Development of Linezolid

Oxazolidinone Structure–Activity Relationships Leading to Linezolid

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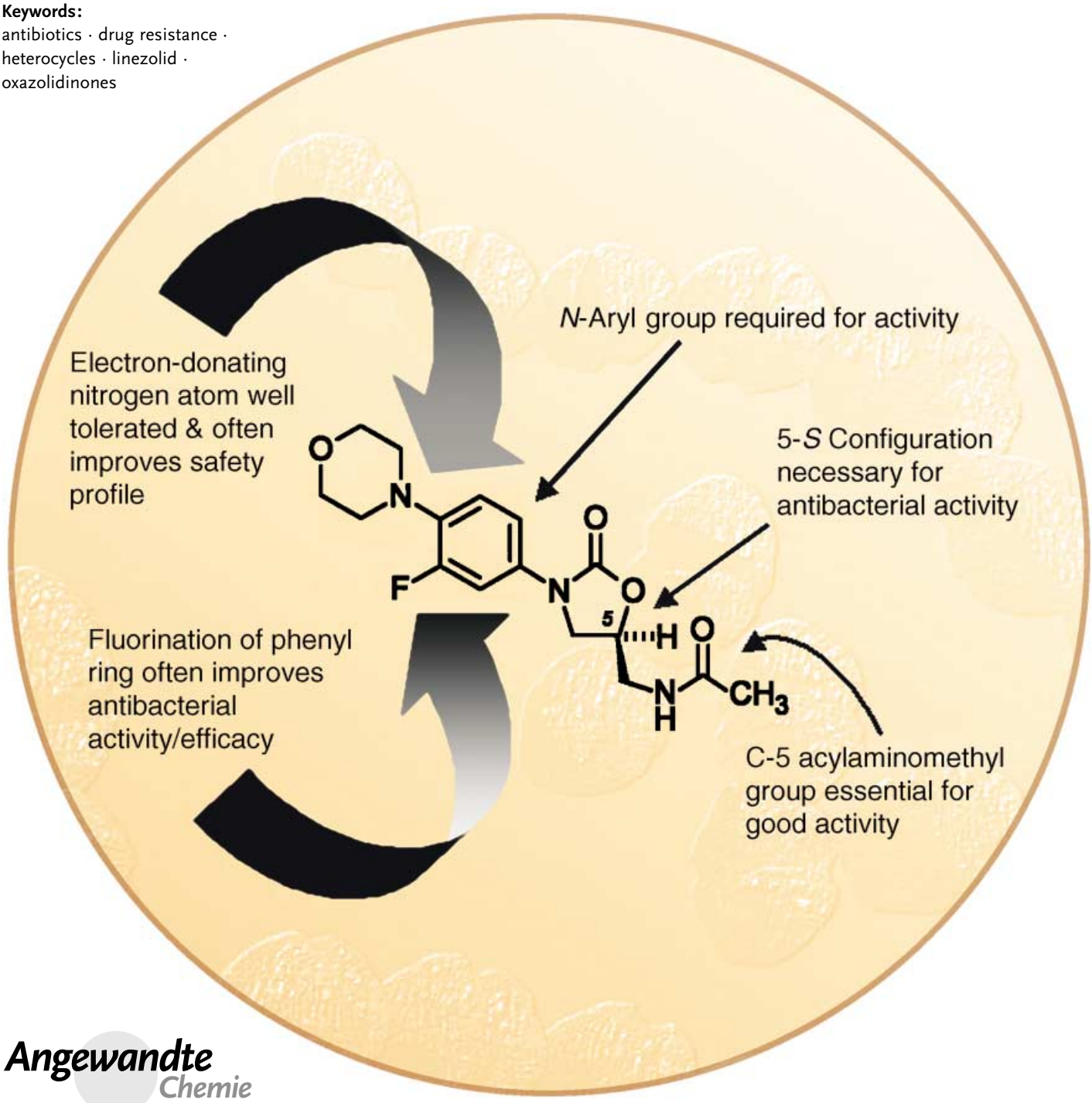

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The development of bacterial resistance to currently available antibacterial agents is a growing global health problem. Of particular concern are infections caused by multidrug-resistant Gram-positive pathogens which are responsible for significant morbidity and mortality in both the hospital and community settings. A number of solutions to the problem of bacterial resistance are possible. The most common approach is to continue modifying existing classes of antibacterial agents to provide new analogues with improved attributes. Other successful strategies are to combine existing antibacterial agents with other drugs as well as the development of improved diagnostic procedures that may lead to rapid identification of the causative pathogen and permit the use of antibacterial agents with a narrow spectrum of activity. Finally, and most importantly, the discovery of novel classes of antibacterial agents employing new mechanisms of action has considerable promise. Such agents would exhibit a lack of cross-resistance with existing antimicrobial drugs. This review describes the work leading to the discovery of linezolid, the first clinically useful oxazolidinone antibacterial agent.

From the Contents

1. Introduction

In the 1980s the antibiotic market and the antibiotic business were often referred to as "mature", in this sense that there were many antibiotics available and few if any bacterial diseases in humans for which antibiotic therapy was not curative. The success of antibiotic research and development by the pharmaceutical companies had become an accepted and expected condition in our society. The antibiotic classes on the market had been primarily discovered through biological screening of fermentation broths, natural product extracts, and collections of research compounds. The large number of antibiotics available on the market occurred through an iterative process wherein structurally related compounds or analogues of any given class of antibiotics were synthesized and introduced to the market when incremental improvements in intrinsic activity, spectrum of antibacterial activity, or safety were realized relative to pre-existing member(s) of the class. These extensive efforts in structure–activity relationships (SARs) led to a very large array of antibiotics within existing classes. By the mid-1980s, the market had not seen a new class of antibiotics introduced in over twenty years and many customers were telling the pharmaceutical companies that there were more than sufficient numbers of available antibiotics for human use.

At the same time in which it was becoming clear that the discovery of new antibiotic classes was going to require major investments in new technologies and the antibiotic market seemed saturated with drugs, the Gram-positive bacteria, such as staphylococci, streptococci, and enterococci, were well on the way to raising havoc with existing drug classes through the development of resistance. The enterococci, particularly *Enterococci faecalis* and *Enterococcus faecium* changed their niche; instead of being found principally in intra-abdominal abscesses and urinary tract infections they began to cause bacteremia particularly in hospitalized and elderly patients. The two enterococci were undesirable pathogens in the sense that they caused virulent disease but they were multidrug-resistant and many strains were vancomycin-resistant,[1] which made them extraordinarily difficult to treat. Until the 1990s *Streptococcus pneumoniae*, which is the most common cause of human bacterial disease, was readily treatable with a variety of older and inexpensive antibiotics. Following at least two individual outbreaks of penicillin-resistant *S. pneumoniae*, the resistant forms of the pathogen have persisted world-wide and have acquired resistance to other antibiotic classes. Today multidrug-resistant *S. pneumoniae* is a major problem in the Pacific Rim countries and is becoming a significant problem for physicians in the United States and parts of Europe.[2–6] Of the Gram-positive pathogens, *Staphylococcus aureus* and *Staphylococcus epidermidis* have caused treatment problems in hospitals because of the development of resistance. Treatment failures with either of these pathogens can easily result in patient mortality, and in recent years the resistance characteristic most frequently associated with multidrug resistance in staphylococci is methicillin resistance. Methicillin resistance does not mean that no drugs will work. It does mean that vancomycin is the only drug which will always work against multidrug-resistant staphylococci, and the determination of whether other antibiotics might be efficacious requires isolation of the infecting organism and identification of drug sensitivity.[7] The very rapid and fatal illnesses caused by staphylococci necessitates that therapy must be initiated before the organism is isolated and sensitivity determination can be conducted. Vancomycin is clearly the empiric choice

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under those circumstances; however, vancomycin resistance in the enterococci realistically threatens transfer of vancomycin resistance to the staphylococci, and a vancomycin- and multidrug-resistant staphylococcus strain threatens to be essentially untreatable.

