

## Manuscript I

### **Modification and Reversal of Resistance in Micro-organisms with Selected Non-antibiotics and their Stereoisomeric Analogs: New Efflux Inhibitors?**

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**Abstract:**

The emergence of bacterial resistance is becoming an increasing problem around the world.

Insight into efflux mechanisms as a major explaining factor of antibiotic resistance demonstrates that active bacterial efflux affects virtually all classes of antibiotics and results in more or less (multi-) drug resistant (MDR) phenotypes. The clear link between efflux systems, microorganisms and the emergence of resistance proves the need to classify efflux as a phenomenon of clinical relevance.

Growing knowledge of the molecular aspects of bacterial efflux mechanisms has encouraged investigation into bacterial efflux as a “target-dimension” for avoiding / ”curing” antibiotic resistance. Non-antibiotics are suggested as “helper compounds” in this process. Stereo-selectivity is introduced as a biological principle to strengthen the anti-resistance effect on prokaryote cells and/or reduce/delete the unwanted “side-effects” on the eukaryote cell system.

This review analyses the potential impact of different non-antibiotic compounds on efflux systems of selected clinical significant pathogens. Especially neurotropic compounds showing anti-resistant activities are presented. Known drugs with known toxicological profiles for reversal of resistance gives excellent possibilities for selecting the best compounds and their analogues for further development.

**Keywords: Bacterial efflux, Non-antibiotics, Helper compounds, Reversal of resistance, Anti-microbial activity, Neuroleptics, Stereo- chemistry.**

## **Background**

### 1.1. Emerging resistance

The rate by which microorganisms and tumors develop resistance against antibiotics and chemotherapeutics is overtaking the development of new drugs for treatment of infectious diseases and malign illnesses (1,2). The result is that it is vital to understand the mechanisms of resistance development in prokaryote and eukaryote cell systems under chemical stress. The most direct and fastest developed kind of resistance has been well described and follows the principles of “survival of the fittest” (3). With the development of synthetic compounds and the classical antibiotics in particular, the chemical pressure on the biological systems - eukaryotes and prokaryotes living together - have increased significantly and the counter pressure from the microorganisms and the mammalian cells have increased correspondingly (4).

The emerge of bacterial resistance is primarily understood as based on the inactivation or modification of antibiotics or target modification. Anyway, increasing insight in bacterial genetics demonstrates that active bacterial efflux results into multidrug resistance, affecting all classes of antibiotics.

### 1.2 Efflux pumps

Passive and active efflux in eukaryotic cell systems (organs, mammalian cells) and in the prokaryotes (microorganisms) is not a new phenomenon. Efflux has been known ever since the first anatomical investigations were carried out (5). Efflux is a fundamental function in mammalian cells and in microorganisms (6). Seen in relationship to the resistance problem it has become increasingly clear that at least some resistance can be explained by the fact that both eukaryotes and prokaryotes are able to pump molecules that are unwanted such as antibiotics/chemotherapeutics out of the cell after entry (7,8,9,10). Different cell systems achieve this by multiple efflux pumps.

In figure 1 the topology of the superfamily efflux pumps are presented. In figure 2a and 2b the different superfamilies of efflux families are presented with examples of different bacterial species belonging to each family. These figures are made to visualize the importance of efflux mechanisms for multiresistance in different microorganisms. The efflux pumps are currently the object of research efforts exploring the possibility of creating drugs that can act as inhibitors of the efflux pumps (11,12). The aim of the research is not just to define a new pharmacological target as a basis for the synthesis of new chemotherapeutics/antibiotics, but also in particular to look for a new

strategy based on the prevention of resistance development and the reversal of existing resistance so that the classic chemotherapeutics/antibiotics can become useful again.

### 1.3 Efflux pumps as a pharmacological target

Following the development of the first antibiotics and chemotherapeutics the challenge has been to develop synthetic or semi-synthetic molecules that retain the antimicrobial effect/anti-tumor effect; compounds the microorganisms/tumor might not be resistant to has been the final goal. This has not been possible up to date especially because of different resistance efflux systems in microorganisms/tumorcells (13).

With regard to the eukaryotic cell system Keld Danø was the first, in 1973, to describe how eukaryotes in the form of Ehrlich`s tumor cell line was able to pump out daunomycine. At the same time he proved how the efflux system in the tumor cell line could be competitive inhibited by the use of Vinca, a toxic plant extract (14).

The importance of this observation was first underlined on prokaryotic cell systems, when the tet-pump and the norA pump was described in *E. coli* and *S. aureus* (15,16).

In 1979 it was shown that membrane stabilizers like anaesthetics, barbiturates, neuroleptics and chloroquine, which inhibit the passive ion effluxes over eukaryotic cell membranes (17,18) also interfered with the prokaryotic cell membranes (19,20,21).

The above mentioned observations opened up the possibilities of using already known drugs as, for example, neuroleptics in conjunction with conventional antibiotics/chemotherapeutics with the object of taking advantage of the synergistic, resistance and virulence modifying effect seen by using these substances together (22,23).

Efflux as a mechanism of resistance may be considered too complex a system to allow for a potential pharmacological use. The efflux pumps are often not the sole cause of the development of the resistance, that is often perceived as being multifactorial (24). It is therefore extremely important to map out the mechanisms of the efflux resistance mechanisms in order to create efflux inhibitors characterized by having more than one point of attack in the resistant cell. Understanding the complex mechanisms of the development of resistance is a great challenge in modern-day science.

## Medical need.

With the rate of infectious diseases caused by resistant and multiresistant bacteria on the increase, the problem of resistance development has become global. In the western world, the problems with resistant infections are especially related to MDR *S. aureus* (MRSA), resistant streptococci, enterococci, and typical as well as atypical mycobacteria (25, 26, 27). Besides these resistant bacterial infections, especially in developing countries resistance development in the intracellular protozoae diseases present serious problems (see later).

The existing national differences in resistance development are expected to be affected by increased human mobility, which characterizes the present time. A restrictive policy in prescribing antibiotics is one of the ways the Nordic countries have tried to curtail the prevalence of multiresistant bacteria. However, this policy has proved to be insufficient in these countries and unsuccessful worldwide. The increase of MRSA in Europe is shown in [figure 3](#) and may serve as an example for the dimension of the problem. The increased prevalence of infectious diseases is in part to be blamed on the, geographically spread of multi-resistant micro-organisms.

### 2.1. Problems caused by resistance in selected Gram positive bacteria.

An increased number of methicillin resistant *S. aureus* (MRSA) in Europe has been documented in the period from 1999 to 2002, as has the pronounced geographical variations for the same time period. This is presented in [table 1](#). No further data is currently available for Europe concerning other relevant clinical Gram positive micro-organisms. The prevalence of *S. pyogenes* M-type, which is resistant to erythromycin because of the presence of the *mefA* gene, has risen considerably all over Europe. The literature quotes resistance frequencies between 38-97%. A German survey covering the 2002-2003 time period show erythromycin resistance rate as 14% (n=381) of which 55.6% was due to *mefA* efflux. The presence of *ermA* (31.5%) and *ermB* (13.5%) is responsible for detected macrolide resistance (28). Statens Serum Institute reports an increase in the use of macrolides in Denmark from 1990 to 1999 ([figure 4](#)) and at the same time an increase in the frequency of erythromycin resistance from 3% to 7% in *Streptococcus pyogenes* and other Gram positive bacteria is seen (29). At the present time enterococci come in third place, sometimes even second place in the surveys defining the frequency of bacterial infections in hospitalised patients (30,31,32).

## 2.2. Problems caused by resistant *Mycobacterium tuberculosis*.

*M. tuberculosis* is estimated to cause infections in more than 2 billion humans. Around 50 million people are thought to be infected with TB-bacteria resistant to one or more of the commonly used antibiotics/chemotherapeutics (WHO report 2000). Tuberculosis is at present a rare disease in the Scandinavian countries, albeit on the increase since 1985. The world scenario and the intense human mobility of today calls for an increased awareness of the problem.

## 2.3. Problems caused by resistance in selected Gram-negative bacteria.

A rise in the prevalence of ciprofloxacin resistant *P. aeruginosa* from 10.3% to 12.1% in the period 2002-2003 has been described by the German GENARS. The most recent reports show a prevalence of efflux related (Mex-opr over production) fluoroquinolone resistance in 14-15 % of the clinical strains investigated (33).

In Denmark approx. 30% of *H. pylori* strains are primarily resistant against metronidazole. This figure has been relatively stable over a number of years; therefore metronidazole is still used in the treatment of *H. pylori* infections. *H. pylori* strains develop resistance against macrolides like claritromycin relatively easily. The rise of the usage of claritromycin has caused a corresponding rise in the resistance over the last few years, from a few percent to more than 20% in many European countries. The claritromycin resistance is often combined with the resistance against metronidazole. Amoxicillin resistance amongst *H. pylori* isolates has also been described, but is still only sporadically found in Denmark (34).

## 2.4. Problems caused by resistant parasites.

The developing countries are dominated by resistant intracellular diseases, caused by parasites like malaria, leishmaniasis and trypanosomiasis.

A rise in the development of resistance in *P. falciparum* against chloroquine can be observed worldwide (35,36). In Asia and South America *P. falciparum* is resistant against combinations of pyrimethamin with sulfadoxine or dapson. A further resistance against proguanil is found in Africa. In the border regions of Thailand the treatment of malaria with mefloquine fail in 50% of the patients, and a reduced sensitivity against quinine has been observed. The mefloquine and quinine resistance has also been sporadically observed in Africa (37).

Leishmaniasis is observed worldwide, but is most commonly found in tropical and subtropical regions with a prevalence of 12 millions cases and an approximal incidence of 0.5 million cases of Visceral Leishmaniasis (VL) and 1.5 million cases of Cutane Leishmaniasis (CL).

(<http://www.who.int/tdr/diseases/leish/diseaseinfo>).

Leishmaniasis is predominant as an infection in children and is also a serious source of infections in dogs in the Mediterranean countries as well as Brazil.

These general aspects of leishmaniasis and the surveillance strategies were recently described (38). Controlling this form of the disease has so far consisted of locating the active incidents and following up with a treatment of chemotherapeutics, but this is under threat by the developing resistance of this parasite.

