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Antimycobacterial activity of 4′-bromo-[1,1′-biphenyl]-4-yl 4-x-phenylmethanone derivatives, and their acute toxicity and cytotoxicity

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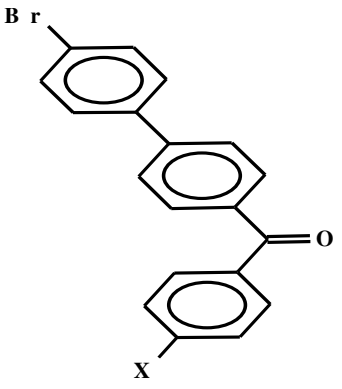
The antimycobacterial activity of nine biphenyl methanone (BPM) derivatives against standard strains of *Mycobacterium kansasii*, *M. avium* and *M. malmoense* was determined by colorimetric assay in microplates with the dye Alamar Blue. Acute toxicity of these compounds was also analyzed by determination of CO_2 concentration in a respirometric assay using *Escherichia coli*. The compounds showed weak antimycobacterial activity with a minimal inhibitory concentration (MIC) over 0.038 mmol l^{-1} and no toxicity was found in *E. coli* up to 400 mmol l^{-1}. No cytotoxicity was observed on V79 cells up to 0.35 mmol l^{-1} with 7 of the BPM derivatives, with two exceptions ($X = SO_2CH_3$, NO_2) that showed some toxicity. The greatest antimycobacterial activity was observed with the SO_2CH_3 derivative and the application of Principal Component Analysis (PCA) showed a relationship between structure and antimycobacterial activity of the compounds. Two descriptors, nucleophilic superdelocalizability of carbon atom and π-hydrophobic constant, were necessary to describe this relationship.

1. Introduction

Tuberculosis remains a major worldwide cause of morbidity and mortality in humans and consequently, there is considerable interest among academic and industrial researchers for a new therapy for this infection [1]. The frequent appearance of strains of drug-resistant *Mycobacterium tuberculosis* and the growing importance of non-tuberculosis mycobacterial strains has accentuated the need for a search for new antimycobacterial drugs [2]. Several studies have clearly shown that the incidence of active tuberculosis is increasing in HIV infected patients [3]. This global situation has underlined the need to identify rapidly new drugs for chemotherapy which are more efficacious and less toxic than the current ones [4].

The balance of the therapeutic versus toxicological effects of a compound is an important parameter when verifying its applicability as a therapeutic drug. Numerous *in vitro* assays have been developed to assess cytotoxicity and these generally take into account different aspects of cellular functions. The reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assesses the functional intactness of mitochondria [5, 6], neutral red uptake (NRU) is a measure of lysosomal integrity [7, 8] and assay of nucleic acid content (NAC) evaluates the total cellular material as an indicator of total cell numbers [9, 10].



Structure of BPM derivatives ($X = -H$, $-Cl$, $-Br$, $-I$, $-NO_2$, $-CH_3$, $-OCH_3$, $-SO_2CH_3$, $-OCOCH_3$)

The antimycobacterial activity of 4′-bromo-[1,1′-biphenyl]-4-yl 4-x-phenyl methanone (BPM) derivatives has been reported against *Mycobacterium tuberculosis* H37Rv ATCC 27294 [11]. In the present study, the potential inhibitory effects of these compounds against the non-tuberculosis mycobacteria, *M. kansasii, M. avium* and *M. malmoense* were investigated and Principal Component Analysis (PCA) was used to determine the relationships between structure and antimycobacterial activity of the compounds. This study also investigated the acute toxicity of BPM derivatives against *Escherichia coli* and cytotoxicity on the V79 cell line derived from Chinese hamster lung fibroblasts by reduction of MTT, NAC and NRU assays.

2. Investigations, results and discussion

BPM derivatives are composed of a biphenyl moiety linked through a carbonyl group to a phenyl ring with one substitution at the *para* position.

