An approach for the in vitro screening of drugs for activity against leprosy

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Summary. Slow growing strains of mycobacteria isolated from leprous tissues present a characteristic resistance pattern to antibacterial agents that is comparable to drug sensitivity of *M. leprae* in man.

The chemotherapy of leprosy is unsatisfactory. The drug of choice, 4-4-diaminodiphenyl sulfone (DDS), is a slow acting bacteriostatic agent which has to be administered for life to control the disease[4]. Resistance against DDS is increasing alarmingly. Rifampicin, with the most potent therapeutic effect in leprosy[5], is available only for a limited number of patients. Since the in vitro cultivation of *Mycobacterium leprae* remains to be proven, no in vitro pharmacological model is available for screening of substances with prospective anti-leprosy effect. The localized disease induced by *M. leprae* in the foot pad of mice is accepted for in vivo trials[6–9]. However, as the table shows, some of the drugs which show high anti-leprosy activity in the mouse foot pad have no therapeutic effect in human leprosy[4,9,10]. Other drugs, active in the foot pad, have deteriorating effect on the disease[8,11]. The only reliable test subject for screening drugs for activity against leprosy is the human lepromatous leprosy patient.

Recently several strains of *M. scrofulaceum* group mycobacteria have been cultured from leprous tissues[13]. These cultures are related to leprosy in a manner which is not yet clear. The cultures, however, present a characteristic resistance pattern to antibacterial agents that is comparable to the drug sensitivity of *M. leprae* in man. This knowledge leads us to propose an in vitro method using *M. scrofulaceum* strains for screening drugs against leprosy.

Comparative data concerning in vivo effect of drugs against *M. leprae* in the foot pad of mice and their therapeutic effect in human leprosy, presented in the table, were collected from the literature. Four strains of *M. scrofulaceum* were tested for drug sensitivity on Middlebrooke and Cohn's 7H10 medium. All drugs, except DDS, were incorporated before solidification into the medium. DDS was dissolved in ethanol and impregnated on the 2 cm^2 surface of the solidified medium. Ethanol was evaporated from the media by incubating at 37 °C for 2 days. *M. scrofulaceum* suspensions were inoculated on the surface of the media. Colonies were counted after 21 days incubation at 34 °C. Results are expressed as resistance (R) or sensitivity (S) to the concentration of drugs recorded in the table. These concentrations are generally accepted in clinical diagnostic laboratories[14,15] as cut-off points for drug sensitivity.

Data presented in the table show that, while all the tested antituberculous drugs[6,8,9] except ethambutol[7] have a complete or partial suppressing effect on *M. leprae* in the foot pad of mice, only DDS has a slow[4] and rifampicin a fast[5] and potent therapeutic effect on leprosy in man. It is evident that the foot pad model[6,8,9] gives useful indications but does not in fact reflect the therapeutic effect of the same drugs when tested in human leprosy, sometimes 'yielding false positive antileprosy activity for new drugs' as pointed out by Chang[16].

Results show that only those drugs which show established clinical efficiency in human leprosy have inhibiting effects on the growth of strains of *M. scrofulaceum* isolated from leprous tissues. Rifampicin, the most potent drug in human leprosy[5] with a minimal bactericidal concentration of 1 to 2 µg/ml in the serum of mice[6], inhibited completely the growth of our cultures in a dose as low as 1 µg/ml. A relatively high concentration of 25 µg/ml of DDS was necessary to inhibit the growth of the same cultures in vitro. The same drug has no significant bactericidal effect in mice[6], though the minimum inhibitory concentration of DDS is 0.01–0.03 µg/ml in the serum of mice against *M. leprae* in the foot pad[6]. DDS, however, is a slow acting drug in man and has only bacteriostatic effects. No information is available as to the concentration of DDS in the macrophage, where DDS has to exert its antileprous action to achieve therapeutic effect. *M. scrofulaceum* isolated from the sputum of a patient with pulmonary mycobacteriosis was resistant to DDS and partially resistant to rifampicin. All the strains isolated from human and armadillo leprous tissues showed resistance against all the other anti-tuberculous drugs tested.

The in vivo foot pad model is expensive and necessitates space and skilled personnel. The test requires observation periods from several months to more than a year. While

Effect of drugs on *M. leprae* in mice and man. Drugs were incorporated in diet (%) for mice, or injected daily (mg). In vivo effects of drugs are compared with in vitro effects expressed as resistance (R) or sensitivity (S) of four strains of *M. scrofulaceum* to drug concentration shown as mcg/ml in the Middlebrook Cohn 7H10 media. Strains H75 and Dakar were cultured from human and strain A6 from armadillo leprous tissues. *M. scrofulaceum* was cultured from the sputum of a patient with pulmonary disease

