In vitro and in vivo antimycobacterial activity of antiinflammatory drug, diclofenac sodium

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The non-steroidal antiinflammatory drug diclofenac sodium exhibited remarkable inhibitory action against both drug-sensitive and drug-resistant clinical isolates of Mycobacterium tuberculosis, as well as other mycobacteria. This agent was tested in vitro against 45 different strains of mycobacteria, most of which were inhibited by the drug at 10-25 μg/ml concentration. When tested in vivo, diclofenac, injected at 10 mg/kg body weight of a Swiss strain of white mice, could significantly protect them when challenged with a 50 median lethal dose of M. tuberculosis H37 Rv02. According to Chi-square test, the in vivo data were highly significant (P<0.01).

Keywords: Antimycobacterial activity, Antiinflammatory drug, Diclofenac sodium

The occurrence of multidrug resistant strains of Mycobacterium tuberculosis in particular and mycobacteria in general needs surveillance and control on a global level. Failure to cure multidrug resistant tuberculosis (MDR-TB) with the currently available antitubercular drugs leads to a search for newer and potent drugs to treat such cases, and thereby prevent an emerging multidimensional problem. Different studies aimed at discovering newer antimycobacterial agents have revealed moderate to powerful action in several compounds belonging to various pharmacological groups, e.g., promethazine1, chlorpromazine2, trifluoperazine3, methdilazine4, thioridazine5 and other phenothiazines6. Many of these agents have exhibited powerful inhibitory action against Gram positive and Gram negative bacteria as well7-9. Such compounds having antimicrobial properties in addition to their predesignated pharmacological action are entitled as “Non-antibiotics”. The antiinflammatory drug diclofenac sodium was seen to possess powerful antibacterial activity against Gram positive and Gram negative bacteria10-11. The present paper describes the antimycobacterial action of diclofenac both through in vitro and in vivo tests.

Materials and Methods

Drugs—The drugs were obtained as pure dry powder from their respective manufacturers in India: Diclofenac sodium (De) and rifampicin (Rf) were obtained from Hindustan Ciba Geigy, streptomycin (Sm) from Sarabhai Chemicals, ethambutol (Eb) from Lyka Laboratories and isonicotinic acid hydrazid (INH) from Glaxo Laboratories. They were preserved at 4°C.

Bacteria—Forty-five strains of mycobacteria were tested. M. tuberculosis Bajaj, J15, N23, H37Rv10 and H37Ra16 were obtained from Tuberculosis Research Center, ICMR, Govt. of India. M. marinum, 50, M. scrofulaceum 1323, M. gordonae 1324 M. flavescence 1541, M. xenopi 160, M. avium 724 M. intracellulare 1406, M. terrae 1450, M. trivat 1453, M. smegmatis 798, M. smegmatis 1546, M fortium 1529 and M. phlei L1 were obtained from Central JALMA Institute for Leprosy, Agra, India M. tuberculosis 912042, 911928, 905574, 911454 910708, 911831, 905358, 911447, 911884, 912234
911677, 90657, 912359, 911337, 912073, 912447, 912056, 911053 and 906909 were obtained from Tuberculosis Research Centre, Chennai, India and M. tuberculosis BTA1, BTA2, BTA3, BTA4, BTA5, BTA6, BTA7 and BTA8 were obtained from Bengal Tuberculosis Association (BTA), Calcutta, India. The strains were identified by Radiometric method (BACTEC 460) and biochemical tests (Niacin, Nitrate, Urease, Catalase, Tween80, Tellurite and 5% NaCl tests).

Media—Liquid medium used was Kirchner’s Liquid medium (KLM)12, which was used to grow and suspend the organisms.

Solid medium was Lowenstein Jensen Medium (LJM), prepared as described by the International Union Against Tuberculosis and Lung Diseases (IUATLD), 1955.

Preparation of inocula for susceptibility tests—The bacterium was first grown in KLM. The inoculum was prepared by homogenizing the KLM culture with glass beads, spinning down the larger particles, and matching the supernate against Mc Farland’s standard13.