2.1. Alamar Blue susceptibility test

The antimycobacterial activities of BPM derivatives were determined against several mycobacteria by the microplate Alamar Blue assay. One week after the inoculums, the results for the visually minimal inhibitory concentrations (MIC) for all the samples were obtained. The Alamar Blue oxidation-reduction dye is a general indicator of cellular growth and/or viability. The blue, nonfluorescent, oxidized form, becomes pink and fluorescent upon reduction in the presence of mycobacteria [12].

The MIC values of the BPM derivatives are given in Table 1. Of the nine samples tested, seven ($X = -H$, $-Cl$, $-Br$, $-I$, $-CH_3$, $-OCH_3$, $-OCOCH_3$) showed weak activities and one ($X = -NO_2$) a very weak activity, while the methylsulphonyl derivative ($X = -SO_2CH_3$), exhibited better activity than all the other compounds tested. The MICs of the nitro ($X = -NO_2$) derivative were

Table 1: Minimal Inhibitory Concentration (MIC – mmol l^{-1}) of BPM derivatives against *M. kansasii, M. avium* and *M. malmoense*

BPM Derivatives (X=)									
Strains	−H	−Cl	−Br	−I	$-NO_2$	$-CH_3$	$-OCH_3$	$-OCOCH_3$	$-SO_2CH_3$
Mk	0.742	0.344	0.307	0.539	1.309	0.712	0.681	0.316	0.038
Ma	0.742	0.344	0.307	0.539	1.309	0.712	0.681	0.316	0.078
Mm	0.742	0.344	0.307	0.539	1.309	0.712	0.681	0.316	0.038

Mk = *M. kansasii*, Ma = *M. avium,* Mm = *M. malmoense*

1.309 mmol l^{-1} for all the test strains and those of the methylsulphonyl derivative were 0.038, 0.078 and 0.038 mmol l^{-1}, against *M. kansasii, M. avium* and *M. malmoense,* respectively.

The antimycobacterial activities of BPM derivatives are in the following sequence:

$-SO_2CH_3 > -Br > -OCOCH_3 > -Cl > -I > -OCH_3 > -CH_3 > -H > -NO_2$ as can be observed in Table 1.

In a previous work these drugs showed moderate activity against *M. tuberculosis* H37Rv [11] which is in agreement with the present results where the methylsulphonyl BPM derivative showed a better antimycobacterial activity. The weak activities against *M. kansasii*, *M. avium* and *M. malmoense* were expected from the characteristics of these strains. These mycobacteria are opportunist pathogens in infections [13] and have natural resistance to almost all drug classes [14]. *M. malmoense* is a clinically relevant, slowly growing, non-pigmented mycobacterial species and recent studies have emphasized the importance of screening drugs against *M. kansasii* [15, 16]. *M. avium* disease is the most widely spread mycobacterial infection which can be responsible for localized [17] or disseminated infection in AIDS patients [18].

2.2. Principal Component Analysis

PCA is an useful statistical method that has been used [11, 19] to classify a series of compounds in relation to their biological activities. PCA studies have shown that the use of both empirical and theoretical descriptors is necessary in the classification process. PCA was able to separate the BPM derivatives into low, very low and high activity compounds as shown in Fig. 1. The two principal components are defined by Equations 1 and 2 (Table 2)

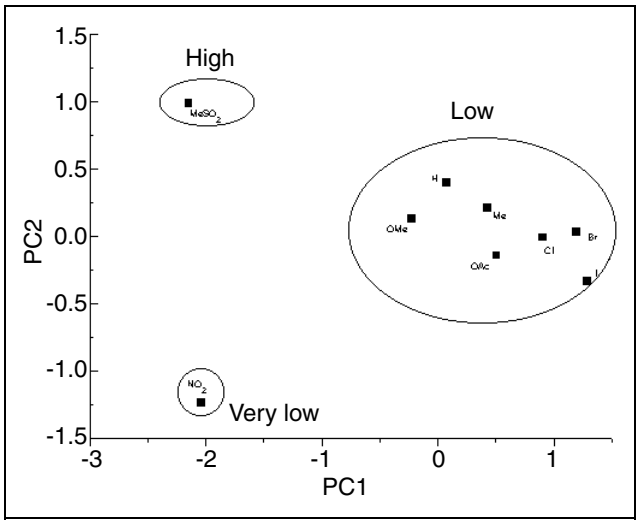


Fig. 1: Plots of the object scores from PCA of BPM derivatives. First (PC1) versus second (PC2) principal component