The development of Gram-positive resistance as a therapeutic problem followed the diminution of interest in antibiotic research and development. During this period we at Pharmacia had conducted a very thorough evaluation of the antibiotic market in the mid-1980s and concluded that there still existed a need for a new class of antibacterial agents which would lend itself to empiric treatment of the staphylococci and other Gram-positive pathogens. In 1987, shortly following this analysis, we attended the presentation of work by scientists of E.I. du Pont de Nemours & Company (DuPont) which covered their cumulative work on a new class of antibacterial agents, the oxazolidinones.[8] The oxazolidinones interested us because they possessed all the characteristics which seemed important in an antibacterial agent at that time. Their spectrum of activity covered the important Gram-positive pathogens, particularly those which had been causing so many problems because of the development of resistance. They also covered *S. pneumoniae* which we believed would be a significant future problem because of the development of resistance. The oxazolidinones appeared to have a unique mechanism of action and, consistent with that observation, they were not cross-resistant with existing resistance mechanisms in bacteria.[8–10] The oxazolidinones were orally active, which we had identified as a key requirement for new antibacterial agents for primary care in the community. It would also be a key cost saving for hospitalized patients in that intravenous therapy could be stopped as soon as possible and oral therapy in hospital or at home could immediately follow. It was extremely difficult to select for resistant mutants in the laboratory, however, studies on the experimental antibacterial activity and pharmacokinetic behavior predicted that the oxazolidinones would work sufficiently well in humans to be able to predict utility in the marketplace.[11–13] We thus committed ourselves to proof-of-concept studies with the oxazolidinones in which we intended to demonstrate that we could make structurally novel oxazolidinones with promising biological activity.

2. The Oxazolidinones

2.1. The Generalized Evaluation Scheme

The generalized scheme of biological evaluation of oxazolidinone analogues we used is shown in Figure 1. Many of us had research experience in the synthesis and evaluation of analogues of existing antibiotic classes, but the oxazolidinones presented a very unusual challenge in that, because they were a new antibiotic class, there was not any pre-existing information about their characteristics and biological behavior. Our testing scheme may seem exceedingly simple, but that is a reflection of our lack of significant

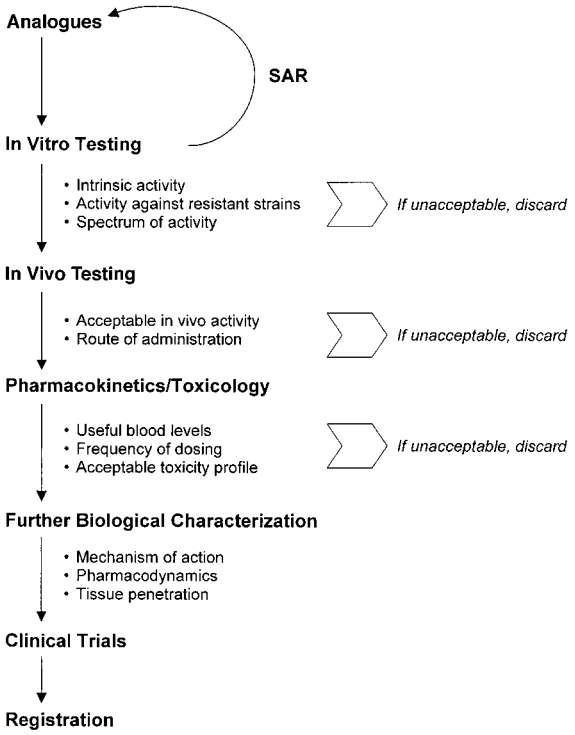


Figure 1. Generalized testing scheme for oxazolidinones showing incorporation of the structure–activity relationship (SAR) component.

Michael R. Barbachyn received his PhD in Organic Chemistry from Wayne State University in 1983, working under the direction of Prof. Carl R. Johnson. After postdoctoral study with Prof. Samuel Danishefsky at Yale University, in 1985 he joined The Upjohn Company (now Pharmacia Corporation). Since late 1990, he has been directly involved with the oxazolidinone discovery effort at Pharmacia and had the good fortune of being involved in the development of linezolid, the first marketed oxazolidinone antibacterial agent. He has 30 US patents and authored numerous publications in the anti-infective field.

Charles W. Ford started his career with a degree in Microbiology from the University of Texas at Austin. After he joined The Upjohn Company (today Pharmacia Corporation) he became the primary microbiologist responsible for the biological component of the oxazolidinone research effort, and functioned as a co-team leader of the oxazolidinone team from the modest inception of the program almost to the introduction of linezolid to the market. He is now a Senior Fellow in research and continues his work on the in vivo evaluation of promising experimental antibiotics.

knowledge of the biological activity of oxazolidinone when we started the program.

Oxazolidinone analogues were submitted for in vitro testing in which the minimum inhibitory concentration values (MIC, the concentration of drug required to kill the bacterial cells or inhibit their growth under standardized conditions) were determined for each drug versus an array of Gram-positive, Gram-negative, and anaerobic bacteria. The panel of bacteria tested included both antibiotic-sensitive and antibiotic-resistant strains. The MIC value was determined as was the activity of the drug against resistant strains and the useful spectrum of antibacterial activity was assessed. The most crucial measure initially used to guide the structure–activity relationship (SAR) was the MIC value, but as we came closer to drugs which might be clinical candidates other characteristics assumed greater importance. We hypothesized, subject to direct testing, that an MIC value of 4 $\mu g\,mL^{-1}$ or less for 90% of the strains would provide us with an oxazolidinone which would compete favorably with vancomycin, which we had designated as our "gold" standard. A compound would pass through the first round of in vitro testing if its MIC_{90} value for the important Gram-positive pathogens was 4 $\mu g\,mL^{-1}$ or less, if it was completely active against antibiotic-resistant bacteria, and if its spectrum of activity covered the important Gram-positive pathogens.

Compounds fulfilling the in vitro criteria would then be submitted for in vivo evaluation, where their oral activity in mouse models of human bacterial diseases would be compared against subcutaneously administered vancomycin. The goal was for our oxazolidinones to have the same in vivo oral activity as subcutaneously administered vancomycin, which is itself not orally active. This was a very ambitious goal, but one we felt was necessary if we were to convince our customers to treat serious Gram-positive infections with an oral agent. We attached great importance to the oral activity of the compounds in question. It was also important that an orally active compound possess sufficient solubility to promise a reasonable intravenous formulation. Hospital use of an oxazolidinone required an intravenous formulation if the drug were to be used against serious infections. Physicians would probably not switch from an intravenous application of vancomycin, for example, to an oral application of oxazolidinone in serious infections or even in clinical trials. After successful treatment with an intravenous oxazolidinone, however, the switch to oral therapy would be logical.

Compounds which were equivalently active to vancomycin in in vivo infection models were then subjected to pharmacokinetic evaluations in rats and dogs, and were submitted for toxicological evaluation. The toxicological evaluations were far too extensive for inclusion in this review and will not be considered here. There were two criteria that the putative clinical candidate had to meet in the pharmacokinetic evaluations to become a clinical candidate. We anticipated that the levels of compound in the blood of rats and dogs following oral dosing should predict a peak serum concentration (C_{max}) in humans of 12–15 $\mu g\,mL^{-1}$ with oral dosing. As we intended our oxazolidinone to be useful in treating serious human bacterial disease, we settled on a C_{max} value of three times the MIC_{90} value for the least sensitive organism (4 $\mu g\,mL^{-1}$) as the target level. A C_{max} value of that magnitude would ensure a significant duration of time for the level of the drug in the blood to be above the MIC value for any bacteria. If the peak serum concentration was the most important characteristic which correlated with antibacterial activity in vivo, then that C_{max} value would be sufficient to predict efficacy in humans. Additionally, with regard to the oral form of the drug, we targeted a daily dosing regimen in humans of twice daily. Three times daily dosing was problematic as we foresaw that parents would have to send their youngsters to school with the drug for the mid-day dose and would themselves have to remember to take drug with them when they were receiving the drug. A twice daily dosing, once in the morning and once at night at home, was thought to be the most convenient and least problematic dosing schedule.