Determination of minimum inhibitory concentration (MIC) of antibiotics/non-antibiotics against different strains of mycobacteria by tube dilution method14—Sm, Rf, Eb and INH each were used in the following concentrations (µg/ml) in KLM: 0 (control), 0.25, 0.5, 1, 2, 4 and 8. De was used in KLM in concentrations of 0 (control), 5, 10, 15, 20, 25 and 50 µg/ml. For some selected strains, the drug was tested in concentrations ± 2 of its MIC value, in order to find out its mean ± SD values with respect to those organisms. Amount of inoculum used to inoculate each tube above was 0.01 ml. Incubation was done at 37°C for 10-20 days as required.

Animal experiments—Systemic infections were produced in groups of 20 inbred Swiss albino male mice (ca. 18-20 g). Animals were maintained in animal house at standard conditions at 21±1°C and 50-60% RH with a photoperiod of 14:10 hr of light-darkness. Water and a dry pellet diet were given ad libitum. Each mouse was administered intraperitoneally 0.05 ml of a suspension (containing 0.5 mg homogenized KLM culture deposit, representing c < 9 x 10³ CFU)13; of these, 10 were administered De (dose 10 mg/kg body weight/day x 6 weeks) while the other 10 did not receive any drug and served as the control. The viscera from the animals autopsied 6 weeks after infection were obtained, taking strict precaution respecting sterility and examined for macroscopic lesions of systemic infections, e.g., tubercles and caseation, both for the treated and untreated groups14. Portions of each organ were processed for histological study of the lesions, while the remainder were homogenized aseptically in sterile glass homogenisers in saline, examined under the microscope as stained smears (Hematoxylin and Eosin, as well as, Ziehl-Neelsen stains) for presence of acid fast bacilli (AFB) and contaminants, and inoculated onto nutrient/blood agar plates to determine rapid growth, if any. Sterile specimens (as well as contaminated specimens after adequate decontamination by Petrov’s method) were plated out on LJM in 0.1 ml amounts and examined for growth of the infecting M. tuberculosis. The growth was confirmed by Radiometric method.

Results Minimum inhibitory concentration (MIC) of Sm/Rf/Eb/INH and De against different strains of mycobacteria by tube dilution method—Out of 45 strains of mycobacteria tested, 5 strains (M. tuberculosis Bajaj, J15, N23, H3/Rv102 and H3/Ra16) were inhibited by diclofenac at 10 µg/ml, while 13 strains (M. marinum 50, M. scrofulaceum 1323, M. gordonae 1324, M. flavescens 1541, M. xenopi 160, M. avium 724, M. intracellulare 1406, M. terrae 1450, M. triviale 1453, M. fortuitum 1529, M. phlei L1, M. smegmatis 798, M. smegmatis 1546) were inhibited at 15 µg/ml of De. These 18 strains were highly to moderately sensitive with respect to conventional antitubercular drugs. Eight strains (M. tuberculosis BTA1, BTA2, BTA3, BTA4, BTA5, BTA6 BTA7, BTA8) were found to be multidrug resistant. They were inhibited by De at 20 µg/ml. Finally, M. tuberculosis 912042, 911928, 905574, 911454, 910708, 911831, 905358, 911447, 911884, 912234, 911677, 90657, 912359, 911337, 912073, 912447, 912056, 911053, 906909 were inhibited by De at 25 µg/ml. These strains were polydrug resistant. The susceptible strains like M. tuberculosis H3/Rv102 were inhibited at lower doses of conventional antitubercular agents (0.5 to 2 µg/ml), while the single-, poly- and multidrug resistant clinical isolates (like M. smegmatis 798, M. tuberculosis 912042 and M. tuberculosis BTA8 and so on) were inhibited at much higher concentrations, and some were even resistant. MIC of De against M. tuberculosis...
H37Rv102 was 10 µg/ml, while it was 25 µg/ml for the drug-resistant strains. The MIC values of Dc in terms of mean ± SD with respect to 5 strains (M. tuberculosis H37Rv102, M. intracellulare 1406, M. smegmatis 798, M. tuberculosis BTA1 and M. tuberculosis 912042) are given in Table 1. The MIC of Dc is much higher (5-6 times) than the MIC of the conventional antimycobacterial drugs (Sm/Rf). It was noticed that even the MDR strains as compared to the sensitive strains. It was noticed that even the multidrug-resistant strains like those obtained from BTA were susceptible to diclofenac, although at a higher concentration (20 µg/ml).