Table 2: Principal components

Derivatives	PC1	PC2	Log 1/MIC
−H	0.0803	0.3936	0.129
−Br	1.1984	0.0242	0.513
−Cl	0.9023	−0.0124	0.463
−I	1.2863	−0.3377	0.268
$-OCH_3$	−0.2214	0.1264	0.167
$-CH_3$	0.5089	−0.1464	0.147
$-SO_2CH_3$	−2.1460	0.9865	1.420
$-NO_2$	−2.0349	−1.2374	−0.117
$-OCOCH_3$	0.4261	0.2032	0.500

and only two descriptors were necessary for the classification of BPM derivatives: nucleophilic superdelocalizability of carbon atom of carbonyl group (S_C^L) and π-hydrophobic constant.

$$PC1 = 0.707\, S_C^L + 0.707\, \pi \quad (1)$$

$$PC2 = 0.707\, S_C^L - 0.707\, \pi \quad (2)$$

The methylsulphonyl BPM derivative was classified as a compound with high activity against mycobacteria. The values of the π-hydrophobic constant and the nucleophilic superdelocalizability of carbon atom are in the following sequence: π-Hydrophobic constant: $-I > -Br > -Cl > -CH_3 > -OCOCH_3 > -H > -OCH_3 > -NO_2 > -SO_2CH_3$; nucleophilic superdelocalizability of carbon atom: $-Br > -I > -Cl > -OCOCH_3 > -H > -CH_3 > -OCH_3 > -SO_2CH_3 > NO_2$ [11].

With a decrease of the π-hydrophobic constant (−1,63 for SO_2CH_3), the antimycobacterial activity increases showing the importance of lipophilicity in anti-tuberculosis drugs. The biological assay data and PCA are in agreement. The methylsulphonyl BPM derivative has a lipophilic character and consequently can penetrate the membrane of the cells causing alterations in cellular viability. However, the nitro BPM derivative has the lowest nucleophilic superdelocalizability of all the compounds studied, which possibly contributes to a low reactivity and consequently a very low activity. This fact could indicate the importance of the reactivity of BPM in the mechanism of action of these derivatives.

2.3. Acute toxicity on E. coli

CO_2 has an important role in microbial metabolism an this product of respiration can be used as a parameter in monitoring the metabolic activities of microorganisms. CO_2 production by bacteria depends on a complete series of biochemical reactions, which constitute the respiration process of the organism. Inhibition of this process will alter the amount of CO_2 produced. The CO_2 produced by treated *E. coli* was in the same range as for the untreated controls. In the presence of tetracycline, there was inhibi-

tion of around 40% (positive control). Bacterial respiration was not inhibited in the presence of our compounds at from 50 to 500 mmol l^{-1}. The BPM derivatives were unable to induce acute toxicity is *E. coli* and these results indicate that the metabolic functions of the bacteria are normal. An important conclusion is that BPM derivatives act to inhibit mycobacteria, but not *E. coli*.