Any drug which succeeded in meeting the in vitro criteria, the in vivo criteria, passed toxicological profiling, and met the pharmacokinetic requirements then became a clinical drug candidate suitable for additional preclinical development and ultimate entry into Phase I human clinical trials. Even when the first oxazolidinones were in clinical development we continued our efforts at uncovering the biological characteristics of the oxazolidinones. We focused on the mechanism of action of the oxazolidinones which we believed was important for the customers to accept this class as completely novel. We also initiated efforts at understanding the basis for the oxazolidinone activity spectrum being limited to Gram-positive bacteria. The key pharmacodynamic parameter of the oxazolidinones which best predicted successful clinical treatment was determined through extensive in vivo testing. This information provided customers with the means of predicting patient response from blood levels. Tissue distribution studies were conducted as a means of predicting which extravascular human bacterial diseases would be amenable to oxazolidinone therapy and a couple of highly specialized efficacy models were undertaken to determine what role if any an oxazolidinone might play in treating endocarditis (inflammation of the lining of the heart) or osteomyelitis (inflammation of the bone). We also began an examination of the effects of the oxazolidinones on the production of virulence factors even when the drug concentrations were below the MIC value for a specific organism.

2.2. Origins and Early Structure–Activity Studies

At the 1987 Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) workers from the DuPont company formally reported the structure and antibacterial activity profiles of two new antibacterial agents, Dup-105 and DuP-721.[13] These clinical candidates were the first significant representatives of a totally novel class of antimicrobial compounds, the oxazolidinones. These compounds originated from an iterative medicinal chemistry effort starting with a series of racemic 5-halomethyl-3-phenyl-2-oxazolidinones with reported utility for treating a variety of plant diseases. Compound **1** is one example of this compound class (Figure 2).[14] Subsequent chemical modification of **1**

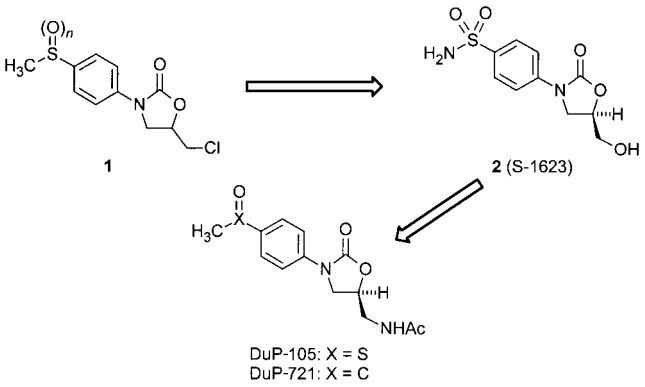


Figure 2. Emergence of the oxazolidinones at DuPont.

eventually led to analogues such as **2** (S-6123), which reportedly exhibited modest in vitro activity and in vivo efficacy against several Gram-positive and Gram-negative organisms.[15] It was at this time that the absolute configuration of the oxazolidinones at C-5 became evident. Further elaboration of analogues such as **2** eventually led to the identification of the prototypical oxazolidinones, Dup-721 and DuP-105,[16] which showed significantly improved characteristics relative to their progenitor compounds.

Some of the activity trends that emerged from the study of the early DuPont structure–activity relationships are summarized in Figure 3. As subsequent events will illustrate, several of these notions now require revision.

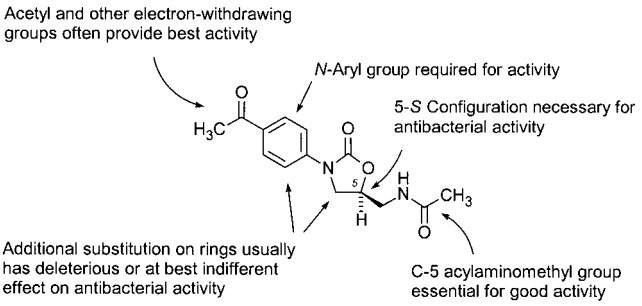
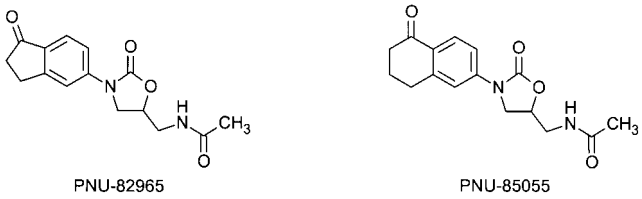


Figure 3. Early structure–activity relationships of oxazolidinones determined at DuPont.

We committed ourselves to proof-of-concept studies with the oxazolidinones. Our goal in these studies was the demonstration that we could synthesize structurally novel oxazolidinones which possessed promising biological characteristics, particularly antibacterial activity and safety profiles.

2.3. Interim Phases of the Development

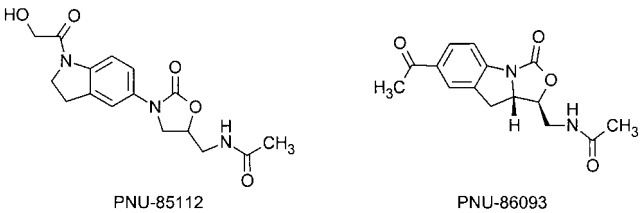
Early chemical modifications at Pharmacia probed a number of structural features of the DuP-721 lead compound to assess their impact on antibacterial activity. A detailed discussion of this effort is beyond the scope of this review, and for the sake of brevity we will focus on analogues wherein the usual 4-substituted phenyl ring is replaced by a fused bicyclic ring system. More specifically, analogues incorporating indanone (PNU-82965)[17] and tetralone (PNU-85055)[18] subunits



were prepared and tested. These compounds were designed to explore the effect(s) of annulating the acetyl moiety of DuP-721 back onto the pendant phenyl ring. It should also be noted that these early analogues were prepared in a racemic form to expedite their preparation. Since only the *S* enantiomer is antibacterially active,[16] the racemic material generally exhibits half the potency of the pure enantiomer. Nevertheless, these racemic analogues were deemed sufficient to probe structure–activity relationships at this early stage of the program.

The compound PNU-82965 eventually assumed a pivotal role for the Pharmacia oxazolidinone program. By 1989 fragmentary reports were obtained that DuPont had removed an oxazolidinone from clinical trials as a result of observed toxicity in several animal models.[19] A comparative in-house safety evaluation of racemic DuP-721 and PNU-82965 was conducted at Pharmacia to test these claims.[20] In this study racemic DuP-721 and PNU-82965 were administered orally at a dose level of 100 mg kg^{-1} body weight twice a day for 30 days to three male and three female Sprague–Dawley rats. The rats treated with racemic DuP-721 fared poorly in the study, one died and two rats were sacrificed in a moribund state. Additional findings for the animals treated with racemic DuP-721 included severe progressive weight loss and evidence of bone marrow atrophy. In contrast, the rats treated with PNU-82965 exhibited only a few adverse findings and these were judged to be very mild in nature. There were no clinical signs, serum or urine chemistries, or histopathological manifestations of drug-related toxicity. The major ramification of this comparative safety study was that a structure–toxicity relationship exists for the oxazolidinones.

