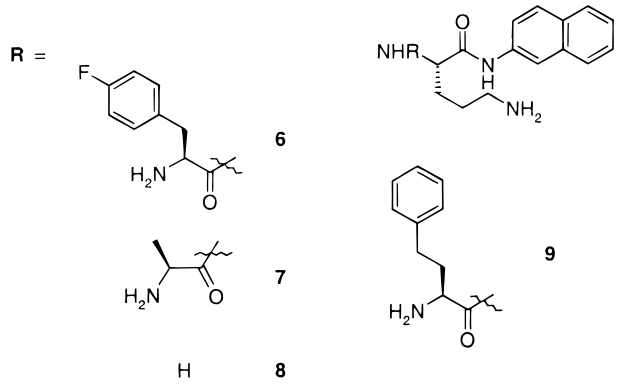
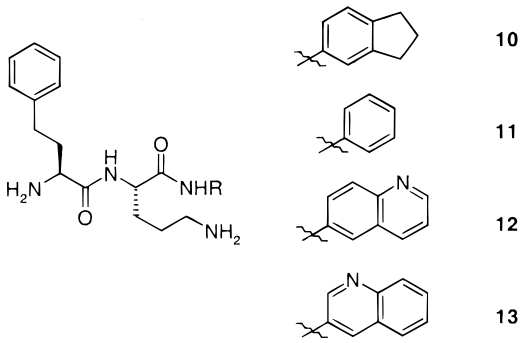


Chart 4



changes at both the aryl amino acid group as well as the capping group (denoted as "aa_1" and "cap", respectively). The chemistry campaign involved both a traditional solution-phase medicinal chemistry program as well as a highly focused solid-phase combinatorial effort, a report of which has recently appeared.[16] Overall, we prepared more than 500 analogues of diverse activity. Whereas substitution of phenylalanine with other aromatic amino acids, a typical example being L-*p*-F-phenylalanine, afforded analogues such as **6**, the alanine derivative **7** and the unsubstituted analogue **8** were much less active, thereby establishing the importance of an appropriately substituted aromatic amino acid at "aa_1" (Chart 3). Replacement of phenylalanine with homophenylalanine (L-hPhe) at "aa_1", such as that found in compound **9**, consistently gave a 2-fold improvement in activity over compounds **3** and **6**. Compound **9**, for example, had an MPC_8 of 2.5 μg/mL vs 5 μg/mL for compound **3** (Table 2).

We probed the SAR of the capping group by maintaining the "aa_1-aa_2" motif at the optimized amino acids, L-hPhe-L-Orn (Chart 4). A variety of groups, such as the 5-aminoindan derivative **10**, were as active as the β-naphthylamine analogue **9**. Aniline or substituted aniline derivatives such as **11** were typically inactive. Several polar capping groups, such as 6-aminoquinoline, afforded compounds such as **12** that were as active as compound **2**. Of the various capping groups studied, compounds incorporating the 3-aminoquinoline moiety had the best overall biological profiles. Thus, whereas compounds containing 3-aminoquinoline were slightly less potent (compare, for example, compounds **9** and **13**, Table 2), they consistently had less intrinsic antibacterial activity and were less cytotoxic to mammalian cells in vitro (data not shown). We confirmed that **13** retained the broad-spectrum pump inhibitory characteristics of the series by demonstrating its activity as a potentiator of levofloxacin vs PAM 1032, PAM 1033, and PAM 1034 (Table 1) and established that, like **1**, it interacted directly with the pump(s).[14] Thus, compound **13**, in PAM 1626, had no effect on the MIC of levofloxacin at concentrations equal to or less than 40 μg/mL.

Because of its stability profile, we chose compound **5** as a prototypical inhibitor to conduct the pivotal in vivo profiling in combination with levofloxacin, assessing its activity in a murine neutropenic thigh model against *P. aeruginosa* PAM 1032. Male CFW mice were immunosuppressed with cyclophosphamide (150 mg/kg) on days 1 and 3. On day 5, the mice were administered 1.0×10^5 colony forming units (cfu) of *P. aeruginosa* PAM 1032 (intramuscularly) into both thighs. After 2 h, animals were treated with levofloxacin (30 mg/kg, subcutaneously) and compound **5** (30 mg/kg, intraperitoneally). Animals were sacrificed at 0, 2, 4, 6, and 8 h following treatment. Both thighs were aseptically removed, homogenized, and plated on drug-free agar, and bacterial counts were measured.

Following the procedure described above, when compound **5** was administered in combination with levofloxacin, a 3-log reduction in cfu was observed for approximately 4 h, followed by regrowth. In contrast, a single dose of either levofloxacin (30 mg/kg) or compound **5** resulted in growth similar to the untreated controls. These results provide clear evidence that the potentiation phenomenon observed in vitro can be demonstrated in vivo.

In summary, we have described the first known class of broad-spectrum efflux pump inhibitors in *P. aeruginosa*. Our efforts are currently focused on optimizing the pharmacological properties of the series, reports of which will be the subject of future publications.

Acknowledgment. This work was principally funded by Daiichi Pharmaceuticals. The authors thank Lubov Filonova, Melissa Jordan, Kristina Mathias, and Deidre Cho for their expert technical assistance. We also thank Will Watkins for his review of the manuscript and Mary Price for analytical support.

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JM9904598