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A Short History of Topoisomerase Inhibitors at Actelion Pharmaceuticals

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Abstract: The discovery of novel antibacterials devoid of cross resistance is of utmost importance. At the same time, biological pathways and processes suitable to be targeted are limited. At Actelion Pharmaceuticals we decided to work on novel bacterial topoisomerase inhibitors (NBTI) to discover new antibiotics with broad spectrum activity and limited resistance development for use against severe hospital infections. This paper summarizes the learnings and results of our efforts in the field, which led to the discovery of multiple chemical classes with potent Gram-negative activity and ultimately to the selection of several compounds that underwent preclinical profiling.

Keywords: Antibiotics · NBTI · Resistance development · Topoisomerase

Cornelia Zumbrunn obtained her diploma in organic chemistry from the Universities of Fribourg and Neuchâtel (CH). After a PhD thesis at F. Hoffmann-La Roche under the guidance of Prof. Klaus Müller (Roche Basel, CH) and Prof. Hans Jürgen Hansen (University of Zürich) and a Postdoc at Cambridge University (UK) in the research group of Prof. Steve Ley, she started her career on antibacterial drug discovery, first at F. Hoffmann-La Roche, later at Morphochem and finally at Actelion Pharmaceuticals Ltd. (Allschwil, CH). As project leader in medicinal chemistry she focused on the discovery of antibiotics with novel modes of action. Since 2018 she works as medicinal chemist and project leader at Idorsia Pharmaceuticals Ltd. in the division of cardiovascular diseases and fibrosis.

1. Introduction

Antimicrobial resistance is a global problem putting the lives of many people at risk. An estimated 1'270'000 deaths every year are currently attributed to infections caused by resistant pathogens and the numbers are expected to rise to 10 Mio in 2050.[1,2] The WHO has defined the most problematic pathogens, the so-called ESKAPE pathogens, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*.[3] Especially the Gram-negative pathogens are of concern, because their cell architecture is composed of two cell membranes rendering them much less susceptible to antibiotics than Gram-positives with a single membrane.

Despite antimicrobial resistance (AMR) being an urgent threat to global health care, the absolute numbers of untreatable infections are still low (about 16 per 100'000[2]). This means that for most infections it is possible to find a treatment option among the old and cheap drugs. The development of new antibacterial agents which will only be used as a last resort is financially not sustainable and is not paying back the initial investment. More and more companies and institutions decided to leave the field for this reason. This fact was true in 2004 when Actelion started its antibacterial research and still is unfortunately today.[4] To maximize the likelihood of success we set ourselves clear objectives:

- Target the highest medical need: severe hospital infections
- Select highly conserved targets to cover a broad spectrum of pathogens and, therefore, several clinical indications and to be suitable for empirical use
- Avoid cross resistance with currently used antibiotics
- Low risk of resistance development
- Allow for iv formulation for hospital use (ICU)

Bacterial topoisomerases, DNA gyrase and topoisomerase IV, are targets that fulfill these requirements. While both enzymes are essential for the regulation of topology of DNA, they differ in function. Gyrase introduces supercoils and topoisomerase IV relaxes DNA and is necessary for decatenation of the chromosomes after replication. Thanks to the high homology between the two enzymes it should be possible to find dual inhibitors. Inhibiting both targets simultaneously and to a similar extent was shown to slow down the emergence of high-level resistance. The reason being that the probability of mutating both targets at the same time is much smaller than for a single target. Several classes of topoisomerase inhibitors have been described over the last 50 years:[5] fluoroquinolones (FQ) such as ciprofloxacin, the only class with several representatives in clinical use; novobiocin and other ATP binding compounds,[6] and finally the Novel Bacterial Topoisomerase Inhibitors (NBTIs)[7] whose first representative, gepotidacin (GSK2140944),[8] is currently in Phase 3 clinical trials.

2. Optimizing Antibacterial Activity

Initially, work from GSK served as inspiration for our own endeavor in this field. Publication of several patents,[9,10] reporting molecules such as GSK299423, led to the discovery of our first tetrahydropyran-based (THP-based) NBTIs such as **1**, **2** and **3** (Fig. 1) with suitable antibacterial spectrum (Table 1) and good properties. In the course of the optimization we realized early on that the major hurdles to be overcome were i) potent hERG blockage leading to QT_c prolongation *in vivo*, ii) duality (activity on both targets DNA gyrase and topoisomerase IV) to limit resistance development as well as iii) penetration and efflux in Gram-negative bacteria leading to reduced potency on these pathogens. Our learnings in these early classes of compounds have been published previously.[11–15]

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Table 1. Early Actelion NBTIs

Compound	Minimal inhibitory concentration [μg/ml][a]								hERG
	S.aureus wt[b]	*S.aureus* QR[c]	*S.aureus* NBTI-R[d]	*E.faecium* QR[e]	*E.coli* wt[f]	*A.baum.* QR[g]	*K.pneum* QR[h]	*P.aerug.* wt[i]	IC_{50} [μM]
cipro	0.25	32	0.25	>8	0.016	>8	>8	0.25	
1	0.03	0.03	1	0.125	8	0.5	>8	>16	19
2	0.03	0.016	1	0.5	16>8	2	>8	32	>60
3	0.25	0.25	8	16	0.25	2	2	1	52

[a]values are means of several independent measurements, [b]ATCC 29213, [c]A-798 (GyrA S84L, GrlA S80F, E84V), [d]laboratory mutant (GyrA: D83N), [e]A-949 (ParC S80R), [f]ATCC25922, [g]T6474 (GyrA S83L, ParC S80L (*e.coli* numbering), [h]T6474 (GyrA S83I, ParC S80I), [i]ATCC 27853; cipro=ciprofloxacin