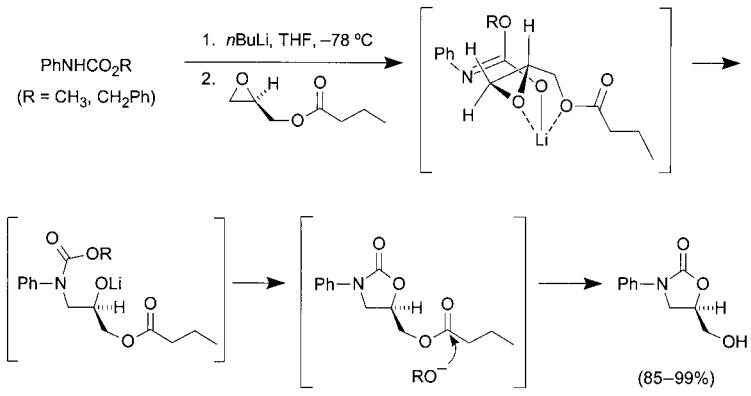
Following up on the favorable safety findings with PNU-82965, and speculating that alternative fused bicyclic inserts might confer favorable properties to the oxazolidinone pharmacophore, the racemic indoline congener PNU-85112 was targeted and eventually synthesized.[21] Like its progenitor, PNU-82965, this indoline analogue was found to exhibit



an excellent safety profile when tested for toxicity in the rat while displaying in vitro activity and in vivo efficacy closely approaching that of (±)-DuP-721.

Continuing investigations into fused-ring analogues of DuP-721 led to the design and synthesis of tricyclic fused systems such as the racemate PNU-86093.[22] This analogue and related congeners (not discussed here) helped to define the optimal dihedral angle between the oxazolidinone and adjacent phenyl rings. (±)-PNU-86093 exhibited in vitro antibacterial activity slightly lower than that of (±)-DuP-721.

With antibacterially active, safe oxazolidinones now in hand it became extremely important to address the preparation of enantiomerically enriched analogues. Previously described synthetic methods involving aryl isocyanates were effective, but not general.[16,17] In the course of extensive studies at Pharmacia it was found that *N*-lithiated carbamate derivatives of anilines could be treated with commercially available (*R*)-glycidyl butyrate under appropriate conditions to directly generate (*R*)-3-aryl-5-(hydroxymethyl)oxazolidinones (Scheme 1).[23] The hydroxymethyl intermediates are readily elaborated to final products.



Scheme 1. Enantiomeric synthesis of phenyloxazolidinones (Manninen reaction).

2.4. Emergence of the Piperazinylphenyloxazolidinones

We wondered whether the 4-pyridyl moiety of the DuPont lead compound, E-3709, might be amenable to replacement by suitable saturated heterocyclic bioisosteres (Figure 4). In connection with this notion, and in an inverse sense, we were cognizant of the successful replacement in the quinolone antibacterial agent area of the piperazine ring system of, for example, ciprofloxacin, with a pyridine fragment, as seen in Win-57273.[24] Another important aspect of the existing quinolone structure–activity relationship was the finding that strategically located fluorine atoms were found to not only increase potency, but also to confer enhanced oral pharmacokinetic performance to the compounds.[25] Contemporaneous to our plans to explore the piperazine surrogate, we speculated that the small but highly electron withdrawing fluorine atom would be tolerated at the *meta* position(s) of the central phenyl ring and confer enhanced antibacterial activity and/or other desirable properties to the targeted oxazolidinones (see generic structure **3** in Figure 4).

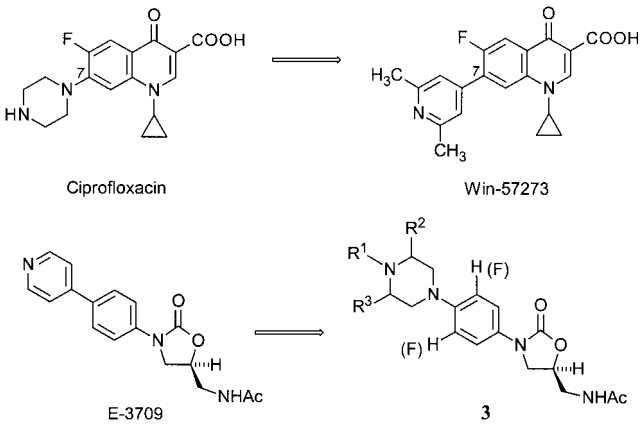
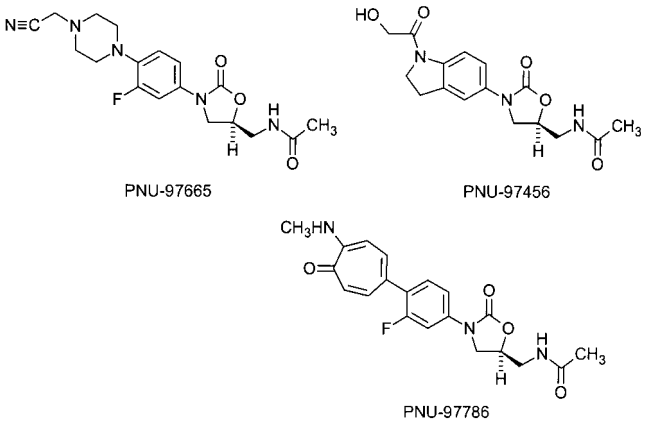


Figure 4. Development of the piperazinylphenyloxazolidinones.

By late 1992 the chemistry effort encompassed primarily three different subclasses of oxazolidinone analogues: 1) piperazinylphenyloxazolidinones (for example, PNU-97665),[26] 2) indolinyloxazolidinones (for example, PNU-97456)[21] and 3) the troponylphenyloxazolidinones (for example, PNU-97786).[27]

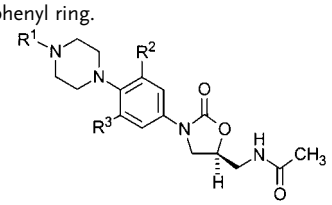
The indolines generally exhibited an excellent safety profile but demonstrated somewhat lower levels of antibacterial activity. The troponyl analogues were generally the most interesting compounds from an antibacterial activity standpoint but displayed poor water solubility and poor pharmacokinetic characteristics. Selected piperazine derivatives exhibited excellent in vitro and in vivo activity while also maintaining an acceptable safety profile, acceptable water solubility, and excellent pharmacokinetic parameters. As a bonus, the piperazine analogues were also the easiest compounds to synthesize. As a consequence of these and other characteristics, the piperazine series became the principal focus of the ongoing chemistry effort.



2.5. Piperazinylphenyloxazolidinones and Isosteres

A number of piperazinylphenyloxazolidinones of generic structure **3** were synthesized. Many of these analogues exhibited interesting levels of in vitro (MIC) and in vivo

Table 1: The potentiating effect of fluorination of the phenyl ring.

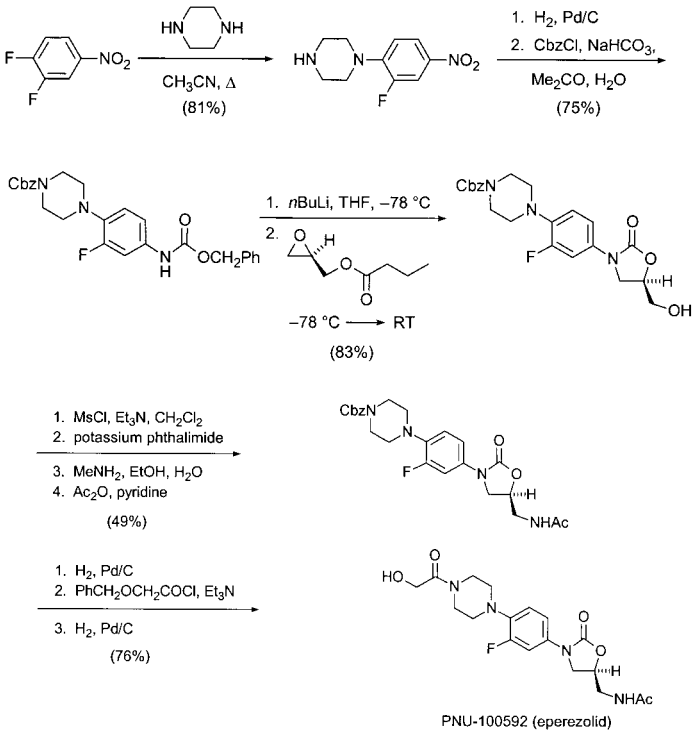


PNU	R^1	R^2	R^3	MIC [μg mL^{-1}] SA[a]	EF[b]	SP[c]	ED_{50} [mg kg^{-1}][d]
107399	CO_2Me	H	H	4	4	2	n.t.[e]
98257	CO_2Me	H	F	4	2	1	3.7 (1.6)
98170	CO_2Me	F	F	2	2	0.5	2.5 (1.3)
108946	$COCH_2OH$	H	H	4	2	1	n.t.
100592	$COCH_2OH$	H	F	4	1	<0.5	3.3 (5.0)
100675	$COCH_2OH$	F	F	2	1	0.25	1.6 (1.8)
143145	$COCH_2OH$	H	OMe	>16	>16	>16	n.t.
97665	CH_2CN	H	F	8	4	1	4.0 (2.2)
98172	CH_2CN	F	F	4	2	1	2.8 (2.7)
99200	$(CH_2)_2OMe$	H	F	16	16	2	7.9 (1.7)
99372	$(CH_2)_2OMe$	F	F	8	4	1	7.7 (2.0)
100762	$(CH_2)_2F$	H	F	16	8	2	8.6 (5.0)
100349	$(CH_2)_2F$	F	F	4	2	1	5.0 (2.8)

