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MexAB-OprM Specific Efflux Pump Inhibitors in *Pseudomonas aeruginosa*. Part 2: Achieving Activity In Vivo Through the Use of Alternative Scaffolds

Kiyoshi Nakayama,[a,*] Yohei Ishida,[a] Masami Ohtsuka,[a] Haruko Kawato,[a] Ken-ichi Yoshida,[a] Yoshihiro Yokomizo,[a] Toshiharu Ohta,[a] Kazuki Hoshino,[b] Tsuyoshi Otani,[b] Yuichi Kurosaka,[b] Kumi Yoshida,[b] Hiroko Ishida,[b] Ving J. Lee,[c] Thomas E. Renau[c] and William J. Watkins[c]

[a]*Medicinal Chemistry Research Laboratory, Daiichi Pharmaceutical Co., Ltd., 1-16-13, Kitakasai, Edogawa, Tokyo 134-8630, Japan*
[b]*New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd., 1-16-13, Kitakasai, Edogawa, Tokyo 134-8630, Japan*
[c]*Essential Therapeutics, Inc., 850 Maude Avenue, Mountain View, CA 94043, USA*

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Abstract—Problems of low solubility, high serum protein binding, and lack of efficacy in vivo in first generation MexAB-OprM specific efflux pump inhibitors were addressed. Through the use of pharmacophore modelling, the key structural elements for pump inhibition were defined. Use of alternative scaffolds upon which the key elements were arrayed gave second generation leads with greatly improved physical properties and activity in the potentiation of antibacterial quinolones (levofloxacin and sitafloxacin) versus *Pseudomonas aeruginosa* in vivo.

A common recent paradigm for drug discovery involves the identification and validation of the drug target, followed by high throughput screening to identify hits.[1] Such starting points for medicinal optimization often do not possess appropriate physicochemical properties for their intended use; for example, the aqueous solubility is frequently far lower than that required for intravenous formulation, or the lipophilicity is inappropriate for efficient permeability across membranes.[2]

We recently described[3] the discovery of the first example of an efflux pump inhibitor (EPI), **1** (Fig. 1), that is specific for the MexAB-OprM system in *Pseudomonas aeruginosa*. This problematic pathogen is characterized by intrinsic resistance to a variety of antimicrobial agents through the expression of several different efflux pumps,[4–7] and **1** was a very effective potentiator, in vitro, of Levofloxacin (LVFX) or Aztreonam (AZT) in strains in which MexAB-OprM was over-expressed. The very high serum protein binding of the compound was recognized as a probable factor limiting its in vivo efficacy, and came as no surprise given the lipophilic and acidic nature of the lead. Unsuccessful attempts to reduce the protein binding through the incorporation of polar groups were described earlier.[3] Herein, we report the application of a second strategy based upon redesign of the molecular scaffold, and the consequent

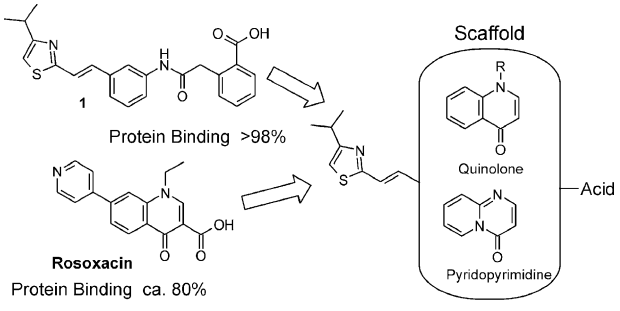

Figure 1. Structures of **1** and rosoxacin, and novel scaffold design.

*Corresponding author. Fax: +81-3-5696-8772; e-mail: nakaygoi@daiichipharm.co.jp

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discovery of a molecule that exhibits in vivo efficacy in the potentiation of quinolones.

The strategy of replacing a molecular scaffold while maintaining key residues of a pharmacophore in appropriate positions has been proven as a method of dramatic alteration of the parent structure without loss of activity (Fig. 1).[8–13] Because the overall structure is changed drastically, the physicochemical properties of the lead will be altered. By choosing a scaffold that is commonly observed in drugs (particularly in the relevant therapeutic area), one might thereby confer molecular properties that are more consistent with the desired pharmacokinetic profile.