Tryposomiasis almost vanished from sub-Saharan Africa by the 1960s due to control programs targeting tsetse flies and trypanosomes. Unfortunately, the disease has since been neglected. It is reemerging since the late 1990s with an estimated number currently of over 500,00 new infections per year (39) [Barrett, 1999]; see <http://www.who.int/tdr/diseases/tryp/> for updates on sleeping sickness. The incidence of Gambian form Trypanosomiasis resistant to melarsoprole treatment has also risen significantly in Northern Uganda, Northern Angola and Southern Sudan, with treatments failing in up to 26,9% of patients (40). .Most of these infections are caused by *T. gambiense*. Without prospect for a vaccine, the treatment of sleeping sickness relies entirely on chemotherapy.

## **Existing treatment**

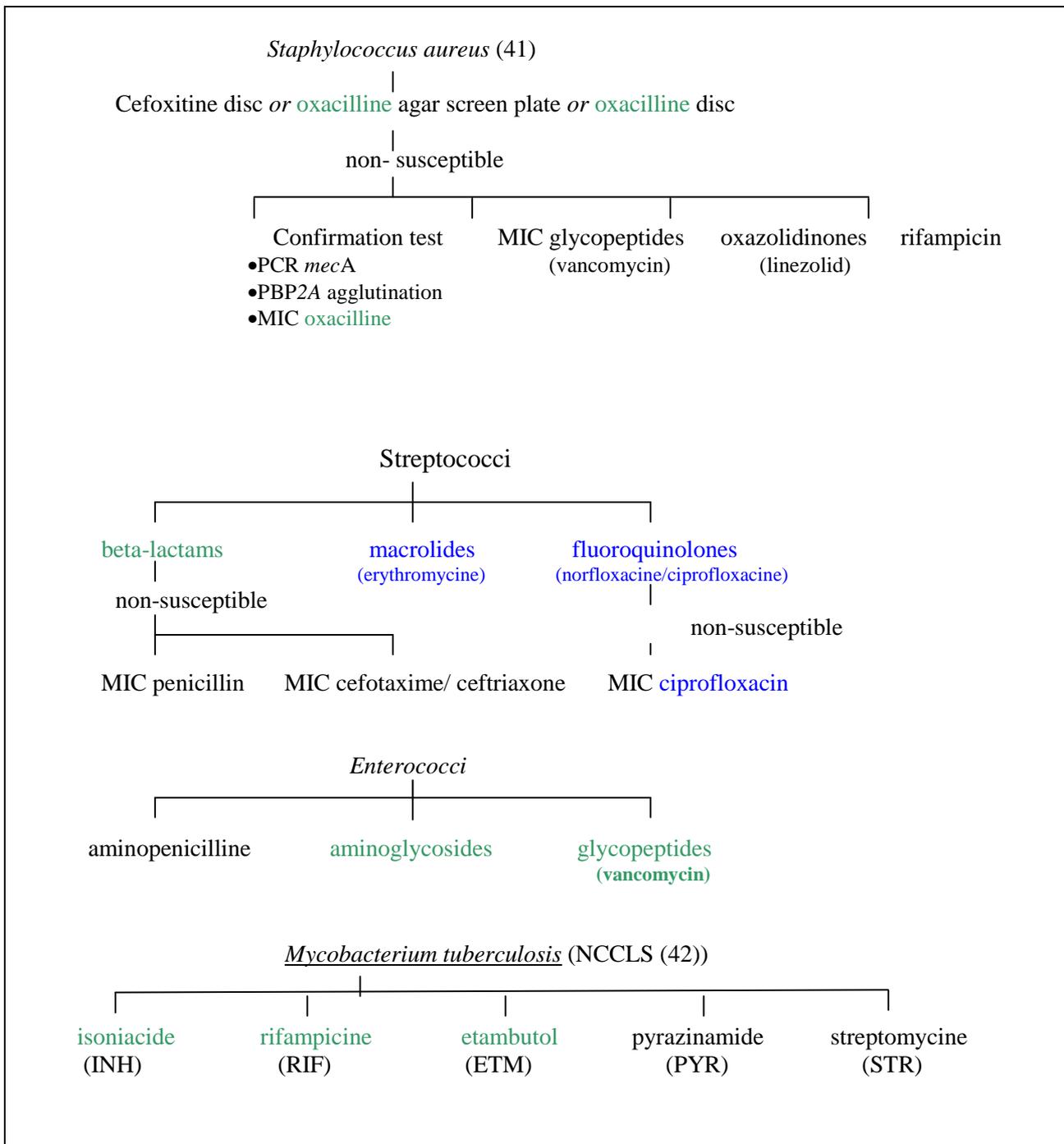
The present strategy for treatment of resistant infections/cancers can best be described as a “tailor made” combination of several antibiotics/chemotherapeutics, the basic concepts are presented in figure 5. The cumulative effect is expected to reduce the development of resistance by influencing several specific antibiotic targets in the respective micro organisms.

### Specific antibiotic strategy – a panoptical description.

A short version of the Antibiotic Sensitivity Testing (AST) guidelines will be presented here for the microorganisms mentioned in this article, which are important due to their clinical relevance and their ability to become multiresistant through efflux mechanisms. A description of these treatment standards is necessary as the possibility of efflux inhibition/reversal of resistance using non-antibiotics presupposes a definition of the given treatment choices and the corresponding resistances. Antibiotics defined as substrates for the efflux mechanisms will in the following be marked in blue. Antibiotics suspected as being substrates for efflux are marked green.

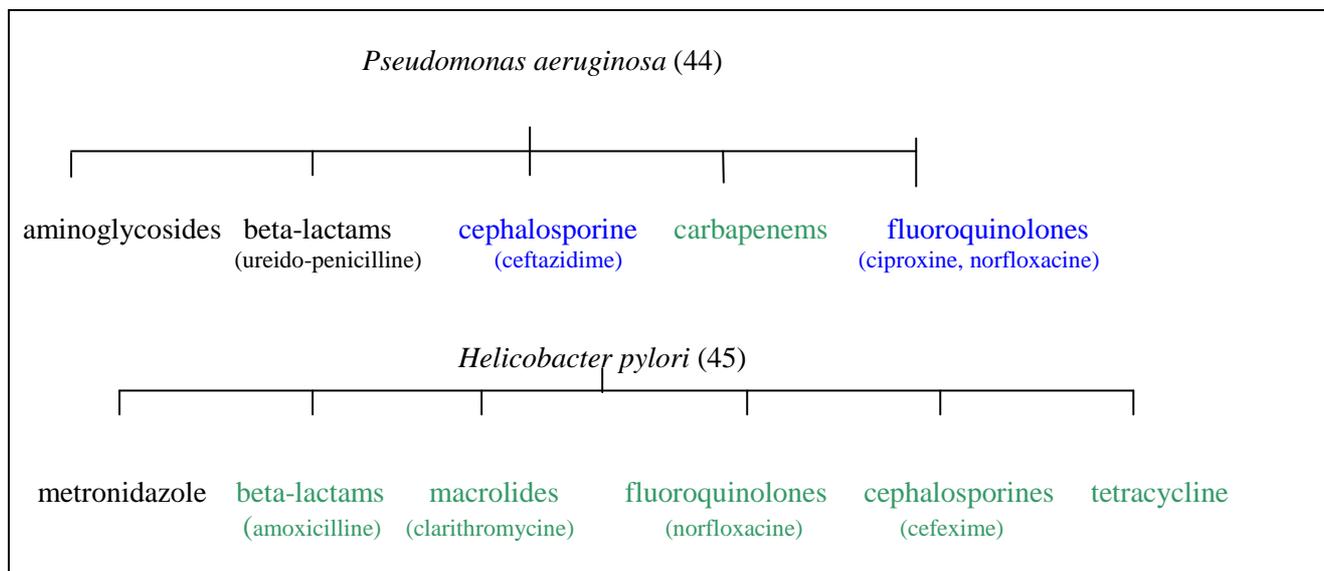
Only those microorganisms presented in the following are included in this review.

Gram-positive bacteria. (EARSS and NCCLS guidelines)

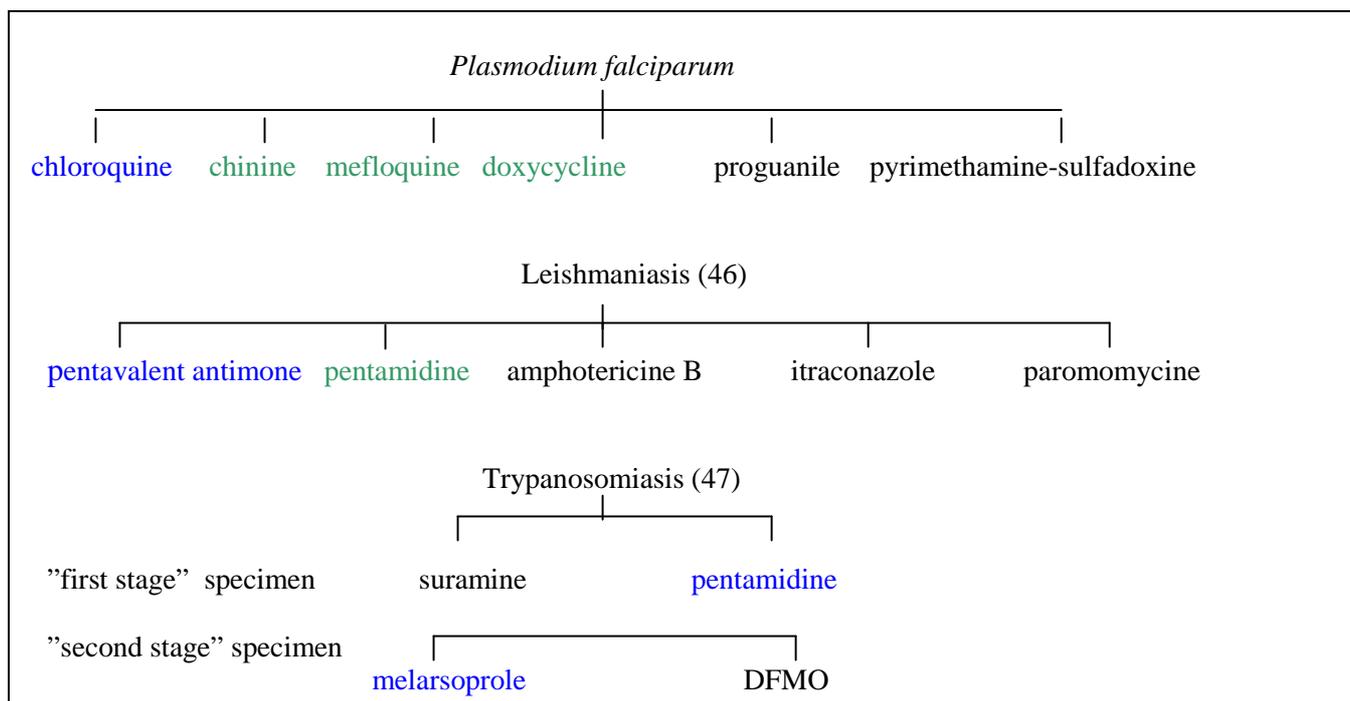


When testing for resistance, this is performed with several substances. (43).