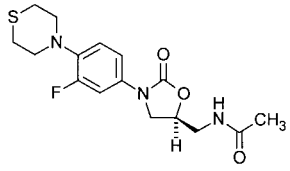
[a] SA = *Staphylococcus aureus* UC 9213. [b] EF = *Enterococcus faecalis* UC 9217. [c] SP = *Streptococcus pneumoniae* UC 9912. [d] ED_{50} = effective dose$_{50}$ (the dose that protects 50% of the animals). Oxazolidinones were administered orally; the results from subcutaneously administered vancomycin are given in parentheses. [e] n.t. = not tested.

(ED_{50}) antibacterial activity. Of particular note was the gratifying finding that one or two fluorine atoms flanking the *para* piperazine group exerted a significant potentiating effect on the antibacterial activity (Table 1); this finding is consistent with observations in other oxazolidinone subclasses.[27] It was found that a wide range of alkyl, acyl, and sulfonyl substituents were tolerated on the distal piperazine nitrogen atom. After a number of synthetic iterations it was found that the hydroxyacetyl moiety was the optimal nitrogen substituent. Ultimately, the monofluorophenyl congener PNU-100592, which was subsequently named eperezolid, emerged as the analogue with the best balance of antibacterial activity, pharmacokinetics, water solubility, and other pertinent properties.[28] An early laboratory synthesis of PNU-100592 is outlined in Scheme 2.

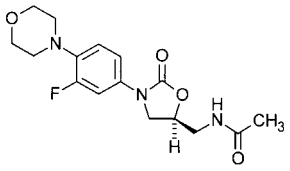
We were cognizant that alternative bioisosteric replacements for the piperazine ring were known, primarily from the literature relating to quinolone antibacterial agents. Systematic modification along these lines led to the identification of the interesting antimycobacterial thiomorpholine derivative PNU-100480[29] and also the morpholine analogue PNU-100766, which subsequently became know as linezolid.[28]



Scheme 2. Laboratory synthesis of eperezolid (PNU-100592). Cbz = benzyloxycarbonyl, Ms = methanesulfonyl.



PNU-100480



Linezolid (PNU-100766)

As depicted in Figure 5, the extensive effort at Pharmacia on the structure–activity relationship of oxazolidinone has prompted several revisions to the dogmas espoused by the earlier DuPont work. Perhaps most interesting was the finding that a suitable electron-donating amino substituent

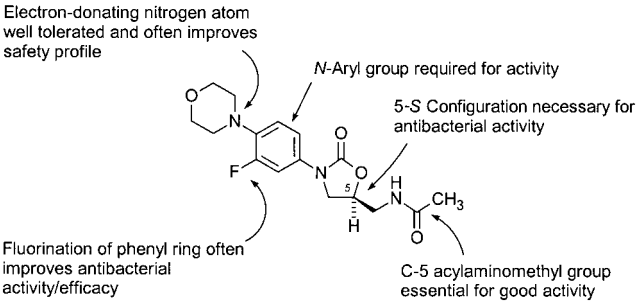
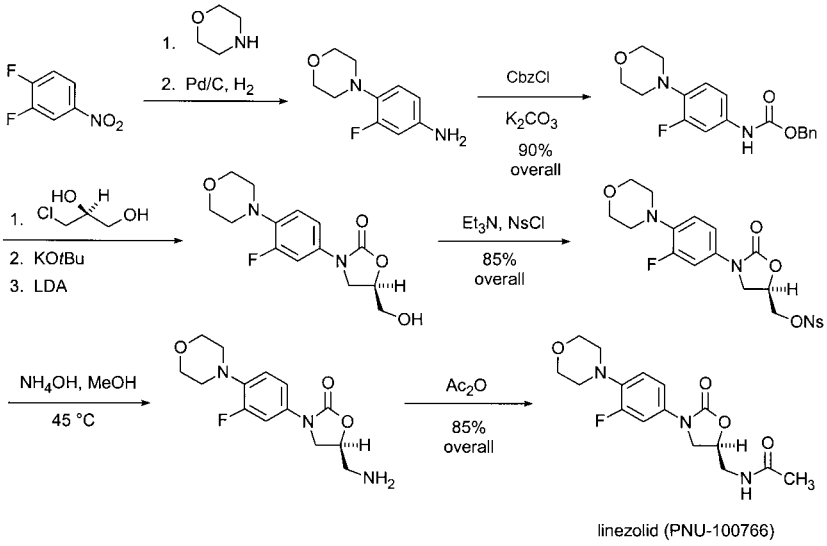


Figure 5. Revised structure–activity relationships identified at Pharmacia.

on the phenyl ring can confer excellent antibacterial activity while helping to maintain a good safety profile. Another important result of this investigation was the identification of the potentiating effect of one or two fluorine atoms flanking the morpholine or piperazine ring.

2.6. Linezolid and Eperezolid

The first oxazolidinones to emerge as potential drug candidates from the testing scheme were eperezolid (PNU-100592) and linezolid (PNU-100766). They were quite unusual in that they were almost identical in our preclinical testing: Their MIC values, their antibacterial spectrum, their ED_{50} values derived from tests with infected mice, and their pharmacokinetic behavior in at least two animal species were virtually identical, within experimental error, of each other. The research team then undertook the unusual strategy of taking both eperezolid and linezolid through a Phase I human clinical trial to determine if a significant difference might exist between the two compounds with respect to their pharmacokinetic behavior in humans. Evaluation of the blood data showed that linezolid would likely need to be dosed twice daily in humans and eperezolid three times daily to provide the same exposure. On the basis of that advantage, linezolid was selected for further development.



Scheme 3. Synthesis of linezolid (PNU-100766) on a process scale. LDA = lithium diisopropylamide, Ns = *meta*-nitophenylsulphonyl.

2.7. Synthesis of Linezolid on a Process Scale

An efficient, cost-effective synthesis of the compound was required to facilitate the clinical study of linezolid. As shown in Scheme 3, a concise large-scale preparation of the compound has been developed.[30]

3. Biological Evaluation of Linezolid

3.1. Antibacterial Activity In Vitro

The determination of the MIC values is really the first key biological data generated in a program aimed at determining the structure–activity relationships of antibiotics. Since there is little or no experience with comparisons of MIC values with blood concentrations and in vivo efficacy studies for members of a new antibiotic class the most prudent evaluation of the MIC values is by comparison with antibiotics on the market. As our program used *Staphylococcus aureus* as the key pathogen for SAR studies we chose the MIC values of vancomycin as our target for the oxazolidinones since vancomycin was the only antibiotic which could be used empirically against staphylococci with assurance. Our customers would, therefore, be used to its MIC values of 1 to 2 $\mu g\,mL^{-1}$ against staphylococci and would have little trouble accepting oxazolidinone values if they were in the same range. In fact linezolid performed comparably to vancomycin against staphylococci (Table 2). The MIC_{90} values of linezolid for methicillin-sensitive and -resistant *S. aureus* ranged from 2 to 4 $\mu g\,mL^{-1}$ and lay within the margin of error of those for vancomycin (1–2 $\mu g\,mL^{-1}$).[31] The MIC_{90} values of both linezolid and vancomycin for *Staphylococcus epidermidis*, a pathogen involved in infections from catheter and invasive devices, was 2 $\mu g\,mL^{-1}$. Other research groups confirmed very early on the activity of linezolid against staphylococci and its approximate equivalence to vancomycin.[32–38] At this time, many staphylococci strains with varying resistance patterns to antibiotics had been tested against linezolid in vitro and two observations were possible: first, linezolid activity was unaffected by pre-existing antibiotic resistance in the staphylococci, thus suggesting that it did have a new mechanism of action, and, second, the MIC values of linezolid for many strains of staphylococci were very similar numerically, which was taken to be the hallmark of a brand new class of antibiotics.