2.4. Cytotoxicity assay

The viability assays which measure the cytotoxicity of the chemicals tested were used with a broad range of concentrations. The cytotoxic effects of BPM derivatives were measured by MTT, NAC and NRU. The results indicated that V79 cell viability after 24 hours exposure was dose-dependent and that detachment and loss of cells were caused at the effective doses. The values of IC_{50} were obtained mathematically from the dose-response curves and are expressed in Table 3, and Figs. 2 and 3. BPM derivatives with methylsulphonyl and nitro groups at the *para* position of the phenyl ring ($X = -SO_2CH_3$ and $-NO_2$) were more toxic to the V79 cells than the other BPM derivatives. The IC_{50} values for $X = -SO_2CH_3$ and $-NO_2$ were 0.160 and 0.128 mmol l^{-1} on NAC and 0.280 and 0.170 mmol l^{-1} for NRU (Table 3). In comparison with other cytotoxic antituberculosis drugs, the BPM methylsulphonyl derivative exhibits low toxicity and in these assays was on an average 4 times less toxic in V79 cells than in the mycobacteria (MIC values). Up to 0.150 mmol l^{-1}, the BPM nitro derivative increased the reduction of MTT (Figure 3). Probably, there is hyperactivation of mitochondrial succinic dehydrogenase and/or an effect on of the cytosolic $NAD(P)^+$/NADP(H) redox balance. The presence of inhibitors or inducers of reactions from the redox balance confirms the involvement of these components on the MTT reduction capacity of V79 cells. This stimulus for MTT reduction observed in cells treated with the nitro BPM derivative may be expected as a result of cytosolic NaDPH levels [10]. As shown for other compounds, nitro derivatives [20] reduced the viability of the cells at doses greater than 0.150 mmol l^{-1}. For the other BPM derivatives, the IC_{50} values were above 0.350 mmol l^{-1} and at these concentrations, the dimethyl sulfoxide (DMSO) necessary to dissolve the compounds was toxic to the cells.

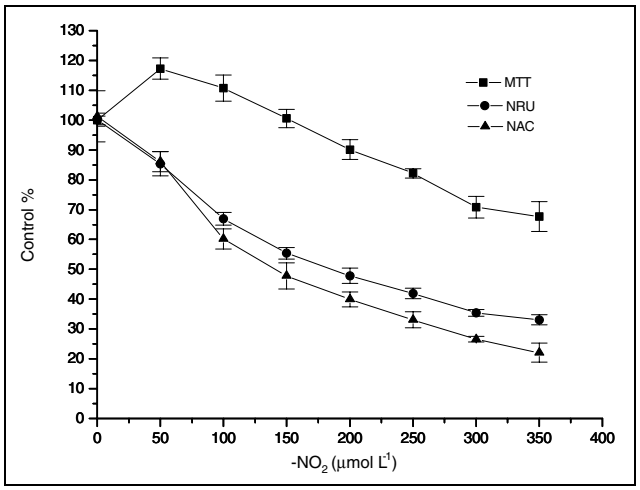


Fig. 2: Cytotoxicity effects of the BPM derivative ($X = NO_2$) on V79 fibroblastics cells. Values are means ± SD ($n = 3$) to neutral red uptake (–●–, NRU), reduction of MTT (–■–, MTT) and nucleic acid content (–▲–, NAC)

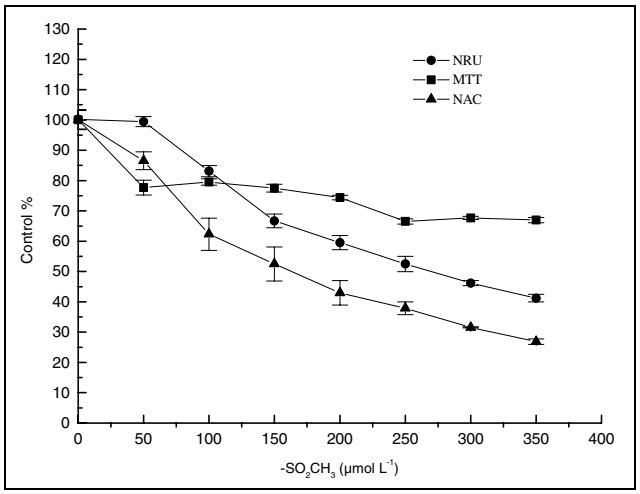


Fig. 3: Cytotoxicity effects of the BPM derivative ($X = SO_2CH_3$) on V79 fibroblastics cells. Values are means ± SD ($n = 3$) to neutral red uptake (–●–, NRU), reduction of MTT (–■–, MTT) and nucleic acid content (–▲–, NAC)