New quinolone antibacterial agents such as LVFX display low protein binding despite the presence of a carboxylic acid. Even the so-called acidic quinolones (early versions of the class lacking a basic substituent such as piperazine at the 7-position of the quinolone nucleus) are not highly bound;[14] for example, the protein binding of Rosoxacin (Fig. 1) is only 80% (ca. 20% free). We wished to transfer such a profile to **1**, without loss of EPI activity, through the use of quinolones and pyridopyrimidines as scaffolds. In order to do so we required a hypothesis as to the bioactive conformation of **1**, so as to be able to design new compounds in which the thiazole moiety, which was essential for activity, was placed properly on the new scaffold. The molecular modelling software CATALYST™[15] was utilized for this purpose.

First, we chose a training set consisting of **1** and seven structurally diverse analogues,[3] encompassing a wide range of efflux pump inhibitory activity. Then, all conformations within 10 kcal/mol of the global minimum for each molecule were calculated and used to generate hypotheses (pharmacophore models). Finally, the model that gave the most reliable prediction of activity of a test set of fifteen analogues not included in the training set was selected. Predictions were binned as inactive, active, or highly active; the chosen model mis-classified only two analogues. The coordinates for the model and a picture in which it is overlaid with compounds **1** and **4** are shown in Table 1 and Figure 2, respectively.

According to this model, the pharmacophore consists of one acidic (purple sphere) and four hydrophobic moieties (white spheres). The presumed bioactive conformation of **1** is shown in black. After a separate conformational search, we concluded that a molecule having the thiazole side chain attached to the C7-position of a quinolone (compound **4**, shown in green) could also fit the pharmacophore satisfactorily, although one lipophilic moiety would clearly be lacking.

In the light of this result, several test cases with bicyclic aromatic scaffolds were synthesized. Concerned that the simple carboxylic acid might be slightly too close to the central lipophilic moiety, we also synthesized some corresponding tetrazoles.[16] The synthesis of quinolone analogues[17] is depicted in Scheme 1, and of pyridopyrimidine variants,[18] exemplified with vinyl tethers, in Scheme 2. The scaffolds were formed by the condensation of aniline or 2-aminopyridine derivatives with ethoxymethylene malonic acid ester or its tetrazole counterpart, followed by removal of the protecting groups.

To quantify the activity of the inhibitors in vitro, we defined the term MPC_n as the minimum concentration

Table 1. Parameters for the most reliable pharmacophore model

	Feature	*X* (Å)	*Y* (Å)	*Z* (Å)	Tolerance (Å)
1	Hydrophobic	−8.486	0.701	0.909	1.7
2	Hydrophobic	−4.926	2.381	0.069	1.7
3	Hydrophobic	0.194	−2.179	−0.451	1.7
4	Hydrophobic	7.114	1.541	0.909	1.7
5	Negative ionizable	6.83	−1.989	−1.591	1.7

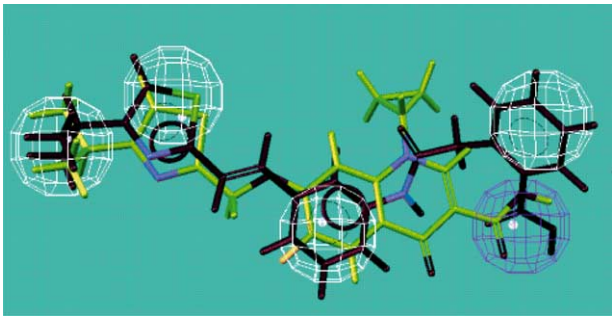
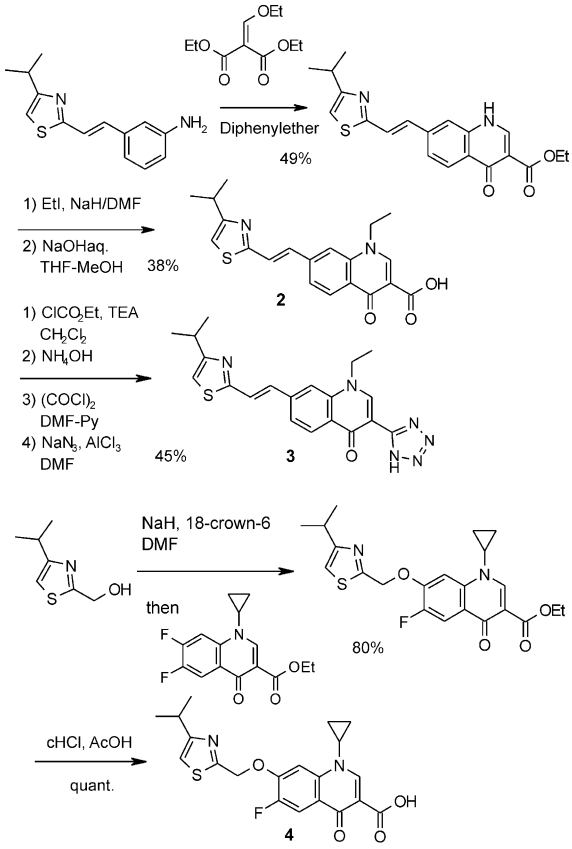
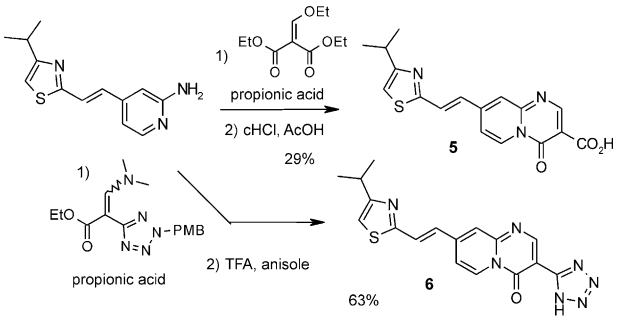