Linezolid also performed well against the enterococci in vitro. Linezolid inhibited all tested strains of *Enterococcus faecalis* and *Enterococcus faecium* at 4 $\mu g\,mL^{-1}$ or less.[31] This

Table 2: In vitro activities of linezolid and vancomycin.

Organism	Antibacterial agent	MIC_{90} [$\mu g\,mL^{-1}$][a]
Staphylococcus aureus	linezolid	4
(methicillin-susceptible)	vancomycin	1
S. aureus	linezolid	4
(methicillin-resistant)	vancomycin	2
Staphylococcus epidermidis	linezolid	2
(methicillin-sensitive)	vancomycin	2
S. epidermidis	linezolid	2
(methicillin-resistant)	vancomycin	2
Enterococcus faecalis	linezolid	4
(methicillin-sensitive)	vancomycin	2
E. faecalis (VanB)	linezolid	4
	vancomycin	>16
Enterococcus faecium	linezolid	2
	vancomycin	≤ 0.5
E. faecium (VanA)	linezolid	4
	vancomycin	>16
E. faecium (VanB)	linezolid	4
	vancomycin	>16
Streptococcus pneumoniae	linezolid	1
	vancomycin	≤ 0.25
S. pneumoniae	linezolid	1
(penicillin-sensitive or -resistant	vancomycin	≤ 0.25
Streptococcus pyogenes	linezolid	2
	vancomycin	0.5
Haemophilus influenzae[b]	linezolid	8
	vancomycin	>16
Moraxella catarrhalis[b]	linezolid	4
	vancomycin	>16
Gram-negative bacilli[c]	linezolid	>64
	vancomycin	>16
Bacteroides fragilis[d]	linezolid	≥ 16
	vancomycin	>16
Clostridium spp.[d]	linezolid	2
	clindamycin	4
Peptostreptococcus spp.[d]	linezolid	2
	clindamycin	2

[a] MIC_{90} = concentration of drug needed to kill or inhibit the growth of 90% of the tested strains under standardized conditions. [b] Fastidious Gram-negative bacteria. [c] Pathogens from the 10th generation. [d] Anaerobic pathogenic bacteria.

observation of enterococcal sensitivity to linezolid was very important as the enterococci had been developing into a therapeutic problem in the 1990s because they were generally antibiotic-resistant and the number of strains possessing vancomycin resistance was growing. The action of linezolid was unaffected by enterococcal resistance to vancomycin and promised welcome relief for physicians with patients who had enterococcal infections untreatable with antibiotics. As with the staphylococci, linezolid performed identically against geographically diverse enterococcal collections with a large array of antibiotic resistance patterns.

Very importantly, in our estimation, linezolid was more than acceptably active against penicillin-sensitive and -resistant *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Table 2). Although the organisms receiving the most attention in terms of fears of resistance development in the 1990s were the staphylococci and enterococci, we also focused our attention on the streptococci. The reasoning was very simple: *S. pneumoniae* is the major cause of bacterial disease in humans, since it is the principal causative agent of upper and lower respiratory tract infections. Since the advent of the antibiotic era, *S. pneumoniae* infections had been effectively treated with inexpensive older line antibiotics, but with the advent of β-lactam (penicillin family) then macrolide and quinolone resistance in *S. pneumoniae*, the treatment of upper and lower respiratory tract infections was becoming a therapeutic problem. Linezolid was quite active against the streptococci and was unaffected by β-lactam resistance. The activity of linezolid against *S. pyogenes* extends the spectrum of the drug to enable treatment of infections following childbirth. It is also interesting to note that, like many Gram-positive antibiotics, linezolid covers both the staphylococci and the streptococci, with more intrinsic activity exhibited against the streptococci.

The spectrum of linezolid activity is also very important since the spectrum of a drugs' activity will determine if the drug will be clinically useful or not. The spectrum of linezolid activity covers the most important Gram-positive pathogens—the staphylococci, the enterococci, and the streptococci. This spectrum of activity indicated that the drug would provide coverage for important medical diseases and would be a welcome addition to the antibiotic armamentarium. Typical of antibiotics active against Gram-positive bacteria, linezolid was inactive against the Gram-negative bacilli and had modest to weak activity against *Haemophilus influenzae* and *Moraxella catarrhalis*, two fastidious Gram-negative organisms found coincident with *S. pneumoniae* in upper and lower respiratory tract infections (Table 2). Linezolid also appeared to have modest in vitro activity against the medically significant anaerobes *Bacteroides fragilis*, *Clostridium* spp., and *Peptostreptococcus* spp., which is consistent with the spectrum of activity of other antibiotics active against Gram-positive bacteria, for example, clindamycin (Table 2).[39]

A very interesting conundrum arose during early SAR work on the oxazolidinones. We were quite aware that the spectrum of oxazolidinone activity did not include the Gram-negative bacilli and yet some of the biologists on the team were measuring oxazolidinone inhibition of bacterial protein synthesis in a cell-free system. The surprise was that the cell-free system was an *Escherichia coli* extract, and *E. coli* was one of the Gram-negative bacilli for which the oxazolidinones, including linezolid, exhibited no appreciable activity. In the absence of cell membranes and the Gram-negative cell wall the oxazolidinones were very active in inhibiting *E. coli* protein synthesis. In the interest of focusing on Gram-positive agents, we assumed that the oxazolidinones lack of Gram-negative activity was some sort of nonspecific transport

activity. The observation was revisited later when it was shown that making the *E. coli* AcrAB transmembrane pump nonfunctional through directed mutagenesis then made whole *E. coli* cells sensitive to linezolid both in vitro and in vivo.[40] The lack of Gram-negative activity on the part of oxazolidinones is therefore a result of the presence of transmembrane pumps which, along other molecules, pump oxazolidinones out of the cell faster than they can accumulate.

3.2. In Vivo Activity of Linezolid

The second step in the evaluation phase of potentially useful oxazolidinone compounds is the determination of antibacterial activity in vivo. This usually took the form of experimentally determining the ED_{50} value (the amount of drug in $mg\,kg^{-1}$ of body weight required to cure 50% of infected animals) in mouse models of bacteremia (bloodstream infection) as the first evaluation of in vivo activity. To cure a bacteremia following oral dosing an antibiotic must remain intact in transit through the stomach and some portion of the intestinal tract, the majority of the antibiotic must be transported across the gut wall, it must appear in the blood in sufficient concentration so as to kill the bacteria without being metabolized or enzymatically converted into an inactive form, and it must remain in the blood stream without being excreted too rapidly. The value of these models is that they put the drug through all of these individual hurdles that a human patient does. Given those requirements, the bacteremia models are the most direct and efficient measure of in vivo activity compared with infection models where the bacteria are localized in a specific tissue.

The results of mouse efficacy studies with linezolid are contained in Table 3. These are representative in vivo tests, many more tests with multiple strains of specific organisms not represented here were performed.[41] In these tests, linezolid given orally to mice performed equivalently to vancomycin administed subcutaneously (vancomycin is not orally available). These tests demonstrated that we had achieved a major goal of the program: a drug has been developed whose oral activity is approximately equal to vancomycin administered subcutaneously.