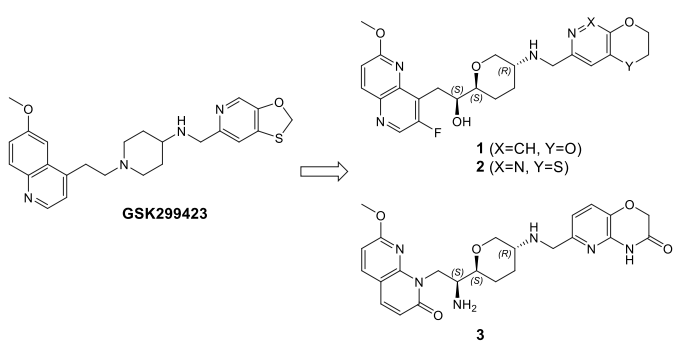

Fig. 1. Early NBTIs.

In 2010, the groundbreaking publication of an X-ray structure of GSK299423 in complex with gyrase and DNA[16] finally showed the binding mode of NBTIs and explained the observed lack of cross resistance with the class of FQ antibiotics. In contrast to FQ which bind in a 2:1 stoichiometry to the DNA/enzyme complex in which DNA is covalently linked to the enzyme (cleaved complex), NBTIs bind in a 1:1 ratio and stabilize the DNA/enzyme complex prior to the cleavage process as shown in Fig. 2.

Despite the proximity of resistance conferring mutation sites (Ser84 for FQ and Asp83 or Met121 for NBTI (*S. aureus* numbering) respectively) no cross resistance is observed for these two classes of antibiotics. This is illustrated in Table 1 for the early Actelion compounds and comparator ciprofloxacin using three strains of *S. aureus* with differing resistance phenotype.

All three NBTIs inhibit the growth of wild-type (wt) *S. aureus* and a FQ-resistant isolate to the same extent, while they are less potent on the NBTI-resistant (NBTI-R) strain. Ciprofloxacin shows the expected profile, active on wt and NBTI-R strains. The reduced or absent activity of NBTIs on the Gram-negative strains *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* has been shown to be linked to penetration and efflux issues rather than lack of inhibitory activity on the enzyme level (see Section 3). Ciprofloxacin in turn is active on wt strains of these species.

The spectrum of **1** and **2** was considered suitable for the treatment of acute bacterial skin and skin structure infections (ABSSSI). Unfortunately, toxicity issues were found (myelosuppression and liver toxicity, as well as CNS side effects) during preclinical development which prevented further development.

The issue we encountered with di-basic compounds such as **3** was high clearance *in vivo* along with low tolerability in rodents and ECG abnormalities in animal models.

The X-ray structure of GSK299423 clearly explains the reduced activity on a GyrA Asp83Asn mutation (Fig. 3). The inhibitors are stabilizing the ternary complex by intercalating with the bicyclic aromatic quinoline moiety (left hand side, LHS) in

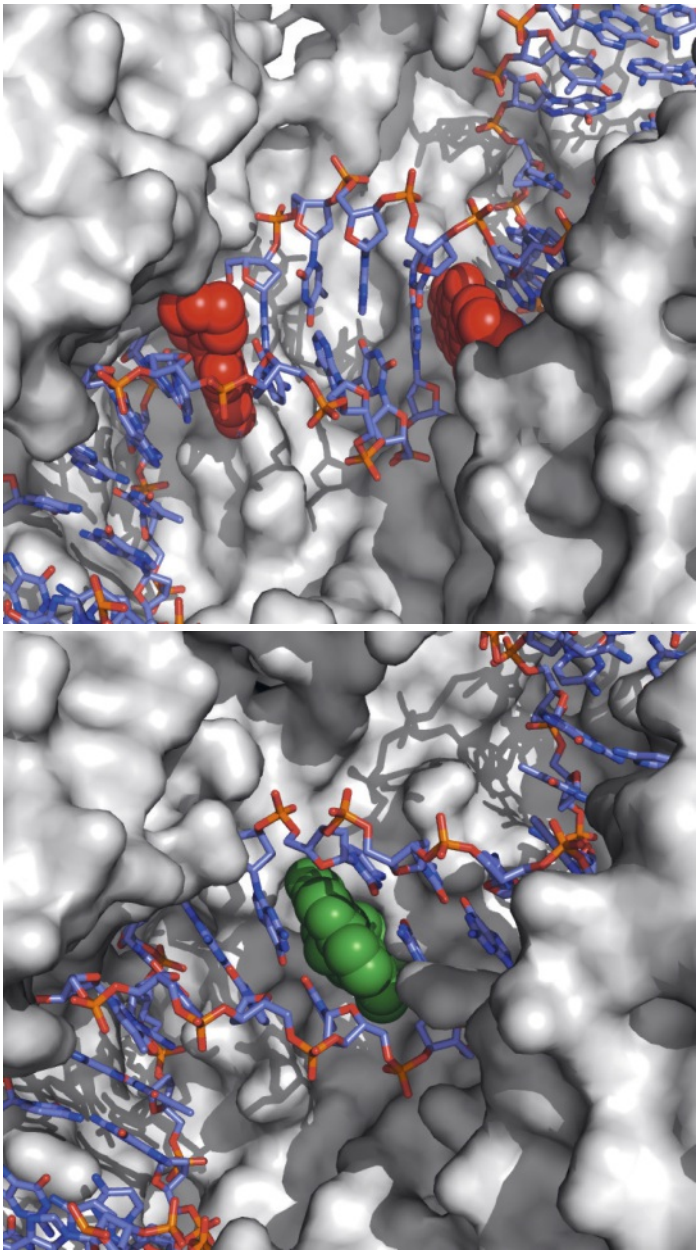

Fig. 2. Comparison of binding modes of FQ (top, 2XCT) and NBTIs (bottom, 2XCS). Enzyme surface shown in grey, DNA in purple, ciprofloxacin in red and NBTI in green.