Figure 2. Pharmacophore model generated by CATALYST™ and overlaid structures of compounds **1** and **4**.



Scheme 1. Synthesis of quinolone derivatives.



Scheme 2. Synthesis of pyridopyrimidine derivatives.

(μg/mL) of inhibitor required to reduce (potentiate) the activity of antibacterial drug *n*-fold. Data for the potentiation of LVFX and AZT in the presence or absence of added horse serum is displayed in Table 2, along with the HPLC retention time (Rt) using an albumin-bound column, which conveniently allows an estimate of relative affinity for human serum albumin (HSA). For compounds with low Rt and reasonable activity that was not dramatically affected by serum, we measured the protein binding (%) and affinity for albumin (K_d, μM).

Although the extremely high potency of **1** was somewhat compromised in novel analogues, the effect of the addition of horse serum was greatly reduced. Of the two novel scaffolds, pyridopyrimidine derivatives gave the best results both in terms of activity and Rt (cf **3** vs **6**). Consistent with the hypothesis for the spatial requirements of the acidic and central lipophilic moieties, tetrazoles were found to be significantly more active than carboxylic acids (**2** vs **3**, **5** vs **6**, **7** vs **8**, **9** vs **10**). Rt proved to be a useful predictor in that the rat serum protein binding of the most promising analogues was indeed significantly reduced. Of the novel analogues, **6** and **8** were selected for further study in the potentiation of LVFX in a murine neutropenic sepsis model (Fig. 3).

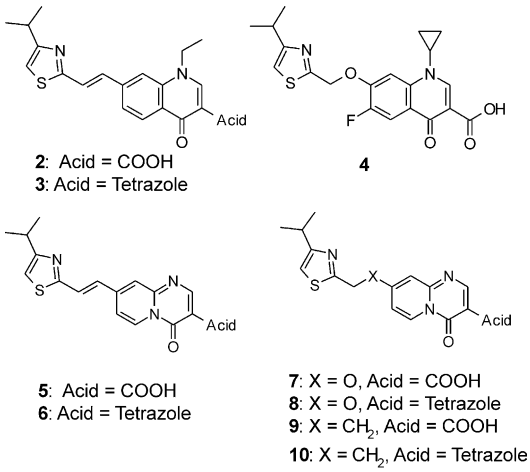


Figure 3. Novel analogues with quinolone or pyridopyrimidine scaffold.

First, we tested LVFX alone to determine the dose response in this model using PAM1723,[20] an experimental strain of *P. aeruginosa* in which the MexAB-OprM pump is overexpressed and MexCD-OprJ and MexEF-OprN are genetically disrupted (MIC of LVFX; 32 μg/mL). As shown in Table 3, a dose of 30 mg/kg gave good survival, whereas no obvious effects were observed at lower doses. The results of potentiation experiments with **6** and **8** are shown in Tables 4 and 5.

Table 3. In vivo activity[a] of LVFX alone[21]

Dose of LVFX (mg/kg)	Number of survivors				% survival on day 7
	Day 0	Day 1	Day 4	Day 7	
7.5	10	0	0	0	0
15	10	1	1	1	10
30	10	9	7	7	70

[a]Organism: PAM1723.