Linezolid and vancomycin performed equivalently against an antibiotic-sensitive *S. aureus* (UC9271) as shown by the two ED_{50} values being within experimental error of each other (data not shown). Very importantly, linezolid was as active as vancomycin in vivo against methicillin- and multiple-drug-resistant *S. aureus* strains. This was also true in an unusual *Staphylococcus epidermidis* bacteremia model where linezolid was again equivalent to vancomycin. Linezolid thus met our originally conceived in vivo requirements for staphylococci activity and was shown to possess excellent in vivo antibacterial activity against a penicillin- and cephalosporin-resistant *Streptococcus pneumoniae* and an *S. pyogenes* strain (Table 3). Multiple strains of *S. pneumoniae* with varying antibiotic resistance could be treated in vivo with ED_{50} values falling between 3 and $10\,mg\,kg^{-1}$ (data not shown). Vancomycin was numerically more active than linezolid in effecting cures in a *Enterococcus faecalis* bacteremia model, however, linezolid performed well orally with an ED_{50} value of $10\,mg\,kg^{-1}$. An oral drug this active against an enterococcal infection is quite unusual. Importantly, linezolid retained its in vivo antibacterial activity when tested against a vancomycin-resistant *E. faecium* bacterium (Table 3) even though the efficacy test was conducted in a neutropenic mouse model which is far more difficult to treat than a model in a normal mouse.

The remaining three in vivo determinations contained in Table 3 are all models for soft tissue infection where the bacteria are localized subcutaneously in the mouse. The cures in these cases are measured by microbiological enumeration of the bacteria from the infected area at the end of therapy. Following oral dosing, linezolid was clearly penetrating to the infection site as it actively cured all three infections. Both linezolid and vancomycin cured the soft tissue infection caused by *S. aureus*, and although their ED_{50} values were different both values would predict utility from this type of a model. The model for soft tissue infection by *E. faecalis* demonstrated very good activity on the part of oral linezolid

Table 3: In vivo activity of linezolid and vancomycin.

Bacterium	Compound[a]	MIC [$\mu g\,mL^{-1}$]	ED_{50} [$mg\,kg^{-1}$][b]
Staphylococcus aureus UC9271	linezolid	9.0	6.9
	vancomycin	1.0	13.2
S. aureus UC6685[c]	linezolid	2.0	3.8
	vancomycin	2.0	2.6
S. aureus UC15080[c]	linezolid	0.5	3.8
	vancomycin	1.0	1.5
Staphylococcus epidermidis UC12084[c]	linezolid	1.0	4.7
	vancomycin	2.0	1.8
Staphylococcus pneumoniae UC15088[c]	linezolid	1.0	2.7
	cefaclor	>32.0	>20.0
Staphylococcus pyogenes UC152	Linezolid	2.0	5.0
	clindamycin	0.6	8.6
Enterococcus faecalis UC12379[d]	linezolid	4.0	10.0
	vancomycin	1.0	0.5
Enterococcus faecium UC15090[e]	linezolid	4.0	24.0
	vancomycin	>69.0	>100.0
S. aureus UC9271[f]	linezolid	4.0	39.7
	vancomycin	1.0	4.7
E. faecalis UC15060[f]	linezolid	4.0	11.0
	vancomycin	2.0	16.3
Bacteroides fragilis UC12199[f]	linezolid	4.0	46.3
	clindamycin	1.0	200.0

[a] Linezolid was administered orally and vancomycin subcutaneously. [b] The amount of drug in mg per kg of body weight required to cure 50% of infected animals. [c] Methicillin- and multidrug-resistant. [d] Penicillin- and cephalosporin-resistant. [e] Vancomycin-resistant; model performed in neutropenic mice. [f] Subcutaneous soft tissue infections.

against this important pathogen and, very interestingly, oral linezolid performed exceptionally well when tested against the anaerobe *Bacteroides fragilis* in a soft tissue infection. The real importance of these models is that they require a drug that penetrates the animals tissues well in order to effect a cure. Linezolid not only penetrates the tissues sufficiently to provide adequate drug concentrations to kill the bacteria but it does so following oral dosing. These observations indicated that linezolid would be very useful in a variety of human infections.

Several other specialty animal models provided very useful specific information concerning efficacy of linezolid prior to or coincident with clinical trials. In a streptococcal pneumonia model in rats[42] linezolid administered twice daily at 50 mg kg^{-1} was found to be equivalent to ceftriaxone, an extended pharmacokinetic cephalosporin, given 100 mg kg^{-1} once daily. In the chinchilla model of otitis media (inflammation of the middle ear), linezolid readily eradicated multidrug-resistant *S. pneumoniae* at 25 mg kg^{-1} twice daily, but was not effective in eradicating *H. influenzae* when the MIC values of linezolid for the strain was 8–16 μg mL^{-1}.[43] Linezolid was weakly effective at 25 mg kg^{-1} in an intraperitoneal abscess model with *E. faecium* but substantively reduced bacterial numbers in the abscesses at 100 mg kg^{-1}.[44]

Physicians hope that any new antibiotic active against Gram-positive agents will be effective against staphylococcal endocarditis as this infection of the heart valves is life-threatening and very hard to eradicate. Our customers were particularly interested in the possibility that oral linezolid would be effective in endocarditis because this would represent a major step forward in ease of drug administration. The action of linezolid was compared directly with that of vancomycin against endocarditis caused by antibiotic-sensitive and methicillin-resistant *S. aureus* in a rabbit model.[45,46] In the first study, oral linezolid at a dose of 50 mg kg^{-1} rendered 50% of infected animals heart-valve culture negative while intravenous vancomycin at 25 mg kg^{-1} cured 8/11. Linezolid reduced blood bacterial counts from 2.3 CFU mL^{-1} (CFU = colony-forming units of bacteria, logarithmic values) to 1.5 CFU mL^{-1} in culture-positive animals at the end of therapy, as did vancomycin. Both linezolid and vancomycin reduced the bacterial counts from heart valve vegetation from 8.4 CFU g^{-1} gram to 4.3 and 4.0 CFU g^{-1}, respectively. Linezolid clearly performed as well as vancomycin in this very important infection model and did so as a drug administered orally—a very important consideration for patient compliance and safety. The key pharmacokinetic parameter identified in this study was simply that in this difficult to treat infection the blood concentration of linezolid had to be maintained at or above the MIC_{90} value for the infecting organism. This key observation was confirmed in a subsequent study[46] of endocarditis caused by methicillin-resistant *S. aureus* where again linezolid administered orally compared favorably with vancomycin administered intravenously. This modeling work with linezolid let us to believe that linezolid could very well have a place in the treatment of human endocarditis and might provide an alternative drug to vancomycin in the treatment of endocarditis caused by antibiotic-resistant *S. aureus*.

3.3. Pharmacokinetic/Pharmacodynamic Properties of Linezolid

One of the key characteristics of antibiotic behavior in a human is its performance in blood. A 600 mg oral dose was chosen for linezolid based on animal studies, and the resultant human blood levels were determined in a clinical trial.[47] The 600 mg dose in humans achieved average maximum serum levels of about 19 μg mL^{-1}, which by 12 h following administration were approximately an average of 6 μg mL^{-1}. These blood levels were very promising for the ultimate use of linezolid in humans as they predicted that following a single 600 mg oral dose of linezolid, the minimum average drug blood level would be above the MIC_{90} value for staphylococci, streptococci, and enterococci. These observations also predicted that linezolid could be dosed twice daily, at about 12 hour intervals, which represented a significant advantage over drugs requiring three times daily dosing. A very satisfying and unusual result from these studies was the determination that linezolid was 103% orally bioavailable. This means that the area under the drug blood level curve for the oral dose in humans was equal to the area under the drug blood level curve for the intravenous dose. Animal studies had hinted this might be true. This situation is extremely rare and means that the patient's exposure following oral dosing was equal to their exposure following intravenous dosing. In a practical sense, it also meant that the oral and intravenous routes of administration used exactly the same amount of drug and that the dose would not have to be changed when switching from intravenous to oral therapy.