**In vivo assessment**—Of the 10 animals in the untreated group (that did not receive any drug), all developed minute tubercles in the liver, 5 in the spleen, 5 in the lungs and 9 in the peritoneum a intestines; microscopic necrosis suggestive caseation was found in the liver of 3 animals and in the spleen, peritoneum and intestines each in 1 animal (Table 2). Smears for acid-fast bacilli (AF by Z-N stain, from centrifuged deposits (for 1 fields) of tissue homogenates, showed all 10 anim: to be smear positive at the time of autopsy, and suggested successful infections in these animals. In contrast, macroscopic examination of the treated (t) received the drug) group (10 animals) showed tubercles to be present in some of the liver specimens (2) and in the spleen, peritoneum, as well as in the intestine (3 each), but in the lungs, no tubercles could be detected: Z-N stained smears of tissue homogenates showed presence of AFB only in cases. In 5 animals of the untreated group M. tuberculosis H37Rv102 could actually be recovered on subculture (as confirmed by BACTEC test) in comparison with only one of the treated group which appeared to be significant (P<0.01). The failure to recover the bacterium in other untreated animals was probably due to a non-viability of the bacilli, although these could readily be detected in all cases. The histopathological sections of liver also revealed a considerable decrease in number of infiltrations in infected mice treated with Dc as compared to the untreated ones (Figs 1a, and 1c).

**Discussion**

When tested against a large number of Gram positive and Gram negative bacteria, the non-steroid anti-inflammatory drug Dc had proved to be a powerful antimicrobial agent, the MIC ranging from 25-100 µg/ml in most of the instances, and even lower in some cases. This bactericidal agent could also offer significant protection to mice, who challenged with a virulent bacterium. The antibacterial activity of Dc was found to be due to inhibition of bacterial DNA synthesis, which was demonstrated using 2µCi (³H) deoxythymidine uptake. The actual factors responsible for attributing antimycobacterial activity to Dc are yet to be ascertained. QSAR studies may reveal the actual moieties responsible for conferring antimycobacterial activity to Dc.

Streptomycin and rifampicin are known conventional antitubercular drugs, and their MIC ranged from 0.5 to 8 µg/ml with respect to most of t
trains tested. The susceptible strains like \emph{M. tuberculosis} H37Rv102 were inhibited at lower oses (0.5 to 2 \(\mu g/ml\)), while the drug resistant atries were inhibited at much higher concentrations, and some were totally resistant. A similar pattern was noticed in the \emph{in vitro} activity of \(D\) as well – while its MIC against \emph{M. tuberculosis} H37Rv102 was 10 ± 0.4 \(\mu g/ml\), it was 25 ± 0.4 \(\mu g/ml\) or the drug resistant strains. It was noticed that even ne multidrug resistant strains like those obtained rom BTA and Tuberculosis Research Centre were susceptible to \(D\), although at a higher concentration 20-25 \(\mu g/ml\). The MIC of \(D\) seems to be high against mycobacteria in the \emph{in vitro} studies. However, the antimycobacterial chemotherapeutics like INH nd pyrazinamide also have quite high MIC values against mycobacteria; such high doses are often toxic o liver and other organs.

\(D\) was found to be bactericidal in action against \emph{M. tuberculosis} H37 Rv102. This may be due to no specific effect or toxic effect.