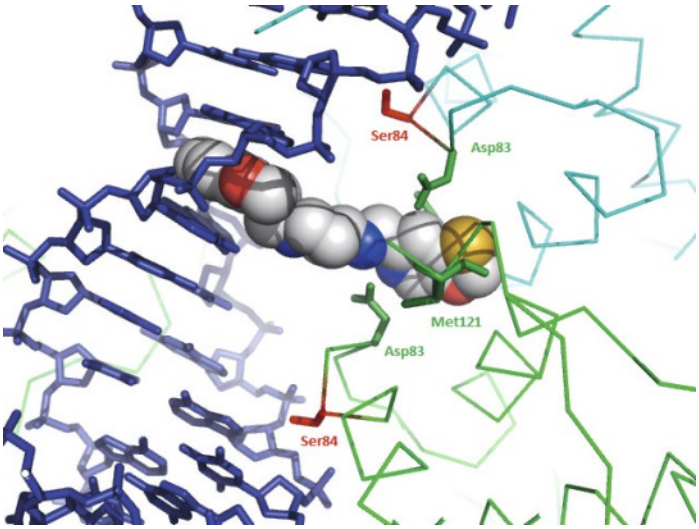

Fig. 3. Side view of GSK299423 (grey) in complex with gyrase (backbone of GyrA subunits in light green and cyan) and DNA (purple) (2XCS). Ser84 (mutation conferring resistance to FQ) in red; Asp83 and Met121 (mutations conferring resistance to NBTIs) in green.

DNA and binding with the right hand side (RHS) in a lipophilic pocket formed by the two GyrA subunits at their center of symmetry, where Asp83 makes a hydrogen bond to the basic amine of the NBTIs.

2.1 *Oxazolidinone Containing NBTIs*[17–31]

Well before any information on the mode of action of NBTIs was published, we decided to modify the linker between cyclic core and RHS of the molecules with the aim to modulate the basicity of our molecules and herewith hERG blockage.

As depicted in Fig. 4 and Table 2, replacement of the benzylic amine in **4** by an amide (**5**) was possible without loss of activity, but replacement with a spiro oxazolidinone led to more potent compounds. While compound **6** with a dioxolane RHS showed only Gram-positive activity, the substitution with a thiazinone (**7**) led to the first compound in this series with activity on *Pseudomonas*. Such rigid molecules however proved very insoluble. To our surprise it turned out that the region of the linker was very amenable to modifications.

Linear as well as cyclic linkers were tolerated and with **8** and **9** we had discovered highly potent and broad spectrum NBTIs with MICs below 1 μg/ml for all species tested in the panel. Unfortunately, also this class of compounds suffered from potent hERG block and other issues such as cytotoxicity and CYP450 inhibition.

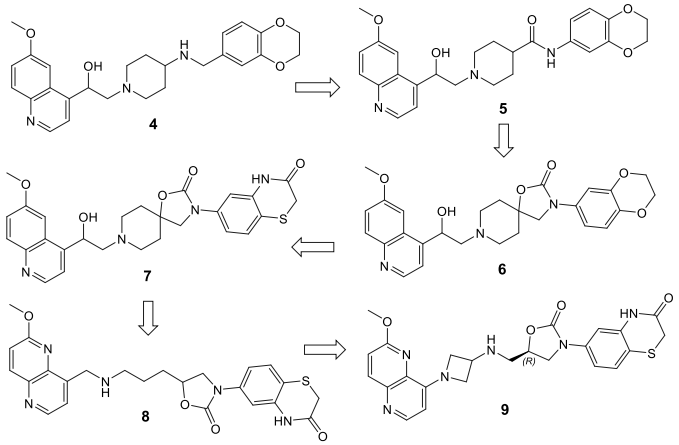

Fig. 4. Discovery of oxazolidinone containing NBTIs.

2.2 *Oxazolidinones with Tricyclic LHS*[22,23]

During the profiling of early oxazolidinones, further issues were identified. Sulfur-containing RHS (benzothiazinones and pyridothiazinones) showed reactive metabolite formation leading to glutathione trapping upon incubation with microsomes. We extensively studied the SAR of the RHS, *i.e.* substituent on the oxazolidinone and realized that many modifications (mono- and bicyclic fused aromatic moieties) led to compounds active in the enzymatic assay and on Gram-positive bacteria. Unfortunately, whole cell activity on Gram-negative pathogens was only achieved with bicyclic moieties like the ones mentioned before as well as with their oxazinone analogs. For this reason we conducted further explorations with the pyridooxazinone-linked oxazolidinone as summarized in Fig. 5 and Table 3 (X = N unless mentioned otherwise).