Other important pharmacokinetic behaviors of linezolid emerged from animal studies and human clinical trials. It was found that the presence of food had little effect upon linezolid absorption, so linezolid could be taken both with meals or at another time.[48] Importantly for prescribing physicians, linezolid is not metabolized by cytochrome P450 nor does it inhibit any of the important P450 isoforms.[49] Metabolism does occur, but it is by non-enzymatic oxidation and the metabolites do not possess antibacterial activity. Between 8 and 8.5% of linezolid is excreted in the urine and 7 to 12% through the gut.[50] Linezolid is a very well behaved antibiotic in terms of its pharmacokinetic behavior, and all of these observations translate to its ease of use in therapy.

Since linezolid was the first member of the oxazolidinone class of antibacterial agents to approach the market we wanted to determine which pharmacodynamic parameter of linezolid best predicted in vivo cure. This information would have a strong bearing on the design of clinical studies and would permit our customers to be able to match the anticipated linezolid pharmacokinetic behavior in the patient with the key pharmacodynamic parameter. Animal studies using a model of a thigh infection caused by *S. aureus* in the mouse were undertaken to answer this question.[51] The parameter which best predicted in vivo cure was determined experimentally to be the time the drug blood level exceeded the MIC_{90} value for the infecting organism. It was further determined that the time the drug blood level exceeded the MIC_{90} value should be a minimum of 40–60% of the dosing interval. The 600 mg dose of linezolid provided blood levels above the MIC_{90} value for 100% of the dosing interval and

thus more than met the minimum requirements of the pharmacodynamic model.

4. Further Biological Findings

4.1. Mechanism of Antibacterial Action of Linezolid

One of the key characteristics of linezolid which we believed would make it very attractive to physicians was the fact it was a brand new class of antibiotic which did not suffer from existing resistance mechanisms in bacteria. We thought it very important to literally demonstrate that the oxazolidinones had a unique mechanism of action and identify that mechanism. Studies done with eperezolid clearly showed that eperezolid bound to the 50S ribosomal subunit and that binding was dosage dependent.[52] It was postulated that the mechanism of action was different from those of chloramphenicol and lincomycin which appeared to bind at sites near the specific site for eperezolid. Linezolid inhibited phage-specific in vitro translation, peptide chain termination, and polypeptide chain elongatin in a cell-free *E. coli* protein synthesis assay.[53] On the basis of these observations it was then determined that linezolid binds the 50S ribosomal subunit and that binding then prevents formation of a functional initiation complex (Figure 6).[54] This discovery clearly demonstrated that linezolid was unique among protein synthesis inhibitors and explained why linezolid retained antibacterial activity against Gram-positive organisms which were resistant to members of existing classes.

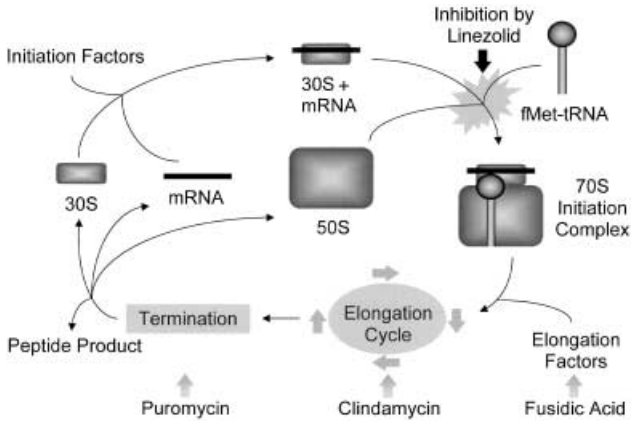


Figure 6. Mechanism of action of linezolid.

4.2. Distribution of Linezolid in Tissue

The in vivo experiments which demonstrated that linezolid effected microbiological cure of soft tissue infections in mice clearly indicated that linezolid was penetrating the tissues from the blood stream in sufficient quantity and with sufficient duration of persistence. A more quantitative and direct measure of linezolid penetration from the blood to tissues was undertaken in the rat[55] and demonstrated that linezolid penetrates tissues very well. Linezolid was radioactively tagged with ^{14}C and administered intravenously to rats at a dose of $10\,mg\,kg^{-1}$. Twenty minutes after administration, linezolid was distributed throughout the entire animal with a tissue level of $10.5\,\mu g\text{-equiv}\,g^{-1}$. The tissues which had at least twofold the MIC_{90} concentration of linezolid for staphylococci ($8\,\mu g\,mL^{-1}$) included the small and large intestine, the bladder, lymph nodes, pancreas, spleen, bone marrow, muscle, thymus, thyroid, heart, lung, liver, and pituitary gland. The concentrations of linezolid in the vitreous humor and brain were about 14% of the blood levels. In fact, the only two tissues which linezolid did not penetrate well following the single dose of drug were the bone matrix and white fat. These findings were, and are, very important as they indicate that linezolid has a very good chance of effecting a cure in humans for Gram-positive infections no matter where the infection may be localized. Most infections in humans are not of the blood alone and linezolid provides the physician with a very effective tool for combating Gram-positive infections.

4.3. Bacteriostatic Versus Bacteriocidal Considerations

An antibiotic is considered static if there is less than a 3-log reduction in bacterial cell numbers in 16–24 h under standardized conditions in the test tube and cidal if it kills $\geq$ 3 logs of bacteria. From this definition, linezolid was determined to be static for staphylococci and enterococci and cidal for pneumococci. The general rule of thumb is that static drugs should not be used for particularly serious infections with Gram-positive infections, such as endocarditis, and under circumstances where the patient is in grave danger. There are several exceptions to these rules and we wondered if linezolid was not an exception when several investigators told us that linezolid was an exceptionally fast acting antibiotic in human infections. Rapidity is not necessarily a hallmark of static drugs. To address this issue, we initiated an investigation into the effect of linezolid upon toxin production in *S. aureus* and *S. pyogenes*.[56] These in vitro studies were limited to linezolid levels below the MIC value of the organism. Linezolid dramatically reduced α-hemolysin, δ-hemolysin, and coagulase levels in *S. aureus* cultures and similarly effected Streptolysin O and DNAase levels in *S. pyogenes* cultures even at levels of 25 or 50% of the MIC concentration. For example, with no linezolid in the culture the titre of α-hemolysin in an *S. aureus* culture was 512 and with the additon of 25% of the MIC value of linezolid, which did not kill the cells, that titre was reduced to 2. Although linezolid is technically a static drug for *S. aureus* it behaves in vivo as though it is a cidal drug through its intervention in toxin production. The rapidity with which linezolid kills the bacterial cells becomes irrelevant to the fact that it inhibits toxin production and thereby inhibits the tissue damage and destruction which is the hallmark of the disease. Linezolid therefore behaves as a cidal drug in vivo although it is clearly static in the test tube.

5. Summary

The development of resistance in the Gram-positive pathogenic bacteria to antibiotics over the last twenty years and continuing today has created a need for new antibiotic classes which are unaffected by existing bacterial resistance. The oxazolidinones were not only a new class with a novel mechanism of action, but importantly were unaffected by existing resistance in Gram-positive agents and were orally active. Our efforts regarding structure–activity relationships had clearly defined biological goals encompassing activity in vitro and in vivo, as well as pharmacokinetic parameters. Early chemistry efforts involved modifications of the indanone and tetranone subunits, with early successes driving the program forward. An enantiomeric-specific synthesis was vital to our continuation of this work and further efforts revolved around piperazinyl-, indolinyl-, and troponylphenyloxazolidinones. Both linezolid and eperezolid, our first clinical candidates, arose from the piperazine subclass with linezolid being chosen for continued development because of its enhanced pharmacokinetic properties. Linezolid subsequently performed exceptionally well in human clinical trials where it performed equivalently to the best comparitive marketed antibiotics. Linezolid was approved in the U.S. by the Food and Drug Administration (FDA) in April 2000.

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