### Gram-negative bacteria (NCCLS, EARSS)



### Parasites (WHO)



In conclusion, the presented abbreviated treatment for the illustrated micro-organisms strongly involves the effectiveness of bacterial efflux/microbial efflux, thus indicating the potential and the need of developing efflux inhibitors.

## Current research goals

### 3.1 The concept of non-antibiotics

The results of scattered observations since the middle of the last century appear to imply that certain eukaryote-directed designed drugs e.g. phenothiazines and chemical related compounds used as psychopharmacological drugs possess properties quite different from their original purpose.

Although they are not designed as antibiotics or chemotherapeutics they exert, for example, antimicrobial, antipathogenetic, antivirulence effects on micro organisms. Such compounds are called non-antibiotics. The concept is elucidated in [figure 6](#).

The main research goals of our group have been:

- to identify unrecognised antimicrobial/resistance combating properties of non-antibiotics belonging to the group of compounds: “neurotropics and their stereo-isomeric analogues”
- to strengthen the antimicrobial/resistance combating and/or reduce/delete the eventual psychopharmacological/toxic properties of the compounds by the help of stereo-isomeric manipulation
- use the resulting compound selectively as an antimicrobial/antiresistance combating agent in the treatment of the specific infection in parts of the body, where it is possible to reach the necessary concentrations

The research has been directed especially against serious MDR-resistant micro organisms in extra- and intracellular infections, which are difficult to treat today.

[Table 2](#) shows the Minimum Inhibition Concentrations (MIC) for a panel of phenothiazines on Gram-positive bacteria. It is noted that although the responses of all of the strains tested was essentially similar for each of the compounds to which they were challenged, the most effective compounds demonstrating greatest activity were the different chemical forms of thioridazine. The clinical significance of the growth inhibiting effect is primarily shown in concentrations of the drug that exceeds the extra-cellular-concentrations, especially in serum. Anyway, the growth inhibiting concentrations and the concentrations need for reversal of resistance of many of these non-antibiotics can be practically reached in specific human organs such as the urinary tract, in the lungs and on the skin (48).

### 3.2. Non-antibiotics and Gram-positive bacteria.

Derivatives of phenothiazines exhibit a direct growth inhibiting effect on *S. aureus* and other Gram-positive bacteria (49). More important is that the above mentioned non-antibiotics (e.g. phenothiazines and their stereoisomeric analogues) furthermore possess an antimicrobial activity that can reverse an already established resistance (22,23,50,51,52,53). The reversing effect of phenothiazine derivatives on methicillin resistant *S. aureus* (MRSA) and erythromycin resistant *S. pyogenes* are presented in table 3a and 3b. Interestingly, the reversal of oxacillin- and erythromycin-resistance, by phenothiazines can be seen at concentrations much lower than needed for the actual growth inhibiting antimicrobial effect of the same compounds, an observation of potential important clinical relevance (54,55,56). Furthermore it has been demonstrated that efflux mechanisms in Gram-positive bacteria are directly influenced by non-antibiotics:

Kaatz *et al.* demonstrated that a panel of phenothiazine derivatives as well as the chemically related thioxanthenes inhibit the NorA mediated fluoroquinolone efflux in *S. aureus* (57). Similar results can be achieved by the phenylpiperidines, another group of neurotropic compounds characterized as serotonin-reuptake inhibitor. These results are presented in table 4 (58).

The antimicrobial activity of the neurotropic compounds is of special interest because these compounds are concentrated in the cells and it might be expected that the substances play a role in the treatment of serious intracellular infections.

The intracellular localization plays a clinically important role in infections e.g. caused by *S. pyogenes* and in certain *S. aureus* and *S. epidermidis* infections and in *M. tuberculosis*.

In 2002, Ordway *et al.* demonstrated how the phenothiazine thioridazine (presented in table 4) at a concentration of 0.1  $\mu$ g/ml inhibited intracellular growth of *S. aureus* in human macrophages. The MIC value for thioridazine in the study was defined as 18  $\mu$ g/ml, which corresponds with the value found in the table 2.

The results cause some immediate confusion as it may be deduced that:

- phenothiazine derivatives reduce efflux mediated fluoroquinolone resistance in vitro
- the same derivatives reduce oxacillin resistance in vitro and in an intracellular model
- oxacillin resistance is defined as a target alteration through PBP2a acquisition

It may be concluded that the effect of the phenothiazines at present must be viewed as multifunctional: even as they block the NorA related efflux (57) they also block a non-NorA-related efflux phenotype in a concentration-dependent manner. It may be that, the results could lead to the conclusion that this group of compounds interacts with both active bacterial efflux mechanisms and/or that the efflux mechanisms in a not as yet elucidated way may be implicated in the methicillin resistance (59). Alternatively, a depolarisation (19, 21) of the bacterial membrane and consecutive disruption (20) of both peptidoglycan-synthetic enzymes and efflux pump activity may be a mode of action of the phenothiazines.

A multifactorial influence on different resistance mechanisms may be involved in these, since resistance development is often a molecular mechanistic multifactorial complex.

The above described observations that phenothiazines modify the resistance against even very different conventional antibiotics could be confirmed by an analogue experiment involving *Enterococcus faecalis*. The vancomycin and ampicillin resistance are also influenced by help of phenothiazines. It is of special interest that the same compounds mentioned above, thioridazine, can cause reversal of both vancomycin and ampicillin resistance in *E. faecalis*.

Table [5a](#) and [5b](#) show the influence of phenothiazine derivatives thioridazine and prochlorperazine on ampicillin and vancomycin resistant *E. faecalis*. Thioridazine has the most potent efflux inhibitory effect among the phenothiazines on the Gram positive micro organisms investigated. How thioridazine exerts its molecular influence is still unclear, although an influence on a putative efflux systems is suspected (60).

### 3.3. Non-antibiotics and mycobacteria.

*M. tuberculosis*, the cause of tuberculosis, is notoriously one of the most difficult diseases to find an effective chemotherapeutical/antibiotic treatment for. Multiresistant strains of tuberculosis became a concern in the late 1980's, and particularly in conjunction with the outbreaks of HIV and AIDS epidemics.

The chemotherapeuticals of the past were reassessed and renewed interest into the earlier investigations with the phenothiazines were seen (61, 62). The phenothiazines and the chemically related thioxanthenes were in 1985 thoroughly examined against typical and atypical mycobacteria, both resistant and sensitive strains.

The work of Crowle *et al.* from 1992 demonstrated clearly that it was possible to obtain non-toxic concentrations of phenothiazines in the lungs, concentrations which were absolutely able to eliminate the *M. tuberculosis* present in the lungs (54). This confirmed previous in vivo results (61). It is here interesting to note that Manion *et al.* as early as 1969 described how the development of resistance in mycobacteria against isoniazid could be delayed by phenothiazine and quinacrine (52). At the time, not enough was known about efflux inhibition or direct inhibition of a membrane sensor (quorum sensing) to connect this to the effect of the membrane-stabilizers on the cell membrane of mycobacterium. Amaral *et al.* and others (62) have recently investigated intensively thioridazine in sensitive and MDR *M. tuberculosis* and MDR *S. aureus* *in vitro* and intracellular and have confirmed the importance of phenothiazines in seriously Gram positive infectious disease.

#### 3.4. Non-antibiotics and Gram-negative bacteria. .

Köhler (personal communication) has in his efflux shown reversal of resistance in tetracycline (MIC 64 µg/ml) resistant *P. aeruginosa* with fluphenazine. It was shown that it was possible to lower the MIC from 64 µg/ml to 4 µg/ml tetracycline. The results found here illustrated the tested phenothiazines, especially fluphenazine being as potent as PHE-ARG-β-naphthylamide, PAN (63). It is expected that among the phenothiazines and related compounds there might be found even better derivatives to influence other antibiotics against which *P. aeruginosa* can develop resistance. These investigations open further possibilities for reversal of resistance in different MDR resistant Gram negative microorganisms by the help of different phenothiazine derivatives. Chen *et al.* described in 2002 that nizatidine, an H<sub>2</sub>-receptor antagonist, is able to reduce the susceptibility of *H. pylori* to metronidazole in vitro, despite having no direct inhibitory effect on the growth of *H. pylori* (64). This agrees with earlier findings that compounds having the ability to reverse antibiotic resistance do not necessarily have an antibiotic or chemotherapeutic effect in the sense of growth inhibition. In addition it could be shown that nizatidine inhibited fumarate reductase in a dose-dependent way, like metronidazole, whereas omeprazole had almost no effect on fumarate reductase. The synergistic effect of nizatidine on metronidazole resistant *H. pylori* strains could be explained by the effect on fumarate reductase, whereas the effect of omeprazole was suggested to be due to an inhibition of a proton pump in *H. pylori*. Reversal of antimicrobial resistance by help of different non-antibiotics seems to be possible by using quite different compounds, and is therefore to be explained by different molecular mechanisms, developed in microorganisms to avoid the use of classical antibiotics/chemotherapeutics as demonstrated in figure 5 (65).

Also among natural compounds such as plant extracts a lot of work has been done looking for new efflux inhibitors but only one example is mentioned here (66): Lewis, Lomovskaya and coworkers demonstrated in 2002 that the application of MDR inhibitors increased the antimicrobial activity of plant extracts, such as Rhein significantly from being completely ineffective against *P. aeruginosa* with a MIC-value  $> 500 \mu\text{g/ml}$ , but in combination with efflux inhibitors potentiate the activity of Rhein 100-fold, resulting in a MIC of  $5 \mu\text{g/ml}$ . Quite different chemical structures among synthetic and natural compounds are able to influence efflux in Gram negative microorganisms. It is promising that the phenothiazines are able to influence resistance in Gram negative microorganisms and Gram positive too.