Replacement of the methoxynaphthyridine (in compound **8**) by N-methylquinolinone (**10**) was tolerated. The subsequent cyclization to a 6,6,5- or 6,6,6-tricyclic systems was also possible (**11**, **15**). The attachment point of the side chain had no big impact on the potency of the compounds (**16** vs. **11**, **17** vs. **15**). The position of the nitrogen in the spacer (**11** vs. **18**, **19**) had no influence on potency but on hERG block. Introduction of an additional nitrogen in the tricyclic system (**20**, **21**) led to a loss in potency. It turned out that the presence of a fluorine in combination with the basic amine in the benzylic position (**11**) was best to reduce hERG block.

The stereochemistry at the two stereogenic centers did not have an impact on the potency on the enzymes (*cf.* **11**–**14**) nor in whole-cell assays. This can be explained by the particular binding mode of BTIs that intercalate with the LHS into DNA and with the RHS

Table 2. Oxazolidinones

Compound	Minimal inhibitory concentration [μg/ml][a]								hERG
	S.aureus wt[b]	*S.aureus* QR[c]	*S.aureus* NBTI-R[d]	*E.faecium* QR[e]	*E.coli* wt[f]	*A.baum.* QR[g]	*K.pneum* QR[h]	*P.aerug.* wt[i]	IC_{50} [μM]
4	<0.03	<0.03	16	1	1	nt	nt	16	nt
5	<0.03	0.06	2	2	>16	nt	nt	>16	6.1
6	<0.03	<0.03	1	0.5	>16	nt	nt	>16	3.7
7	<0.03	<0.03	0.125	0.25	1	nt	nt	4	nt
8	0.008	0.008	0.5	<0.06	0.125	<0.03	0.25	1	1.5
9	<0.003	<0.03	<0.03	<0.03	0.06	<0.03	0.25	0.125	1.5

[a]values are means of several independent measurements, [b]ATCC 29213, [c]A-798 (GyrA S84L, GrlA S80F, E84V), [d]laboratory mutant (GyrA D83N), [e]A-949 (ParC S80R), [f]ATCC25922, [g]T6474 (GyrA S83L, ParC S80L (*e.coli* numbering), [h]T6474 (GyrA S83I, ParC S80I), [i]ATCC 27853;

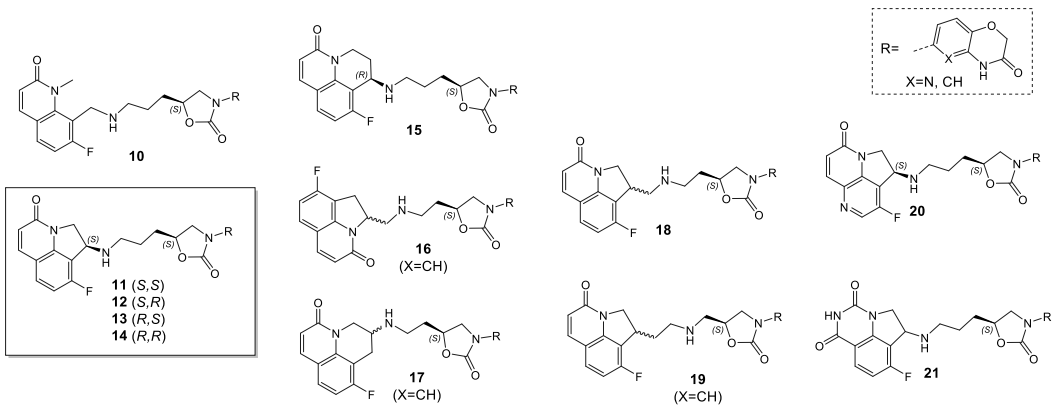

Fig. 5. Oxazolidinones with tricyclic LHS.

Table 3. Tricyclic LHS

Compound	Minimal inhibitory concentration [µg/ml] [a]								hERG IC_{50} [µM] (%inh@10µM)
	S.aureus wt[b]	*S.aureus* QR [c]	*S.aureus* NBTI-R[d]	*E.faecium* QR[e]	*E.coli* wt[f]	*A.baum.* QR[g]	*K.pneum* QR[h]	*P.aerug.* wt[i]	
10	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	0.25	0.25	
11 (*S,S*)	0.004	<0.008	0.125	<0.125	0.25	0.06	0.5	1	37
12 (*S,R*)	<0.016	<0.016	0.125	<0.125	0.25	<0.06	0.5	1	24
13 (*R,S*)	<0.016	<0.016	0.25	<0.125	0.25	<0.06	1	1	26
14 (*R,R*)	<0.016	<0.016	0.125	<0.06	0.125	<0.03	0.25	0.5	30
15	<0.03	<0.03	0.5	0.06	0.25	0.06	1	2	11
16	<0.03	<0.03	0.5	0.25	<0.03	<0.03	0.125	0.25	5.8
17	<0.03	<0.03	0.125	0.06	0.125	<0.03	nt	1	(98%)
18	<0.016	<0.016	0.125	0.125	0.125	0.03	0.25	0.25	(88%)
19	<0.03	<0.03	0.25	0.125	0.06	<0.03	nt	0.5	2.5
20	<0.016	<0.016	0.5	0.125	0.5	0.125	0.5	0.5	6.3
21	0.125	0.125	4	1	2	0.25	2	2	>60

[a]values are means of several independent measurements, [b]ATCC 29213, [c]A-798 (GyrA S84L, GrlA S80F, E84V), [d]laboratory mutant (GyrA D83N), [e]A-949 (ParC S80R), [f]ATCC25922, [g]T6474 (GyrA S83L, ParC S80L (*E. coli* numbering), [h]T6474 (GyrA S83I, ParC S80I), [i]ATCC 27853;

stick in the lipophilic pocket formed by the two GyrA subunits (Fig. 3). The linker itself seems to make no direct contacts. Rather it is responsible for the correct positioning of the aromatic moieties. The linker length could be reduced by one atom at the expense of potency (data not shown).