In the animal experiments with \emph{M. tuberculosis} H37 Rv102 in mice, several minute tubercles were observed in the liver, spleen, lungs, peritoneum and ntestines of infected mice. However, there was a definite reduction in these macroscopic lesions in De-reated animals. Smears for acid-fast bacilli by Z-N tain of tissue homogenates of untreated mice showed all animals to be smear-positive, in contrast with 4 animals out of 10 in case of De-treated mice. In 5 animals of the untreated group, \emph{M. tuberculosis} H37 Rv102 could be recovered from subculture. The tubercle bacilli could not be recovered from the rest of the untreated animals; possibly because of the relatively few bacilli that mouse lesions had during autopsy, with even fewer survivors some weeks after infection, since it is known that compared to mice, guinea pig is a better animal model for producing experimental tuberculosis infection.

Most antimycobacterial non-antibiotics reported so far have shown \emph{in vitro} MIC values ranging from 10 to 25 \(\mu g/ml\), which seems to be in accordance with that of diclofenac. Phenothiazines such as chlorpromazine\(^2\), thiourazine\(^5\) and promethazine\(^1\) have been shown to have \emph{in vitro} activity against clinical strains of \emph{M. tuberculosis}. This activity required concentrations that are beyond those that are clinically achievable (like 1 mg/l). However, such antitubercular non-antibiotics may be concentrated more than 10-fold by macrophages that have phagocytosed \emph{M. tuberculosis}. Thus, clinically acceptable dosing of a tuberculosis patient may result in an inhibitory effect \emph{in situ} intracellularly similar to that observed \emph{in vitro}. This suggests that such drugs, including diclofenac, may be used as adjuvants to current regimens used for the management of freshly diagnosed tuberculosis.
Diclofenac was further tested for synergism with the conventional antimycobacterial drug streptomycin against *M. smegmatis* 798. When compared with their individual effects, synergism was found to be statistically significant (*P*<0.05). By the checkerboard assessment procedure, the fractional inhibitory concentration index of this combination was found to be 0.37, confirming synergism.

Although De is reported to be a rather toxic agent for human consumption, this drug could be tolerated by mice for the entire period of 6 weeks when this was administered intraperitoneally everyday in the dose of 10 mg/kg body weight. Protection at such low concentration could be achieved possibly due to the fact that diclofenac is rapidly and completely absorbed after oral administration. There is a substantial first pass effect, such that only about 50% of the drug is available systematically. Its half-life in plasma is 1 to 2 hours. Diclofenac produces side effects in only 20% of patients when used as an antiinflammatory agent, and only 2% of them discontinue therapy as a result. This depends upon genetic factors, nutritional factors and physiological state of the patient.

Earlier studies by Amaral and Kristiansen had proved the efficacy of chlorpromazine in combating tuberculosis *in vivo*, along with a significant *in vitro* action. Apparently, the drug De has remarkable structural correlation with chlorpromazine in having two complete benzene rings attached to each other as phenyl acetic acid derivative through an NH group, and two halogen (Cl) atoms.

Although the sensitivity of mycobacteria towards known antitubercular drugs could be higher than that towards De (MIC ranging from 10-25 μg/ml), it corroborates the work of Amaral and Kristiansen on chlorpromazine.

Thus, the presence of antitubercular function in De may prove to be a significant finding in the treatment.

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Fig. 1—(a) Thin section of mouse liver showing normal hepatic architecture with a large central vein, sinusoids without any detectable staining intensity difference in the hepatic parenchyma. (b) Thin section of mouse liver infected with *M. tuberculosis* for 6 weeks, representing a detectable abnormal pathology showing expansion of normal portal tract with inflammatory cells, mostly lymphocytes, spilling into the surrounding hepatic lobules and destroying them by apoptosis. Infiltration of neutrophils around hepatic sinus and central vein also noted. (c) Thin section of infected mouse liver treated with De for 6 weeks, representing a gross recovery compared with untreated (a×cx100).
of tuberculosis, acting via various pathways involved in immunological mediated activity. Further, in course of time, it may be possible to make a new generation of potential antitubercular drugs, by obtaining effective synergistic combinations between Xe and conventional antitubercular agents.

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References