### 3.5. Non-antibiotics and resistant parasite infections.

The reversal of resistance in plasmodium using methylene blue was suggested by Guttman and Ehrlich as early as 1891 (67). Also the more recently developed phenothiazines and the chemically related thioxanthenes possess antimalarial activity (68). It is interesting to note that the use of modern neuroleptic drugs on chloroquine resistant *P. falciparum* answers a question proposed in 1891. It is possible to study the reversal of the resistance in *P. falciparum* in vitro and in vivo with the use of different efflux inhibitors such as chlorpromazine (69), promethazine (70) and verapamil (71).

Derivatives of e.g. phenothiazine and reserpine are capable of reversing drug resistance of mammalian cells. Each of these compounds has potential use against leishmania parasites. Essodaigui and coworkers reported the inhibition of an efflux system by two phenothiazines derivatives, thioridazine and chlorpromazine on three leishmania species: *L. braziliensis*, *L. guyanensis* and *L. mexicana* (72). The treatment of trypanosomiasis by another brilliant example for the use of non-antibiotics: Trypanocidal drugs used today were developed from synthetic dyes, organic arsenicals, or diamidines. The development of synthetic dyes against African trypanosomes was initiated by Paul Ehrlich in 1907 (73). By performing staining reactions with acridine dyes, he could demonstrate that drug-resistant trypanosomes did not stain as readily as sensitive ones. The compound suramin, a symmetrical, urea-linked benzoyl analog of Trypan red, is still used for the treatment of early-stage sleeping sickness. The cellular target of suramin still remains unknown (74).

### 3.6. Non-antibiotics and cancer.

Insufficiency of chemotherapeutics is interpreted as a result of factors concerning both the entire human organism (absorption, metabolism, elimination of the drug, etc.) and the individual cancer cell (75).

Cellular drug resistance is one important reason for treatment failure. P-glycoprotein (PGP) mediated multidrug resistance (MDR) was identified in the early 1970's (76) and characterised by cross resistance between unrelated drugs, increased expression of PGP and reversal of the phenotypic resistance by a variety of different compounds (77). PGP is a member of the ABC transporters. As mentioned in table 2a, this family includes a variety of bacterial efflux transporters as well as the *P. falciparum* resistance mechanism. The structural similarity between bacterial efflux and efflux in cancer cells led to the extension of the hypothetical use of non-antibiotics as "helper compound" in chemotherapy. Molnár and co-workers demonstrated the activity of different classes of non-antibiotics as possible resistance modifiers in cancer chemotherapy (78). In vivo investigations with prochlorperazine as a "helper compounds" is already in progress.

### 3.7 Expectations and ultimate goals: Selected non-antibiotics and their stereoisomeric analogs

A promising group of non-antibiotic “helper” drugs are under investigation in the author’s laboratory, with the aim of using them to reverse, remove or inhibit resistance in order to extend the use of the conventional antibiotics. The drugs presented in Table 4 are neurotropics, i.e. they are defined as a group by their affinity to the peripheral and/or central nervous system. Our research has focused on stereo-chemistry, with the view of exploiting the so-called side effects of known psychotropic drugs, while removing or minimizing the psychotropic activity, which was the original primary use of the drug (79, 80, 81, 82).

This has been possible by using the +/- stereochemistry in the phenothiazines, the cis/trans stereochemistry in the thioxanthenes and the cis +/-, trans +/- stereo-chemistry in the phenylpiperidines (see Scientific rationale). So far the antimicrobial effect has been reduced successfully, whilst retaining the efflux inhibiting effect of selected chemical substances. It might be of interest to find compounds having as low potency as possible for growth inhibition on microorganisms and still have a strong efflux inhibitory activity. The index of efflux inhibition/antimicrobial activity of an investigated compound will then be as small as possible. The idea is that resistance development from the cell systems under influence of chemically stress might then be very low for these new “helper compounds”.

To address the problem of the development of the resistance and the reversal of it, research has turned to increasing the efficiency of the targeting molecules by incorporating them into dendrimers (a class of synthetic macromolecules).

## 4. Scientific rationale

For elucidating the usefulness of the different stereo-chemical possibilities of different psychotherapeutics to be developed for minimizing the CNS activity and maximizing the antimicrobial activity in modern neurotropics we have investigated the antimicrobial activity and the anti resistance activities especially of the phenothiazines, the chemically related thioxanthenes and the phenylpiperidines including different stereoisomeric analogs in each group.(see Table 6).

### 4.4.1. Phenothiazines.

Phenothiazine is a tricyclic ring system with sulfur- and a nitrogen atom bridging the middle ring. One of the earliest known derivatives of phenothiazine was methylene blue, one of the early dyes, first tested by Ehrlich on the peripheral and central nerve system, and later against malaria. More complicated derivatives of the phenothiazines were introduced in the 1950's for the treatment of mental disorders. Some of these compounds, shown in Table X has been investigated by our group with respect to antimicrobial activity and reversal of resistance. Promethazine and thioridazine are compounds, existing in two mirror image forms. Such enantiomers, exhibit chirality (chiro means "hand" in Greek) due the lack of symmetry in the molecule usually at a carbon atom bound to four different substituents. The chiralcenter in the molecules in the table is marked with an "\*". Enantiomers have the same physical properties except for their interaction with polarized light, leading to rotation of the plane of polarization. This property is used for characterization of chiral compounds, and the direction of the rotation is given by (+) for right-rotation and (-) for left, (anti- and clockwise rotation) respectively. The sum of the degree of polarization of the enantiomers may be zero for a racemic mixture (83).

The phenomenon of chirality has been known since the work on tartaric acids by Pasteur (1848), but the importance of chirality in connection with biological systems was first really recognized in the 1960's with the discovery of the toxic side effects of thalidomide, which was marketed as a racemic mixture. There are in many other differences in the activity or side effects of two enantiomers of a drug, but especially in the case of the older drugs, marketed as racemic mixtures. Classical organic synthesis will in most cases give a racemic mixture. Separation of a racemic mixture is costly because 50% of the material will be the unwanted enantiomer to be discarded unless it is possible to convert it into either the desired chiral compound or the racemate. The drugs promethazine and thioridazine are stereo chemical mixtures.

In the thioridazine mixture the – compound has the lowest potency on the brain and all three chiral compounds rac, +, - are equal in antimicrobial activity. These compounds are among the most potent of the phenothiazine group. Selection of the – compound of thioridazine as an antimicrobial compound the side effect on the brain will be minimized.

#### 4.4.2. Thioxanthenes

The thioxanthenes differ from phenothiazines by having a carbon atom instead of a nitrogen atom in the central ring, to which the side chain is attached by a double bond. As a result thioxanthenes have two geometric-isomeric forms, one of which has the side chain on the same side as the substituted benzene ring (cis- or Z-isomerism) while the other has the side chain on the other side of the substituted benzene ring (trans- or E-isomerism). The thioxanthenes examined are mainly pure trans- or cis- compounds. However, thioxanthenes are also found to be mixtures. Trans-/cis-CPT (chlorprothixene) sulfoxide is an exception in this respect. Since the S atom is trisubstituted, and has a lone-pair of electrons, it constitutes a chiral center giving rise to two enantiomers for each of the two cis/trans-isomers. (Niels Lassen, Lundbeck A/S, personal communication) (103,104).

The CNS activity of the thioxanthenes is linked to the cis (Z) compounds.

The trans (E)-compounds of the thioxanthenes are of interest for antimicrobial activity. The antimicrobial activity of the trans (E)-compounds are at least double potency of the cis(Z) compounds and the trans (E)- compounds are without or little CNS activity.

#### 4.4.3. Phenylpiperidines

A third type of psychotherapeutic drug (with antidepressant effects) is the series of 4-Phenylpiperidines, femoxetine and paroxetine and their three stereo-isomeric analogues. These drugs inhibit the re-uptake of serotonin, which inhibition is presumably associated with the antidepressant effect, while no neuroleptic activity is observed (Mogens Engelstoft, Ferrosan A/S, personal communication). For example femoxetine and paroxetine have two chiral centers and can therefore be found in four stereo-isomeric forms: (+) trans, (-) trans, (+)cis and (-) cis. The following illustration presents two of each. In the phenylpiperidines it is also possible to minimize the CNS activity and strengthen the antimicrobial and antiresistance activity.

## Conclusion:

The chemical groups investigated describe the pharmacological possibilities in a more general manner, but will lead to the best stereo isomeric analogue for further development as a new class of compounds (drugs) for antimicrobial/reversal of resistance, with as low potency as possible on the brain. To search for the desired pharmacological actions in an already established drug with as low toxicity as possible and a low activity on the brain, and a high antireversal potency on micro organisms holds promise. The procedure for development of the drug developing should be easier because of the already available toxicological data of the selected chemical structure.

### 4.5. A new anti reversal drug -Thioridazine:

Theoretically and practically it is possible to reduce the neurotropic activity of a neurotropic compound by stereo chemical modulation. The drug thioridazine consists of three compounds: the racemate, a + compound characterized by a higher neurotropic activity compared to the – compound. It is here very important to point out that the antimicrobial activity, e.g. a growth inhibitory activity and a reversal of resistance activity is found in all 3 compounds.

The pharmacological selection of these compounds is based on a reduction of the neurotropic activity: selection of the – compound in order to get rid or minimize the neurotropic activity.

Detailed studies with thioridazine and its enantiomers have been performed in an attempt to define the properties of these substances in the context of reversal of drug resistance, more specifically reversal of resistance to antimicrobial agents, such as antibacterial drugs.

Furthermore these investigations were performed in order to evaluate the hypothetical implication of efflux inhibition as an optional mode of action.

Interaction of compounds with antibiotics is analyzed in established microbiological models, such as agar- and microdilution. Under certain experimental settings, racemic or enantiopure thioridazine displayed equipotent bacteriostatic or bactericidal properties when given alone or in combination with traditional antibiotics. Invariably, and surprisingly, racemic or enantiopure thioridazine acted in a synergistic manner to lower the MIC of the other antibiotic.