Compound **11** was chosen as preclinical candidate because of its favorable PK in rodents, good aqueous solubility as a mesylate salt (12 mg/ml) and excellent *in vivo* efficacy. Unfortunately, genotoxicity *in vivo* and *in vitro* (micronucleus formation assay) prevented its development in the indications of complicated urinary infection (cUTI) and complicated intra-abdominal infections (cIAI).

2.3 *Biaryl LHS*[24,25]

As mentioned previously, the pyrido-oxazinone oxazolidinone RHS was crucial for Gram-negative activity and therefore to be kept constant. In addition, we hypothesized that the culprit for genotoxicity was the tricyclic moiety of **11**, as we had not encountered this issue with earlier compounds. We therefore turned our attention to this region to further modulate the properties of our inhibitors. We found that simple biphenyl moieties retained good *in vitro* potency as long as a planar arrangement was possible for intercalation.

Introduction of heteroatoms was tolerated as well as polar and basic sidechains as depicted in Fig. 6. The optimal linker length was four atoms, the basic amine in benzylic position. Any substitution on the linker led to reduced activity. As observed previously, the stereochemistry on the oxazolidinone had no impact on activity.

Compounds with potent and broad-spectrum activity (**22**, **23**) were discovered. A basic side chain on the LHS was detrimental for activity on *Acinetobacter baumannii* (**24**) and despite high IC_{50}

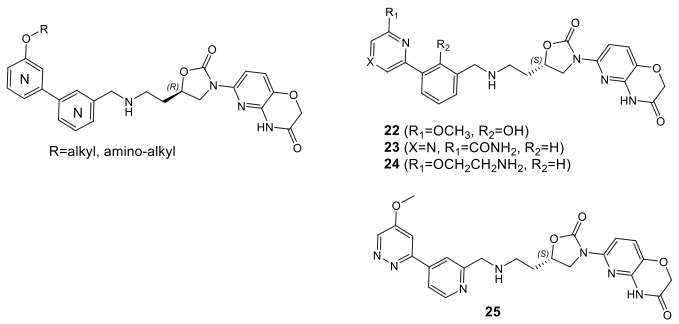

Fig. 6. Examples of biaryl LHS containing NBTIs.

Table 4. Biaryl NBTIs

Compound	Minimal inhibitory concentration [μg/ml] [a]								hERG IC$_{50}$ [μM] (%inh@10μM)
	S.aureus wt[b]	*S.aureus* QR [c]	*S.aureus* NBTI-R[d]	*E.faecium* QR[e]	*E.coli* wt[f]	*A.baum.* QR[g]	*K.pneum* QR[h]	*P.aerug.* wt[i]	
22	<0.016	<0.016	0.5	0.25	0.25	0.06	1	1	(99%)
23	0.5	0.25	>32	8	0.5	1	1	1	40
24	1	0.25	>8	>8	0.5	6	1	0.25	>60
25	0.25	0.5	>8	>8	0.25	0.5	0.5	2	46

[a]values are means of several independent measurements, [b]ATCC 29213, [c]A-798 (GyrA S84L, GrlA S80F, E84V), [d]laboratory mutant (GyrA: D83N), [e]A-949 (ParC S80R), [f]ATCC25922, [g]T6474 (GyrA S83L, ParC S80L (*e.coli* numbering), [h]T6474 (GyrA S83I, ParC S80I), [i]ATCC 27853;

on hERG, the compounds prolonged QT$_c$ interval *in vivo* and were not tolerated (Table 4). For unknown reasons, many compounds were not able to control the growth of *Enterococcus faecium*.

Antibacterial spectrum, hERG block, cytotoxicity and solubility greatly differed between derivatives and a compromise on antibacterial activity had to be accepted. **25** is a good compound in this respect, lacking in addition the genotoxicity liability and showing good *in vivo* efficacy in murine animal models.

Unfortunately, the pyridazine moiety which was essential for good solubility is a substrate for metabolism by aldehyde oxidase in humans and the compound therefore did not qualify for development. The risk for unfavorable and variable PK in patients was considered too high.

2.4 *Fluoroquinolone Hybrids*[26–28]

Over the course of the project it became clearer that the class of oxazolidinone NBTIs suffered from penetration and efflux issues in Gram-negative bacteria. This is in contrast to the clinically used FQ antibiotics which penetrate well and even accumulate in the cytoplasm thanks to their zwitterionic character, the weakly acidic nature of the carboxylic acid (pKa ~6.3) and the basic amine (pKa ~8.5). The pH gradient between periplasm (pH 6.1) and cytoplasm (pH 7.8) in Gram-negatives such as *E. coli* favor entry (as 'overall neutral' zwitterion) and hinder exit of the charged species.[29] Fluoroquinolones also intercalate into DNA as mentioned in the introduction.

We therefore explored the idea, shown in Fig. 7, to use the quinolone (*e.g.* ciprofloxacin) as intercalating moiety in NBTIs, thereby creating hybrid molecules (*e.g.* with **9**) such as **26**. It is known that the piperazine region in fluoroquinolones tolerates many variations and we therefore wondered if compounds with a double mode of action (*i.e.* acting at the same time as FQ and as NBTI) were feasible.