	SM	INH	PAS	TBI	EMB	CY	ETH	DDS	RFP
M. leprae in mice, foot pad	2 mg/day complete suppression[9]	0.01% complete suppression[8]	0.6% complete suppression[8]	0.1% partial suppression[8]	0.25% none[9]	0.5% partial suppression[8]	0.01% complete suppression[7]	0.01% complete suppression[8]	0.001% complete suppression[6]
M. leprae in man	Weak slow[4]	Temporary then deteriorating[11]	None[4]	Temporary then deteriorating[12]	Report limited	Weak slow[16]	None[10]	Slow[4]	Fast potent[5]
In vitro mcg/ml	10	5	10	4	16	40	40	25	1
Strain H-75	R	R	R	R	R	R	R	S	S
Strain Dakar	R	R	R	R	R	R	R	S	S
Strain A6	R	R	R	R	R	R	R	S	S
M. scrofulaceum	R	R	R	S	R	R	R	R	S/R

SM, streptomycin; INH, isonicotinic acid hydrazide; PAS, para-aminosalicylic acid; TB1, 4-acetaminobenzaldehyde thiosemicarbazone; EMB, ethambutol; CY, cycloserine; ETH, ethionamide; DDS, 4-4-diaminodiphenyl sulfone; RFP, rifampicin.

other pathogenic or non-pathogenic slow or fast growing mycobacteria show a wide range and variety of sensitivity to drugs[14,15], the drug sensitivity of these *M. scrofulaceum* type strains seems to reflect better the therapeutic effect of drugs in leprosy.

The proposed in vitro model is inexpensive and permits the in vitro testing of unlimited number of compounds in a short period of time. The drug sensitivity profile of these cultures seems to provide a genetic marker similar to the drug sensitivity of *M. leprae* in the diseased man[6-9,16]. The results obtained illustrate the possibility that potent therapeutic agents in the fight against leprosy might be sought among drugs which have a molecular mechanism of antibiotic action similar to rifampicin, interacting directly with DNA-directed RNA polymerase. Drugs affecting cell wall synthesis such as cycloserine, or interfering with protein synthesis similar to streptomycin, as well as other antituberculous agents, have practically no therapeutic effect in leprosy. Drugs with insignificant bactericidal effect, such as DDS and others, should be considered for second choice only and this mainly for economic reasons. An in vitro technique is necessary for rapid and large scale screening of drugs having efficiency similar to that of rifampicin. Compounds selected with the proposed in vitro techniques might then be subject to testing in the in vivo foot pad model.

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Species-dependent stereospecific serum protein binding of the oral anticoagulant drug phenprocoumon[1]

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Summary. 13 mammalian species are classified into 3 clearcut groups with respect to the stereospecific serum protein-binding of phenprocoumon: 2 groups showing opposed stereospecific binding characteristics and a 3rd group exhibiting no stereospecific binding. Structural differences in the albumin molecule account for these stereospecific differences in serum protein-binding.

It has been shown that optical isomers of drugs can interact in a stereoselective manner with mammalian serum proteins[2-5]. There is also evidence that the relative proportion of the binding capacity of serum proteins for enantiomers may vary from one species to another[6]. The order of binding strength of serum proteins for the enantiomers, however, was similar in all species (e.g. the enantiomer which showed the strongest binding in one species also did so in the others).

We now report that with respect to the stereospecific serum protein-binding of phenprocoumon mammalian species can be classified into 3 clearcut groups: 2 groups showing opposed stereospecific binding characteristics and a 3rd group exhibiting no stereospecific binding.

Material and methods. Blood samples were obtained from a peripheral vein (man, monkey, horse, goat, dog), carotid artery (cow, pig, sheep, cat, rabbit) or abdominal aorta (rat, mouse, guinea-pig). The freshly drawn blood was permitted to clot, centrifuged, and the serum was removed. Human and bovine albumin (dried, purified, electrophoretic purity of 100%) was purchased from Behring-Werke AG, Marburg/Lahn, BRD, and rat albumin (fraction V powder) from Sigma Chemie GmbH, Munich, BRD. The enantiomers of phenprocoumon (S(-)phenprocoumon $-113.8°$, 3.8% in methanol; R(+) phenprocoumon $+114.0°$, 3.4% in methanol) were gifts from Hoffmann-La Roche, Basel, Switzerland.

Protein binding was determined in undiluted serum or pure albumin solution (3.45 g albumin per 100 ml phosphate buffer, 0.15 M, pH 7.4) by equilibrium dialysis using custom made plexiglas cells of 1 ml separated by a cellophane dialysis membrane (Union Carbide Corp., Chicago, Ill.). Serum or albumin solution was dialysed for 18 h at 37 °C against phosphate buffer (0.15 M, pH 7.4) containing the desired drug concentration. Only fresh serum (stored no longer than 8 h at 4 °C) was used. Drug concentrations were measured fluorimetrically after extraction with n-heptane[7].

Results and discussion. The table shows the binding of R(+) and S(−)phenprocoumon to blood serum of 13 mammalian species at a total drug concentration of $3 \cdot 10^{-5}$ M. It is evident that the species under investigation can be arranged into 3 clearcut groups with respect to the stereospecific binding of phenprocoumon. Whereas in the serum of rat, mouse, guinea-pig and rabbit, the fraction of unbound S(−)phenprocoumon was markedly higher than that of R(+)phenprocoumon, the opposite was true in the serum of man, monkey, cat, dog, pig and horse. The serum of bovine, goat and sheep exhibited apparently the same