Under certain bacterial strains and/or when combined with specific antibiotic drugs, one of the enantiomers showed superior activity. For example, the levorotatory enantiomer was more efficient in reversing resistance towards erythromycin in *Streptococcus* strains (56). Incidentally, the levorotatory enantiomer has earlier been reported to display less challenging CNS pharmacodynamic activity, e. g. weaker blockade of dopamine D2-receptors, than the

dextrorotatory enantiomer (87). Furthermore, the levorotatory enantiomer has been reported to be the compound that is concentrated in human tissue at higher levels than the dextrorotatory form (88). Taken together, these facts suggest that the levorotatory form of thioridazine should clearly be superior in the context of reversal of drug resistance due to potentially fewer side effects.

We have used an intracellular infection model to study the effect of non-antibiotics and combinations of non-antibiotics and antibiotics on intracellular located *S. aureus* in macrophages. The model is extended by the application of different epithelial cell lines in order to study the effect on invasive pathogens, such as *Klebsiella pneumoniae*, *Salmonella typhimurium* and *S. pyogenes*. Strains to be investigated are chosen on the background of high level antibiotic resistance based on the existence of well described efflux pumps. Preliminary results indicate reduction of invasion capacity of both Gram-positive and Gram-negative bacteria in different epithelial cell lines (55).

Compounds that appear to demonstrate promising antibiotic properties, in the sense of direct anti-microbial effect, reversal of resistance or only effect on efflux-related resistance will be tested in relevant animal models. Furthermore it is planned to investigate the mode of action of our test-compounds: Based on the hypothesis of an influence of non-antibiotics at the level of genetic transcription, the quantitative expression of genes coding for MDR efflux pumps will be investigated. Analysis of the antibacterial effect of non-antibiotics on the level on the gene is to be performed by RT-PCR methods. Based on the hypothesis of an influence of non-antibiotics on the bacterial membrane, we are creating a mathematical model in order to define the effect on selected membrane structures, e.g. PBP's, utilizing photo-optical nano-techniques.

The mathematical model might help us not only being descriptive but also to try to understand the function in the reversal of resistance systems in cells. By knowing more about how the neurotropic compounds interfere with the resistance mechanisms in microorganisms it seems possible to find a common molecular tool target in the cell systems to cure resistance.

It is interesting that different chemical structures developed for treatment of psychiatric diseases can interfere with different resistance mechanisms in microorganisms (bacteria, protozoae and even in tumor cells). It is to be expected that the interference might be on the cell membranes and the energy systems. The transport proteins and the energy systems in the different efflux super families in the different cell systems seem to be more specific than expected.

The hope and goal for us is utilizing already known non-antibiotics especially among the neurotropic compounds for further development. Especially the stereo isomeric compounds of known drugs are important tools for further investigations in reversal of resistance both *in vitro* and *in vivo*.

It may be possible to find different groups of “helper compounds” which might be classified to interfere with the transporter super families. These helper compounds may be used together with an antibiotic/chemotherapeutic from the beginning of a treatment to overcome resistance development.

In the mean time the new possibilities for reversal of resistance by using stereo isomeric analogues of known drugs (by avoiding the main pharmacological activity of the neuroleptic drugs) could help us in the battle against resistance. The development and the possibilities for using “helper compounds” together with the classical antibiotics seem to be in reach.

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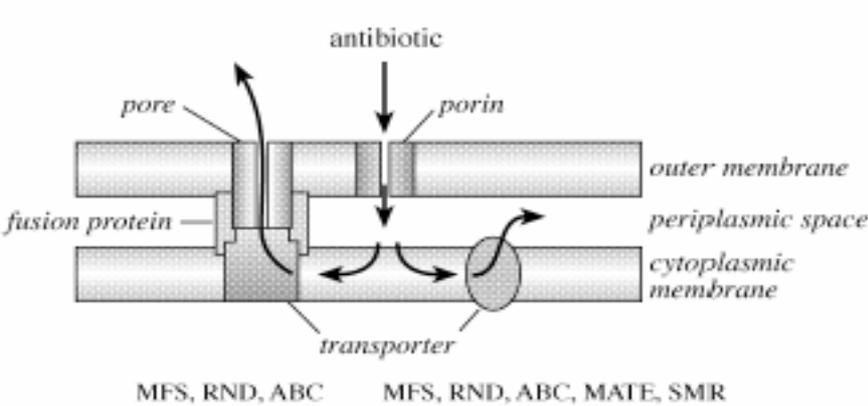
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Figures and Tables

Figure 1.

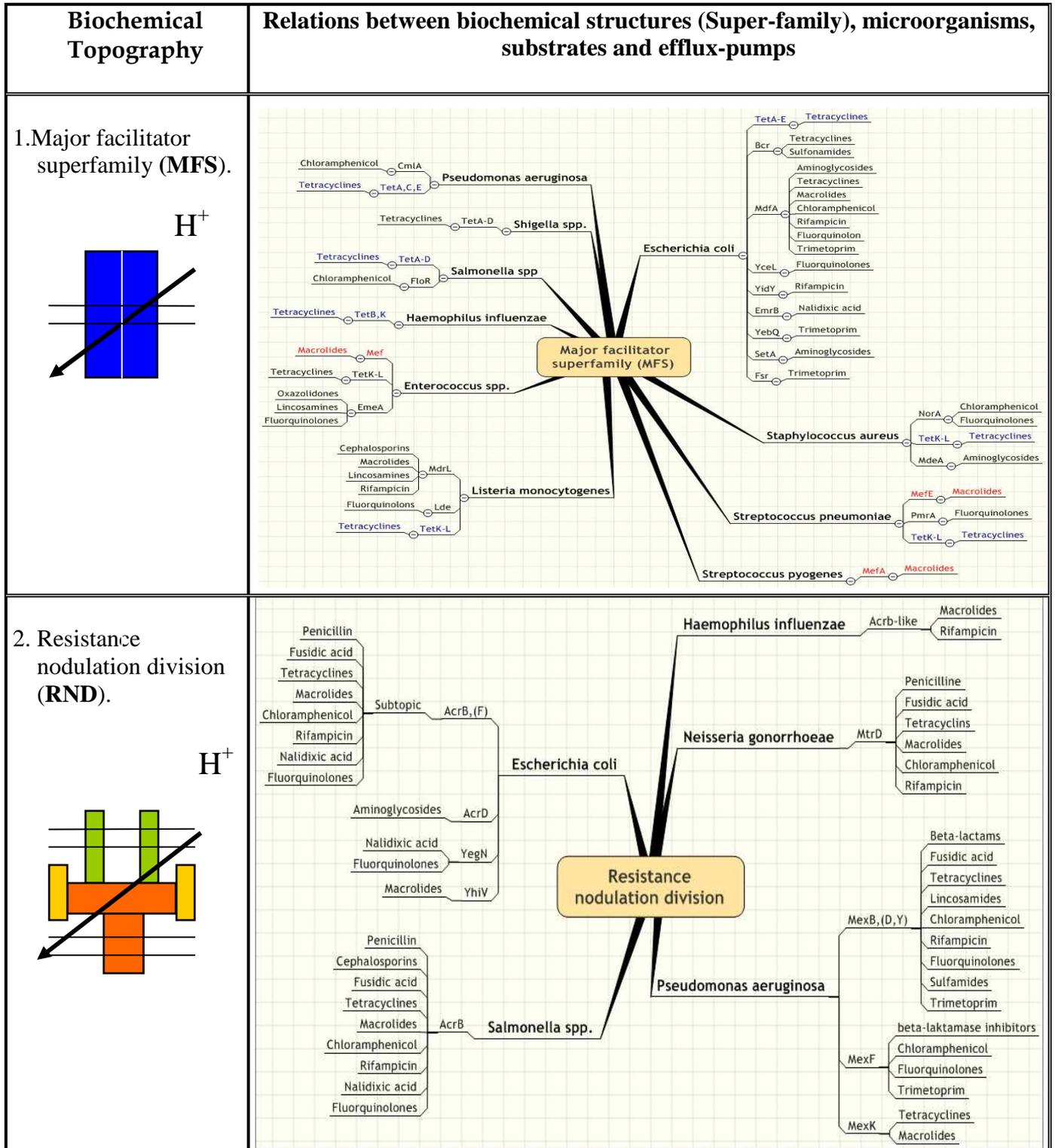
The topology of superfamily efflux pumps



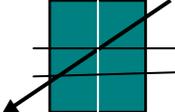
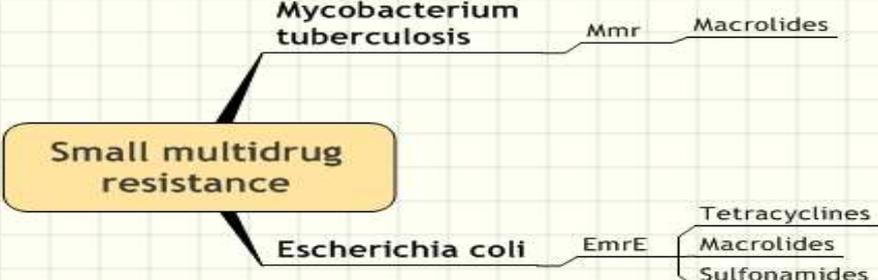
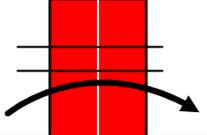
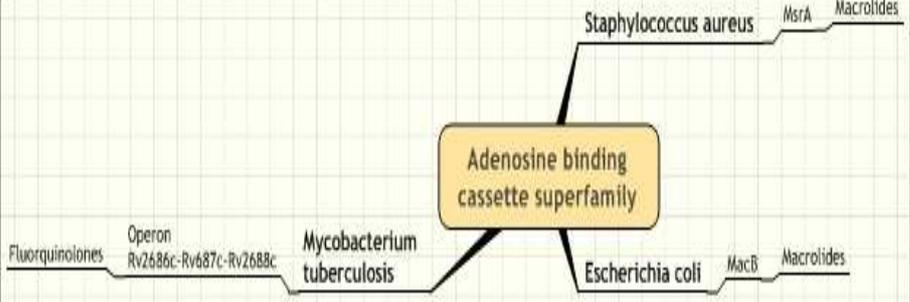
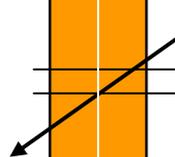
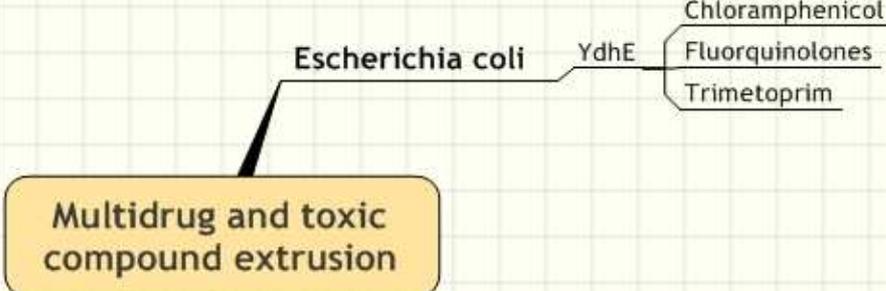
**Figure 2.**

Anti-bacterial resistance sorted by microbiological efflux systems. A road map for the identification of the antimicrobial activity of phenothiazines as potential efflux inhibitors.