To our surprise, **26** and all subsequently produced FQ-oxazolidinone hybrids with various spacers act exclusively *via* the NBTI mechanism. We showed this by extensive analysis of MICs on clinical isolates with known mutations as well as on the enzyme level with gyrase and topoisomerases containing the Ser84 and Asp83 mutations (data not shown). Indeed, such FQ-oxazolidinone hybrids show exquisite antibacterial spectrum and potency and thanks to the carboxylic acid also reduced potential to block the hERG channel (Table 5). Linked to the flat and rigid nature of the molecules we were faced with very low solubilities and lack of oral bioavailability. Unfortunately, despite big synthetic efforts we were unable to fix this issue.

Solubility increased when we switched back to the 'traditional' RHS having a benzylic amine instead of the oxazolidinone. Compound **27** (Fig. 8) only showed weak but balanced antibacterial

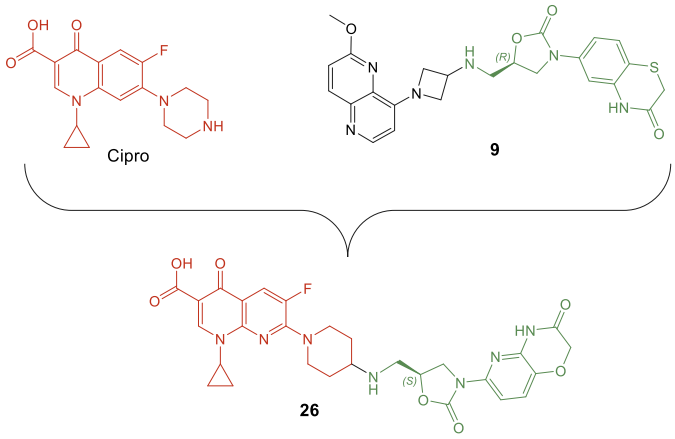

Fig. 7. FQ-NBTI hybrids: the concept.

activity across the panel with the exception of *Enterococcus faecium*. Replacement of the cyclopropyl moiety by a methyl group (**28**) increased the potency by a factor 2–8. Changing from a piperidine spacer to a *trans*-configured cyclohexyl group in analogy to the 'traditional' NBTIs, completely abolished antibacterial activity (**29**).

Surprisingly however, shifting the cyclic spacer to the left, ending up with **30** and its analog **31**, we were able to regain potency, especially against the difficult to treat Gram-negative pathogens with MICs between 0.25 and 4. In addition, **31** exhibited good efficacy in murine animal models which correlated with the large free exposure *in vivo*. Unfortunately, it was observed that **31** precipitated *in vivo* in the kidneys and liver of the animals at exposures only slightly above the one needed for efficacy against *K. pneumoniae* and the compound could not be selected for development.

3. Characterization of Actelion's NBTIs

3.1 *Enzyme Inhibition and Selectivity*

Gel-based assays were used to characterize compounds for their ability to inhibit supercoiling activity of gyrase and relaxation activity of topoisomerase IV.[13] Wild-type enzymes as well as mutant enzymes from clinical isolates with resistance phenotypes of several bacterial species were studied. In Table 6 the results for *S. aureus* and *E. coli* are listed to illustrate the general findings. NBTIs are more active on gyrase than on topo IV in Gram-positive isolates, especially *S. aureus*, while the opposite is true for Gram-negatives. The mutation typically conferring resistance to FQ (Ser84) does not affect the potency of our

Table 5. FQ hybrids

Compound	Minimal inhibitory concentration [μg/ml] [a]								hERG
	S.aureus wt[b]	*S.aureus* QR [c]	*S.aureus* NBTI-R[d]	*E.faecium* QR[e]	*E.coli* wt[f]	*A.baum.* QR[g]	*K.pneum* QR[h]	*P.aerug.* wt[i]	IC_{50} [μM]
26	<0.016	<0.016	0.5	0.25	0.125	<0.016	0.25	0.5	>60
27	4	16	4	>16	1	4	8	8	nt
28	2	2	8	>8	0.25	0.5	1	4	380
29	>8	>8	>8	>8	>8	>8	>8	>8	nt
30	8	4	>16	>16	0.25	0.25	2	4	>60
31	4	2	32	>32	0.25	1	2	1	>60

[a]values are means of several independent measurements, [b]ATCC 29213, [c]A-798 (GyrA S84L, GrlA S80F, E84V), [d]laboratory mutant (GyrA: D83N), [e]A-949 (ParC S80R), [f]ATCC25922, [g]T6474 (GyrA S83L, ParC S80L (*e.coli* numbering), [h]T6474 (GyrA S83I, ParC S80I), [i]ATCC 27853;

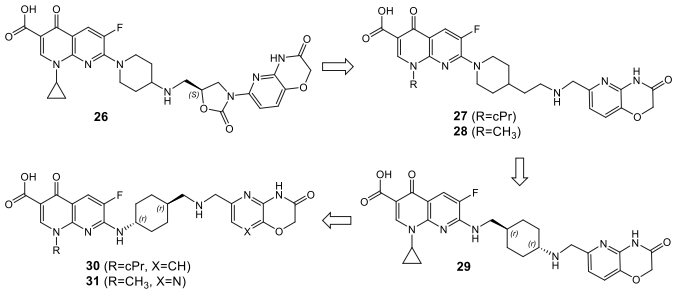

Fig. 8. FQ hybrids.

compounds. The mutation Asp83Asn reduces potency by several orders of magnitude as illustrated for *S. aureus* in Table 6. MIC measurement on resistant isolates of *S. aureus* therefore is a rapid assessment of on-target activity and confirmation of the desired mode of action. With the exception of **31**, which is significantly weaker on *S. aureus* gyrase and topo IV, the compounds are equipotent on enzymes from Gram-negative and Gram-positive pathogens.