Table 2. In vitro potentiation activity of novel EPIs[a]

	MPC_8 (AZT); μg/mL[b]		Ratio	MPC_8 (LVFX) (μg/mL[c])	Rt on HSA-HPLC (min)[d]	Protein binding (%) (K_d; μM)
	Without serum	With 10% horse serum				
1	≤0.63	10	>16	≤0.63	>60	>99.9 (0.28)
2	10	10	1	>160	>60	—
3	5	20	4	1.25	>60	—
4	10	10	1	320	13.9	90.5
5	80[e]	40	0.5	>320	3.0	—
6	2.5	2.5	1	1.25	13.0	90.8 (22.0)
7	40	80	2	320	1.2	—
8	5	5	1	10	4.7	88.4 (66.4)
9	>40	>40	—	>40	0.9	—
10	10	10	1	>80	3.1	81.5 (327)

[a]Versus PAM1032.[19] All the analogues were devoid of intrinsic antibacterial activity (MIC >320 μg/mL).
[b]Values are for 100% growth inhibition.
[c]Values are for 70% growth inhibition.
[d]Hypersil chiral and drug binding column (HSA) (length, 30×4.6 mm) was used; mobile phase, M/15 phosphate buffer solution–H_2O–*i*PrOH (5:10:1); flow rate, 1 mL/min. A DMSO solution (6.4 mg/mL) of each compound was diluted to 40 μg/mL with phosphate buffer (pH 7.0), and 10 μL of the resulting solution was injected.
[e]Compound precipitated in well.

Table 4. In vivo activity[a] of LVFX + **6** (50 mg/kg)[21]

Dose of LVFX (mg/kg)	Number of survivors				% survival on day 7
	Day 0	Day 1	Day 4	Day 7	
7.5	10	1	1	1	10
15	10	10	7	7	70

[a]Organism: PAM1723.

Table 5. In vivo activity[a] of LVFX + **8** (50 mg/kg)[21]

Dose of LVFX (mg/kg)	Number of survivors				% survival on day 7
	day 0	day 1	day 4	Day 7	
7.5	10	4	4	4	40
15	10	10	10	10	100

[a]Organism: PAM1723.

The combination of **6** with 15 mg/kg of LVFX gave a result equivalent to that of 30 mg/kg LVFX alone. More striking effects were observed with compound **8**, which in combination with 15 mg of LVFX gave greater survival than for 30 mg/kg LVFX alone. Furthermore, an increase in survival at a dose of 7.5 mg/kg LVFX was achieved.

It is possible that the observed potentiation in these studies arises from a pharmacokinetic interaction of the potentiator with the antibiotic, leading to higher LVFX exposures in the combination regimen. While this cannot be ruled out for the above experiments, supplementary work provided further circumstantial evidence for in vivo activity due to MexAB-OprM inhibition. Two clinical strains were identified that displayed similar susceptibilities to sitafloxacin (STFX), one of which (MIC 2 μg/mL) responded to potentiation by **8** in vitro (MPC_4 2.5 μg/mL), whereas the other (MIC to STFX 1 μg/mL) did not. For the first strain, an obvious effect was observed in vivo. Thus, STFX given alone[21] at doses of 100 and 150 mg/kg gave 20 and 90% survival, respectively, whereas at 75 mg/kg in combination with **8**[21] (50 mg/kg iv), survival was 100%. In contrast, no differential effects were seen upon combination of STFX with **8** in vivo for the second strain that was unresponsive to potentiation in vitro.

In conclusion, the problems of low solubility, high serum protein binding and lack of efficacy in vivo in the first generation of MexAB-OprM specific efflux pump inhibitors were addressed by the design of alternative scaffolds using a pharmacophore model. By this means activity was retained and the serum protein binding was successfully reduced, resulting in **8**, which clearly showed in vivo efficacy as a potentiator of two quinolones (LVFX and STFX). The results of further optimization of this series, together with more detailed analysis of pharmacokinetic profiles of combination regimens, will be reported in due course.