2 a. MFS and RND systems



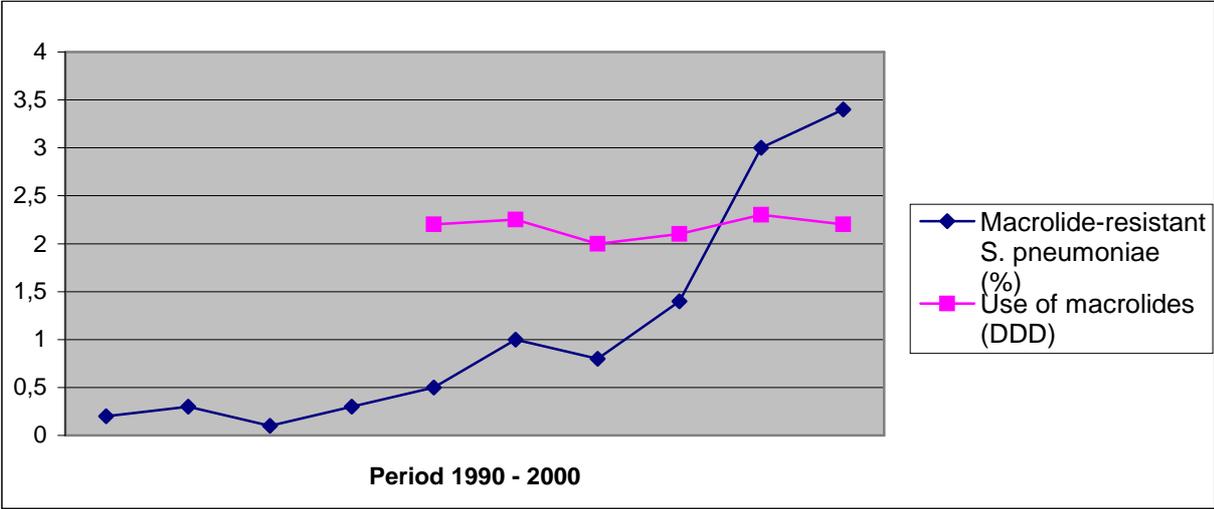
2b. SMR, ABC and MATE systems

Biochemical Topography	Relations between biochemical structures (Super-family), microorganisms, substrates and efflux-pumps
<p>3. Small multidrug resistance (SMR). <math>H^+</math></p> 	
<p>4. Adenosine binding cassette super family (ABC).</p> 	
<p>5. Multidrug and toxic compound extrusion (MATE). <math>H^+</math></p> 	



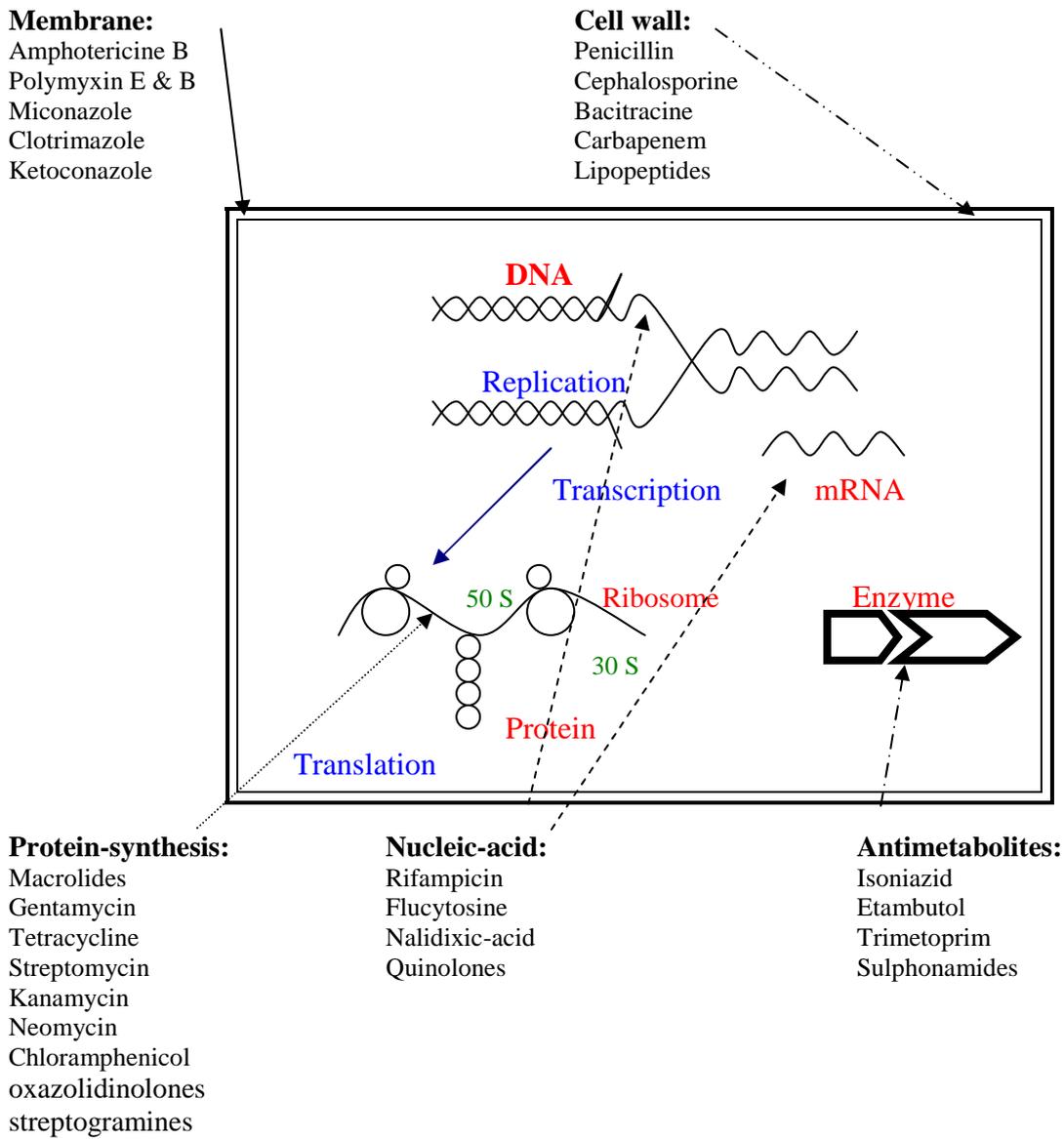
**Figure 4.**

Development of macrolide consumption and macrolide-resistance in *S. pneumoniae* in Denmark



**Figure 5.**

Antibiotics and targets.

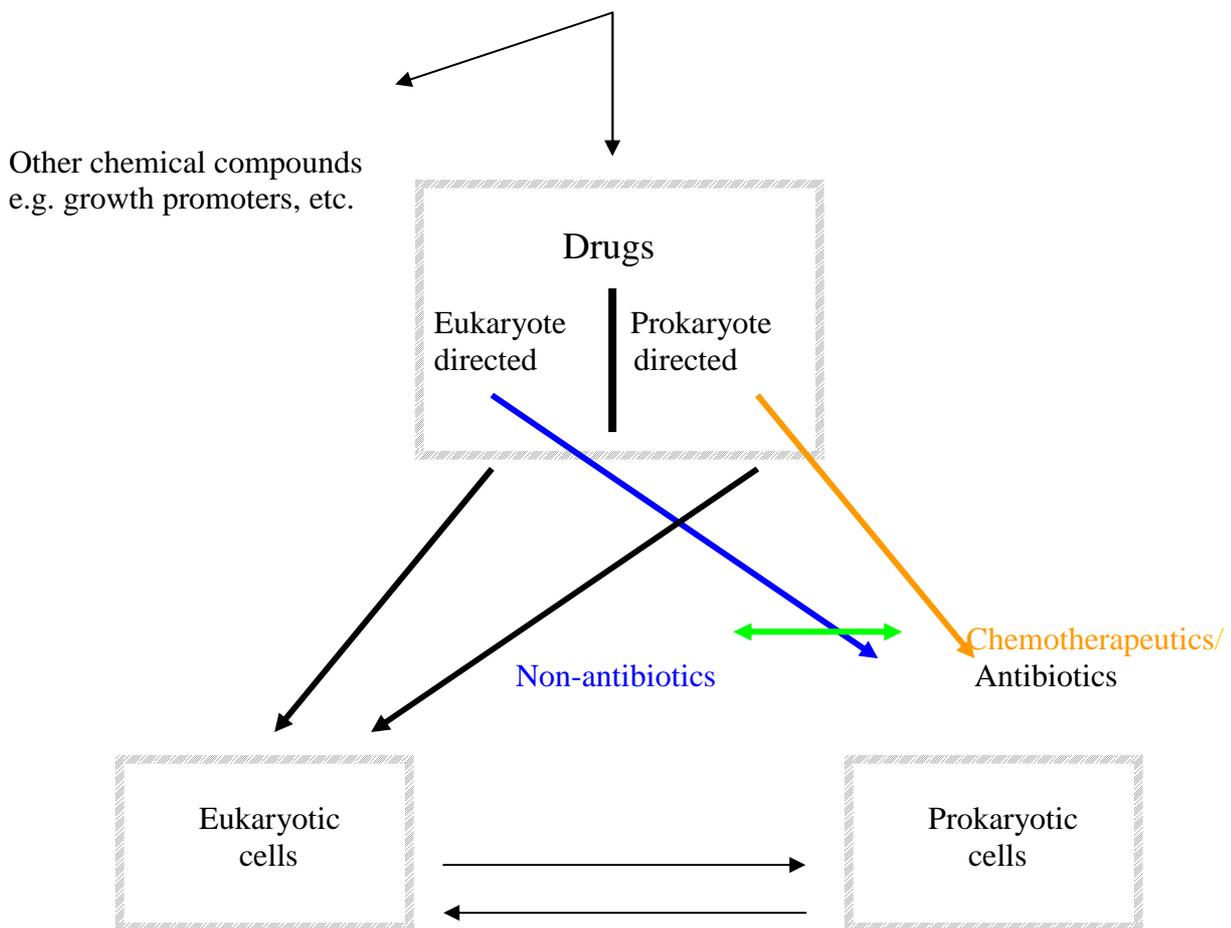


**Figure 6:**

Classification of compounds according to their action on different biological systems:

The concept of non-antibiotics

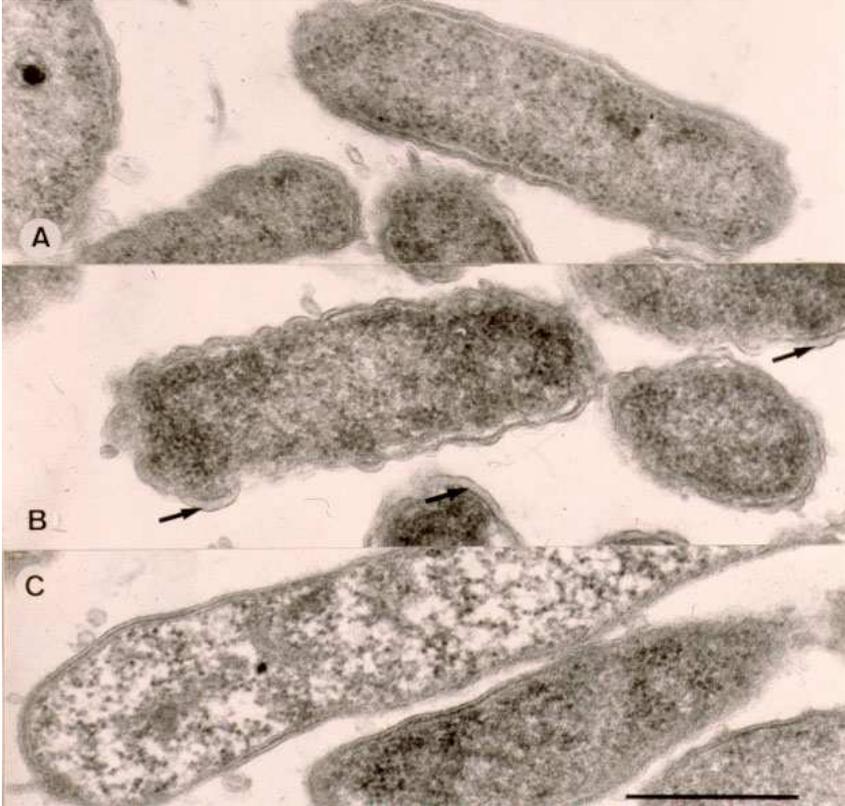
Compounds which are chemically foreign or hostile to a biological system



( J.E. Kristiansen 1990)

**Figure 7.**

Influence of Omeprazole on *H. pylori* strain Cag A Hp 007.





## Figure Legends

### Figure 1:

The topology of Superfamily efflux pumps

The topology of multi component (left) and mono component (right) superfamily efflux pumps like the Major facilitator superfamily (MFS), Resistance nodulation division (RND) and adenosine binding cassette (ABC) super family, Multidrug and toxic compound extrusion (MATE) and Small multidrug resistance (SMR) superfamily. The multi component efflux pumps are specific to the Gram negative bacteria as their membrane structure allows for the extrusion of chemotherapeutics/ antibiotics straight into the extra cellular media. The arrows indicate the direction of the substrate transport.

### Figure 2:

Relation between biochemical structures (Super-family), microorganisms, substrates and efflux-pumps

The topology of superfamily efflux pumps MFS, RND, SMR, ABC and MATE are related to identified efflux pumps. Efflux pumps are sorted by bacterial species in order to visualize the degree of multiresistance in different bacterial species.

### Figure 3:

Proportionale geographical variation of methicillin resistant *Staphylococcus aureus*.

Figure 3 shows the proportion of MRSA as percentage.

Source is the results of hospital antimicrobial susceptibility tests of *S. aureus* blood isolates from 1999 to 2002 in Europe recorded by the EARSS.

### Figure 4:

Figure 4 shows the proportion of macrolide-resistant *S. pneumoniae* as percentage.

Source are the results of *S. pneumoniae* isolates from blood and spinal punctures in Denmark in the period from 1990 – 1999 according to data from the States Serum Institute, Epi-news, week 4, 2001.

The consumption of macrolide antibiotics is given for the period 1995 –1999 (Defined Daily Dose (DDD) / 10.000 inhabitants).

**Figure 5:**

## Antibiotics and targets

The mode of action of antibiotics in accordance with bacterial targets

- cell membrane
- cell wall
- protein synthesis
- nucleid acid
- antimetabolites

**Figure 6:**

## The concept of non-antibiotics

Classification of compounds relates the technical term "non-antibiotics" to the general conception that determines the mode of action of any compound according to the action on different biological systems. The hypothetical synergy and reversal of resistance between non-antibiotics and antibiotics is illustrated.

**Figure 7.**

Representative electron micrographs of *H. pylori* strain Cag A Hp 007 grown for 24 hours on 7 % lysed horse agar plates without (A) and with 3.2 µg/ml and 100 µg/ml of omeprazol (B, C).

- A. show several *H. pylori* profiles with two tightly adjacent layers in the outer plasma membrane.
- B. show profiles of *H. pylori* bacteria with dilation between the layers in the outer membrane (arrows). The periplasmic space is also dilated.
- C. show two profiles of *H. pylori* bacteria with no ultrastructural changes in the plasma membrane or in the periplasmic space. The cytoplasm of one of the cells is characterized by edema.

**Figure 8:**

The efflux protein HP 1092 has also been successfully purified and a model of its possible topology has been constructed.

**Table 1.**

Relative change in MRSA proportion per country per year and 95% confidence intervals (CI) as calculated from a Poisson regression model.

Country	reported % MRSA at start	reported % MRSA in 2002	relative change per year, ratio	95% CI of estimated change	p-value
Austria	7.0	7.6	0.80	0.48-1.34	0.39
Belgium	22.1	27.2	1.25	1.12-1.41	< 0.01
Bulgaria	35.1	37.7	1.11	0.59-2.09	0.76
Czech Republic	4.5	6.2	1.15	0.89-1.50	0.29
Denmark	0.3	1.0	1.64	0.97-2.75	0.06
Finland	1.5	0.8	0.69	0.43-1.11	0.13
Germany	9.4	19.2	1.72	1.54-1.93	< 0.01
Greece	37.0	48.6	1.23	0.89-1.71	0.21
Iceland	0.0	0.0	0.52	0.07-3.67	0.51
Ireland	39.4	45.0	1.36	1.17-1.58	< 0.01
Italy	35.2	40.0	1.11	0.94-1.30	0.23
Luxembourg	15.0	18.3	1.09	0.71-1.67	0.70
Malta	34.7	42.5	1.58	0.92-2.74	0.10
Netherlands	0.4	1.0	1.62	1.01-2.58	0.04
Portugal	39.7	38.9	0.91	0.75-1.09	0.32
Slovenia	22.3	14.7	0.69	0.51-0.93	0.02
Spain	28.4	23.5	1.03	0.87-1.21	0.74
Sweden	1.1	0.7	0.95	0.73-1.23	0.68
United Kingdom	30.5	44.5	1.48	1.31-1.66	< 0.01

**Table 2.**

MIC-values of phenothiazine-derivatives for pathogenic Gram-positive species.

Phenothiazin- derivative	<u>S. pneumoniae</u> ( n = 10)	<u>S. pyogenes</u> ( n = 13)	<u>E. faecalis</u> ( n = 11)	<u>E. faecium</u> ( n = 9)	<u>S. aureus</u> ( n = 20)	<u>S. epidermidis</u> ( n = 4)
Chlorpromazine	8 – 16	8 – 32	64 – 128	64	32 – 64	16 – 32
Prochlorperazine	16 – 32	16 – 32	32 – 64	32 – 64	32 – 64	32
Thioridazine (+)	4 – 16	4 – 16	16	16	16 – 32	16
Thioridazine(-)	4 – 16	4 – 16	16	16	16 – 32	16
Thioridazine (rac)	4 – 16	4 – 16	16	16	16 – 32	16

Legend.

MIC in µg/ml, MIC values were defined by agardilution-techniques.

n: number of tested strains.

Ref:

**Table 3a.**

The influence of phenothiazine derivatives on methicilline resistant *S. aureus* (MRSA).

Compound/ combination of compounds	MIC
Oxacillin:	> 256
Thioridazine (rac), (+), (-)	32
Prochlorperazine	32- 64
Chlorpromazine	32- 64
Oxacillin + 8- 12 µg/ml Thioridazine (rac)	1- 4
Oxacillin + 8- 12 µg/ml Thioridazine (+), (-)	1- 8
Oxacillin + 8- 12 µg/ml Prochlorperazine	16-32
Oxacillin + 12 µg/ml Chlorpromazine	4-16

**Legend:** number of strains = 4.

MIC values were defined by Microdilution/checkerboard and E-test.

MIC in µg/ml each test was performed 4 fold.

**Table 3 b.**

The influence of phenothiazine derivatives on erythromycin-resistant *S. pyogenes*.

Compound/ combination of compounds	MIC
Erythromycin:	24
Thioridazine (rac), (+), (-)	16
Prochlorperazine	64 - 128
Chlorpromazine	32- 64
Erythromycin + 8 µg/ml Thioridazine (rac)	< 8
Erythromycin + 8 µg/ml Thioridazine (+)	< 8
Erythromycin + 12 µg/ml Thioridazine (-)	1
Erythromycin + 12 µg/ml Prochlorperazine	<i>no effect</i>
Erythromycin + 12 µg/ml Chlorpromazine	8-16

**Legend:** number of strains = 4.

MIC values were defined by microdilution/checkerboard and E-test.