One clear differentiation between the modes of action of FQ and NBTI is the formation of the cleaved complex with quinolones. The fact that FQ trap the complex at the stage where DNA is covalently linked to the enzyme leads to accumulation of double strand breaks which is highly toxic to the cells and can be easily detected in gel-based assays, usually already at sub-MIC concentrations. With our NBTIs, the cleaved complex was not observed, again confirming 'on-target' activity.

Selectivity vs human topoisomerase II was also assessed. With the exception of **9** (IC_{50} = 16 μM), IC_{50} were above 50 μM, if measurable at all.

3.2 *Permeability and Efflux in E. coli*[30–32]

To study the discrepancy of *in vitro* potency (IC_{50}) vs. Gram-negative whole cell activity (MIC), a panel of isogenic *E. coli* strains containing either a *rfaC* mutation[33] rendering the LPS layer more penetrable or a *TolC* knock-out[34] eliminating the most important efflux pump were used. As summarized in Table 6, while the compounds are more active on both mutants, the *TolC* deletion had the greater impact. The whole-cell activity in *E. coli* is therefore more affected by efflux than by penetration. As can be estimated from the calculated efflux ratios (ratio between wt and *TolC* mutant), especially the early compounds (**1** and **2**) are efficiently pumped out of the cells. The FQ hybrids (**26** and **31**) as well as very polar compounds (**3** and **25**) are less good substrates for *TolC*. Similar conclusions can be drawn for *P. aeruginosa* (data not shown).

3.3 *Resistance Development*

Resistance development is a major problem for antibacterials and therefore assessed early on. Antibiotics with a single target have a high risk that a single mutation (first step mutants) can lead to highly resistant clones. By inhibiting two targets, as is the case for our dual inhibitors of gyrase and topoIV we anticipated that a single mutation would only lead to a small increase in MIC and that mutations in both targets would be needed for high level resistance.

Indeed, the frequency of resistance measured at a concentration of 4*MIC was comparable to the one of FQ and the increase of MIC was moderate as shown for the example of *S. aureus* in Table 6. Several clones were sequenced and mutations in GyrA (mostly Asp83 and Met121) were detected. To obtain highly resistant clones, several passages were needed. This parameter also turned out to be in the range of FQ antibiotics.

In Gram-negatives the situation looked differently. While resistance frequencies as well as MICs of first step mutants were in a similar range than for *S. aureus*, no mutations were found in the topoisomerase enzymes. We suspected upregulation of efflux or other metabolic changes to be the reason for reduced susceptibility. Whole genome sequencing confirmed this for **11** and **31** in *Pseudomonas*, *Klebsiella* and *Acinetobacter* (unpublished data).

3.4 *Efficacy in Murine Models of Infection*

Thanks to the reliability and predictivity of animal models of infection, doses and dose regimens for human use can be estimated with high confidence.[35,36] The compounds in Table 6 were assessed in animal models of infection. Compounds proved efficacious in models of ABSSSI (acute bacterial skin and skin structure infections), RTI (respiratory tract infections), cUTI (complicated urinary tract infections) and cIAI (complicated intra-abdominal infections) in infections caused by susceptible organisms. The frontrunner compounds are metabolically stable in microsomes and hepatocytes of rodents and human and exhibit low to medium clearance *in vivo* (Table 6).

3.5 *Toxicity (hERG/QTc, genotoxicity)*

Inhibition of the hERG K-channel is thought to be linked with QTc prolongation *in vivo*. The potential of our compounds to block hERG was routinely assessed as described previously.[13] As a trend, the more polar the compounds were, the higher the IC_{50} on hERG. Reducing the pKa of the basic amines below 7,