References and Notes

1. Chanda, S. K.; Caldwell, J. S. *Drug Discov. Today* **2003**, *8*, 168.
2. Lipinski, C. A. *J. Pharmacol. Toxicol. Methods* **2000**, *44*, 235.
3. Nakayma, K.; Ishida, Y.; Ohtsuka, M.; Kawato, H.; Yoshida, K.; Yokomizo, Y.; Hosono, S.; Ohta, T.; Hoshino, K.; Ishida, H.; Yoshida, K.; Renau, T. E.; Léger, R.; Zhang, J. Z.; Lee, V. J.; Watkins, W. J. *Bioorg. Med. Chem. Lett*. See preceding paper in this issue doi: 10.1016/j.bmcl.2003.07.024.
4. Poole, K.; Krebes, K.; McNally, C.; Neshat, S. *J. Bacteriol*. **1993**, *175*, 7363.
5. Poole, K.; Gotoh, N.; Tsujimoto, H.; Zhao, Q.; Wada, A.; Yamasaki, T.; Neshat, S.; Yamagishi, J.; Li, X. Z. *Nishino Mol. Microbiol*. **1996**, *21*, 713.
6. Koehler, T.; Michea-Hamzehpour, M.; Henze, U.; Gotoh, N.; Curty, L. K.; Pechere, J. C. *Mol. Microbiol*. **1997**, *23*, 345.
7. Mine, T.; Morita, Y.; Kataoka, A.; Mizushima, T.; Tsuchiya, T. *Antimicrob. Agents Chemother*. **1999**, *43*, 415.
8. Belanger, P. C.; Dufresne, C. *Can. J. Chem*. **1986**, *64*, 1514.
9. Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Salvino, J.; Leahy, E. M.; Sprengeler, P. A.; Furst, G.; Smith, A. B., III; Strader, C. D.; Cascieri, M. A.; Candelore, M. R.; Donaldson, C.; Vale, W.; Maechler, L. *J. Am. Chem. Soc*. **1992**, *114*, 9217.
10. Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Leahy, E. M.; Salvino, J.; Arison, B.; Cichy, M. A.; Spoors, P. G.; Shakespeare, W. C.; Sprengeler, P. A.; Hamley, P.; Smith, A. B., III; Reisine, T.; Raynor, K.; Maechler, L.; Donaldson, C.; Vale, W.; Freidinger, R. M.; Cascieri, M. A.; Strader, C. D. *J. Am. Chem. Soc*. **1993**, *115*, 12550.
11. Hirschmann, R.; Hynes, J., Jr.; Cichy, M. A.; van Rijn, R. D.; Sprengeler, P. A.; Spoors, P. G.; Shakespeare, W. C.; Pietranico, S.; Barbosa, J.; Liu, J.; Yao, W.; Rohrer, S.; Smith, A. B., III *J. Med. Chem*. **1998**, *41*, 1382.
12. Olson, G. L.; Cheung, H.-C.; Chiang, E.; Madison, V. S.; Sepinwall, J.; Vincent, G. P.; Winokur, A.; Gary, K. A. *J. Med. Chem*. **1995**, *38*, 2866.
13. Nakayama, K.; Kawato, H. C.; Inagaki, H.; Ohta, T. *Org. Lett*. **2001**, *3*, 3447.
14. Zlotos, G.; Bucker, B.; Kinzig-Schippers, M.; Sorgel, F.; Holzgrabe, U. *J. Pharma. Sci*. **1998**, *87*, 215.
15. Catalyst™, version 4.6, Accelrys, Inc., San Diego, CA, 2002.
16. Carini, D. J.; Duncia, J. V.; Pierce, M. E. *Pharm. Biotechnol*. **1998**, *11*, 29.
17. Mozek, I.; Sket, B. *J. Heterocycl. Chem*. **1994**, *31*, 1293.
18. Kanno, H.; Yamaguchi, H.; Ichikawa, Y.; Isoda, S. *Chem. Pharm. Bull*. **1991**, *39*, 1099.
19. Lomovskaya, O.; Lee, A.; Hoshino, K.; Ishida, H.; Mistry, A.; Warren, M. S.; Boyer, E.; Chamberland, S.; Lee, V. *J. Antimicrob. Agents Chemother*. **1999**, *43*, 1340.
20. Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; Léger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. *Antimicrob. Agents Chemother*. **2001**, *45*, 105.
21. Four-week-old male Slc: ddY mice (Japan SLC, Shizuoka, Japan) were used in groups of 10. Mice were inoculated ip with a single 0.2-mL portion of bacterial suspension with 3% mucin (1.8×10^6 CFU/mouse). All drugs were administered as a bolus, iv, via the tail vein. LVFX and STFX were dosed as a solution in 0.1 N NaOH immediately after inoculation, followed (in potentiation experiments) by the EPI. **6** was formulated in EtOH/Cremaphore EL/phosphate buffer (pH 7) (5:5:45:45); **8** was formulated in EtOH/Cremaphore EL/0.1 N NaOH/phosphate buffer (pH 7) (5:5:45:45) or EtOH/PEG400/0.1 M NaOH (5:5:90).