MIC in µg/ml each test was performed 4 replicates

**Table 4**

The influence of phenylpiperidine derivatives on fluoroquinolone-resistant *S. aureus* (147,148).

Compound \ Strain	SA-1199 MIC in µg/ml	SA-1199B MIC in µg/ml
NNC 20-4962	500	500
NNC 20-4963 (= femoxetine)	1000	1000
NNC 20-7051	125	125
NNC 20-7052 (= paroxetine)	125	125
Reserpine	>100	>100
Norfloxacin	1.25	50

Effect of inhibitors on norfloxacin susceptibility of strain 1199 B (µg/ml).

No inhibitor	50
Reserpine	12,5 (4)
NNC 20-4962	12,5 (4)
NNC 20-4963 (= femoxetine)	6,3 (8)
NNC 20-7051 (= paroxetine)	6,3 (8)
NNC 20-7052	6,3 (8)

#### Strains

SA-1199: Clinical isolate, methicillin susceptible

SA-1199B: NorA-overproducing derivative of SA-1199 also has A116E GrIA substitution

**Table 5 a.**

The influence of the phenothiazine derivatives thioridazine (Thio rac) and prochlorperazine (PCP) on vancomycin resistant *Enterococcus faecalis* (VRE).

Strain Drug	A	C	D
Vancomycin	192	128	512
Thio <u>rac</u>	32	32	32
PCP	32	32	32
Vancomycin + Thio ( <u>rac</u> )	VAN (6) + THIO (8)	VAN (64) + THIO (4)	VAN (32) + THIO (8)
Vancomycin + PCP	VAN (48) + PCP (16)	VAN (64) + PCP (8)	VAN (32) + PCP (8)

**Legend:** number of strains = 3; Van: Vancomycin;  
THIO: Thioridazine, PCP : Prochlorperazine  
MIC values were obtained by Microdilution/ checkerboard and E-test  
MIC in µg/ml, each test was performed 4 times.  
Numbers in brackets  
present the specific MIC of each compound, when used in the checkerboard.

**Table 5 b.**

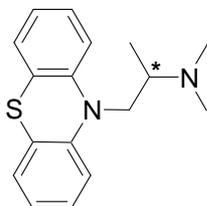
The influence of phenothiazine derivatives thioridazine (thio rac) and prochlorperazine (PCP) on ampicillin resistant *Enterococcus faecalis*.

Strain Drug	A	C	E
Ampicillin	32	16	128
Thio (rac)	32	32	32
PCP	32	32	32
Ampicillin + Thio (rac)	AMP (1) + THIO (16)	AMP (4) + THIO (4)	AMP (16) + THIO (8)
Ampicillin + PCP	AMP (32) + PCP(0.25)	AMP (8) + PCP (0.5)	AMP (64) + PCP (16)

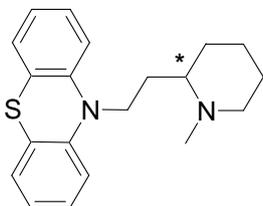
**Legend:** number of strains = 3; AMP: Ampicillin;  
THIO: Thioridazine, PCP: Prochlorperazine  
MIC values were defined by microdilution/ checkerboard and E-test  
MIC in µg/ml each test was performed 4 times.  
Numbers in brackets present the specific MIC of each compound, when used  
in the checkerboard test.

**Table 6.**

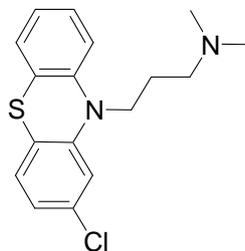
The psychotherapeutic drugs studied.



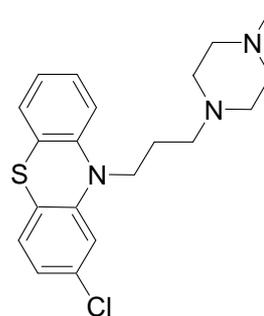
**1: Promethazine.**  
Registered in 1956.



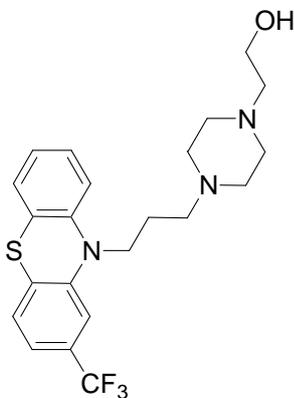
**2: Thioridazine.**  
Registered in 1958.



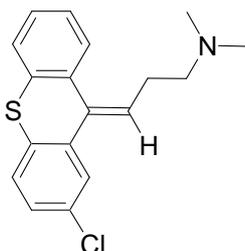
**3: Chlorpromazine.**  
Registered in 1956.



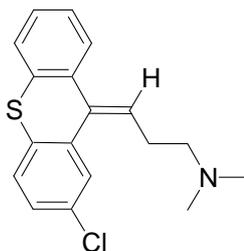
**4: Prochlorperazine.**  
Registered in 1956.



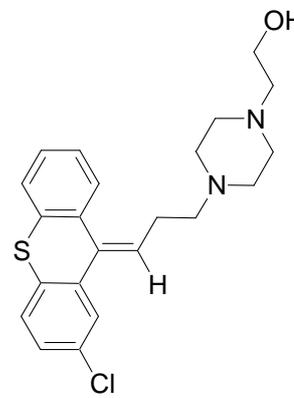
**5: Fluphenazine.**



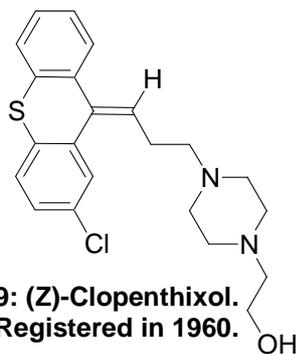
**6: (E)-Chlorprothixen.**  
Not registered.



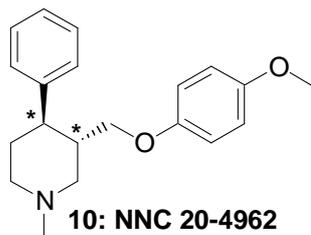
**7: (Z)-Chlorprothixen.**  
Registered in 1960.



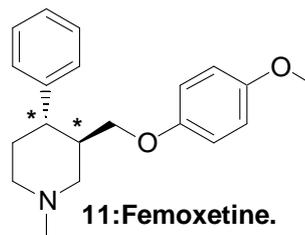
**8: (E)-Clopenthixen.**  
Not registered.



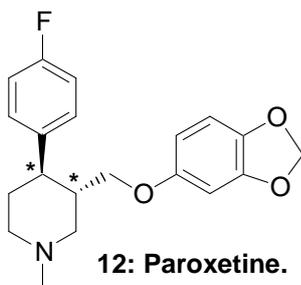
**9: (Z)-Clopenthixol.**  
Registered in 1960.



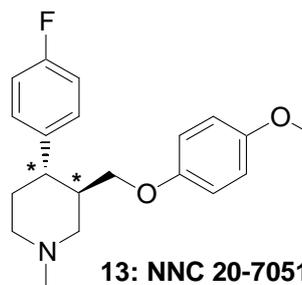
**10: NNC 20-4962**



**11: Femoxetine.**



**12: Paroxetine.**



**13: NNC 20-7051**

## Conclusion

The antimicrobial activity of nearly all neuroleptic drugs investigated so far seems to be quite different from that of most classic antimicrobial agents. It is possible to develop new antibiotics by structure-activity-based manipulations of non-antibiotic drugs to change their activity, but this approach is probably not easy. It might therefore be more interesting to develop "helper compounds"- and use non-antibiotics to act in cooperation with known antimicrobial agents. This strategy is characterized by an intriguing advantage, as the toxicological implications and the pharmacological criteria are already defined in well known compounds!

The reversal of resistant MRSA, streptococci, enterococci and *Plasmodium falciparum* with the aid of psychotherapeutic drugs and their analogs are examples mentioned above.

This phenomenon may have therapeutic significance, particular because psychotherapeutically nonactive analogs potentiate the activity of classic antibiotics against pathogenic species that cannot be inhibited by current drugs.

Can pathogenicity and virulence of microbes be reduced by psychotherapeutic drugs and their analogs? By using subinhibitory concentrations, this goal may be realized as in vitro investigations demonstrated in Gram positive-, Gram negative bacteria and parasites. Can "purer" drugs be developed by utilizing stereoisomeric specificity? The antimicrobial activity of psychopharmacological agents is independent of the neuroleptic effect. This finding raises the question of whether the antimicrobial effect can be reduced to create purer psychotherapeutic drugs, i.e., drugs with fewer side effects, or antimicrobial drugs with less neuroleptic effect, but since the antimicrobial activity (growth inhibition) of the investigated non-antibiotics is independent of the influence on the efflux pumps, we might have quite new possibilities, in the field of molecular biology. If the basis for these differences is understood in detail, more stereo selective drugs might be designed.

Chiral analogs lack pharmaco-dynamic activity, even though they are otherwise identical to their active drug counterparts from the point of view of physics and chemistry. Thus, chirality has to be interpreted as a chemical property implicating biological specificity, which dictates that racemic drugs should be excluded from therapeutic use, leaving only well-characterized, stereo isomerically pure compounds. Finally non-antibiotic drugs can be used as tools in studying both eukaryotic and prokaryotic cell systems, especially the ones that are clinically well established, and where the knowledge about side effects is well known.

All these questions might lead us to the need for a general theory of the interactions between host, microorganisms and drugs (compounds which are chemically foreign or hostile to a biological system). Fig 1 showed that the variation in the eukaryotic cell systems and variation in the prokaryotic cell systems opens up a complex world of chemical –interactions between host organism and microorganisms, and drugs. Utilizing the interactions between drugs used for non-infectious diseases having antimicrobial activities (non-antibiotics) and chemotherapeutics/ antibiotics as here described by the examples of reversal of drug resistance in different cell systems (44), might give us quite new pharmacological possibilities according to bacterial resistance as well as modulation of bacterial virulence (27).

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