Table 6. Characterization of selected compounds

	1	2	3	8	9	11	25	26	31
Enzyme inhibition									
S.aureus gyr wt IC_{50}[µM]	0.07	<0.08	0.031	0.02	<0.007	0.031	0.125	0.031	8
S.aureus gyr QR IC_{50}[µM][a]	0.05	<0.07	nt	0.016	<0.006	0.031	0.125	0.031	8
S.aureus gyr NBTI-R[b] IC_{50}[µM]	34	5	nt	4	>65	22	128	32	>256
S.aureus topoIV wt IC_{50}[µM]	4.75	5	0.5	0.5	25	2	20	2	16
E.coli gyr wt IC_{50}[µM]	9	15	0.125	0.063	0.02	0.031	1.25	0.5	0.18
E.coli topoIV wt IC_{50}[µM]	0.1	0.125	0.03	0.03	0.048	<0.015	0.03	0.125	0.03
Human topoII IC_{50}[µM]	>180	>320	384	178	15	96-256	>340	>50	>50
***E. coli* permeability panel :**									
E.c. wt MIC [µg/mL]	4	12	0.25	0.125	0.25	0.18	0.25	0.031	0.25
E.c. ΔrfaC MIC [µg/mL]	0.06	0.5	0.25	0.004	0.016	0.016	0.125	0.004	0.125
E.c. ΔTolC MIC [µg/mL]	0.016	<0.031	0.016	<0.002	0.004	0.001	0.008	0.002	<0.003
Efflux ratio (wt/ΔTolC)	250	>380	16	>64	64	180	32	16	>8
Resistance frequencies									
S.aureus ATCC 29213	$1*10^{-7}$	$1*10^{-7}$	nt	$2*10^{-9}$	$2*10^{-8}$	$2*10^{-8}$	$1*10^{-7}$	$2*10^{-8}$	$3*10^{-8}$
(MIC of mutant, [µg/mL])	(0.25-0.5)	(0.25-2)	nt	(0.25-0.5)	(0.5)	(0.25)	(16)	(4-8)	(8)
E.coli ATCC 25922	nt	nt	nt	$<8*10^{-10}$	nt	$<2*10^{-9}$	$<2*10^{-10}$	$<2*10^{-9}$	$5*10^{-8}$
P.aeruginosa ATCC 27853	nt	nt	nt	$<3*10^{-10}$	$3*10^{-6}$	$2*10^{-9}$	$<2*10^{-10}$	$1*10^{-9}$	$4*10^{-7}$
ADME/DMPK									
Solubility pH 7 [µg/mL]	789	679	>810	15	167	50	312	2	34
pKa	6.8	6.4	7, 8.7	8	8.4	6.2	7.4	nt	8.2 (basic) 6.7 (acidic)
Plasma protein binding (m/r/h/gp)[c] [%]	94/ 97/ 94/ 89	90/ 96/ 93/ 80	63/ 76/ 49/ na	99/ >99/ 99/ 99	>99/ 99/ 99/ 99	94/ 98/ 92/ 84	88/ 98/ 90/ 82	99/ 99.5/ 99.6/ na	61/ 68/ 61/ 55
Microsomal stability (r/h) [µL/(min*mg)]	220/36	146/20	106/6	>1250/387	62/<1	351/37	30/6	28/13	13/<10
Hepatocyte stability (r/h) [µL/min*1E6 cells)]	18.8/nt	13/nt	9.5/<2	21/nt	nt	30/2	20/46	6.3/nt	<2/<2
PK rat									
CL [mL/min/kg]	23	13	110	23	50	4	12	20	38
Vss [L/kg]	1.1	0.75	21	2.3	7.1	0.4	0.36	0.8	2.7
% bioavailability (dose)	33 % (5mg/kg)	47% (1mg/kg)	0 (10mg/kg)	25% (10mg/kg)	0 (10mg/kg)	34% (25mg/kg)	5% (10mg/kg)	1% (2mg/kg)	0.2% (10mg/kg)
QT_c prolongation in GP									
Dose (iv infusion/20min)	30mg/kg	30mg/kg	30mg/kg	15mg/kg	15mg/kg	75mg/kg	30mg/kg		60mg/kg
QT_c [%]	2	–2	20	8	0	5	3	nt	5
tC_{max} (fC_{max}) [µM]	36 (4)	39 (9)	53 (28)	16	6.7 (0.07)	172 (28)	145 (22)		395 (179)

[a]GrlA S84 L; [b]GyrA D83N; [c]m: mouse, r: rat, h: human, gp: guinea pig

or the introduction of an acidic group reduced the liability of our compounds to interact with hERG. Selected compounds were further assessed for QTc prolongation in anesthetized guinea pigs[13] at increasing doses infused over a 20 min period. The observed effect on the QTc interval and the corresponding total and free concentrations observed at the top dose are listed in Table 6.

Genotoxicity was assessed using a microscope based assay[37,38] determining the number of micronuclei in the cells at concentrations close to toxicity (*ca.* 50% remaining viable cells). Micronucleus formation did not correlate with inhibition of eukaryotic topoisomerase and did not seem to be target related. The cause of the genotoxicity of certain NBTIs is still unclear. Micronucleus formation was observed for compounds **8**, **9**, **11**, but not for **1**, **2**, **25** and **31**.

3.6 *Solubility*

The need of a formulation for intravenous application requires very high solubility of the drug. This aspect of drug development was initially underestimated. Antibiotics are given in doses up to several grams per day. Dissolving this dose in a reasonable amount of fluid (*e.g.* not more than *ca*. 500ml/day) requires solubilities of the drugs around 10mg/ml in an aqueous formulation. This is much higher than what is usually required for oral drugs.

We early on included solubility measurements and salt screening efforts in our optimization strategy. Still, many compounds had to be dropped despite potent and broad antibacterial spectrum because it was not possible to find a suitable formulation.

4. Conclusions

Over the 13 years of the program, several compounds mentioned in this article were selected for preclinical development. All of them had to be stopped, unfortunately, at different stages of the process for tolerability reasons. The observed toxicity was different for each compound (not target related) and ranged from myelosuppression (**1**), liver toxicity due to reactive metabolite formation (**2**), genotoxicity (**11**) to precipitation *in vivo* (**31**). Sadly, we had to accept that the ambitious objective we had set ourselves, namely to cover all of the difficult-to-treat Gram-negative pathogens, required very high doses that left no safety margin between exposure needed for efficacy and occurrence of toxicity.

The innovation void in the development of antibiotics has several reasons, the economic situation being just one of them. New drugs are developed in the same way than 50 years ago, *i.e.* for a certain clinical indication requiring a broad antibacterial spectrum rather than against a specific pathogen.

Today's availability of novel diagnostic tools should allow the development of narrower spectrum compounds suitable to treat infections caused by pathogens including recent resistant isolates.

The problem of AMR needs to be tackled globally and with urgency. Indeed, global initiatives exist, be it in the form of antibiotic stewardship or private/public partnerships to foster innovation (Carb-X,[39] GARDP,[40] BARDA,[41] and others) or by initiatives from industry (AMR action fund[42]) to find sustainable ways to develop novel antibiotics.[43] Thanks to these initiatives we will hopefully witness the turnaround in this crisis, the availability of effective antibiotics being the basis of our health care system and modern society.

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