Table 3: Viability inhibition

Assays	Compounds IC (mmol l^{-1})	
	$-NO_2$	$-SO_2CH_3$
NRU	0.170*	0.280*
MTT	0.350**	0.350**
NAC	0.128*	0.160*

*IC_{50}; **IC_{30}

Antimycobacterial activity and cytotoxicity induced by the methylsulphonyl BPM derivative showed that although it is cytotoxic this derivative is also the most active antimycobacterial compound. These results confirm the theoretical studies of BPM derivatives, which showed a relationship between the lipophilic characteristics and biological activities, and are similar to the results found by Klopman et al. [21] for antimycobacterial fluorquinolones.

3. Experimental

3.1. Synthesis and drug preparation

The compounds were synthesized by a classical Friedel-Crafts reaction between 4-bromobiphenyl and the corresponding acid chloride to give BPM derivatives following a previously described procedure [22].

3.2. Biological assay

BPM derivatives ($X = -H$, $-Cl$, $-Br$, $-I$, $-CH_3$, $-OCH_3$, $-OCOCH_3$, $-SO_2CH_3$, $-NO_2$) were assayed against *M. kansasii* ATCC 12478, *M. avium* ATCC 15769 and *M. malmoense* ATCC 29571 strains to determine the antimycobacterial activity and also with *E. coli* to verify the acute toxicity.

3.2.1. Antimycobacterial susceptibility test

Antimycobacterial susceptibility assay was performed through the microplate Alamar Blue Assay [23]. The test strains of mycobacteria were kindly supplied by Dr. Robert G. Cooksey from the Center for Disease Control and Prevention (CDC-Atlanta/GE/USA) and were cultured in Lowenstein-Jensen egg medium (Difco) at 37 °C for 3 weeks and then subcultured in Middlebrook 7H9 broth medium at 37 °C for 10 days. At a bacterial density corresponding to a McFarland standard Nº1 turbidity standard (1×10^7 cell/ml), the suspensions were further diluted 1 : 25 in Middlebrook 7H9 broth medium to give 4×10^5 cell/ml. Stock solutions of the samples were prepared in dimethyl sulfoxide (DMSO – Sigma) (1 g l^{-1}) and were diluted in Middlebrook 7H9 (Difco) broth medium in the range from 0.001 to 2.0 mmol l^{-1}. The control wells consisting of either bacterium only (MB) or medium only (M) and those containing different drug concentrations (100 µl) were inoculated with an aliquot of 100 µl of the diluted suspensions of the strain (4×10^5 cell/ml). The plates were incu-

bated at 37 °C for 6 days and after that 25 µl of a 1 : 1 (v/v) mixture of 10x Alamar Blue reagent and 10% Tween 80 were added to the wells and the plates were reincubated at 37 °C. After 24 hours, a change in color from blue to pink was observed in the wells where the mycobacteria grew. The visual minimal inhibitory concentration (MIC) was defined as the lowest drug concentration which prevented a color change from blue to pink.

3.2.2. Acute toxicity on E. coli

The acute toxicity was determined experimentally by assay of the CO_2 concentration in the *E. coli* culture. The experimental procedure used was a flow injection analysis (FIA) with a conductometric detector [24]. The change in conductance is proportional to the total CO_2 concentration present in the sample. The *E. coli* (ATCC 25922) was kindly provided by the Fundação Tropical de Pesquisas e Tecnologia "André Tosello" (Campinas-SP/Brazil) and the culture was grown under aerobic conditions.
All bioassays were conducted at 37 °C. Before adding the drugs, 48 ml aliquots were withdrawn from a 1000 ml of bacterial stock solution (when the initial level of CO_2 was in the range of 0.4–0.5 mmol l^{-1}, carefully transferred to 150 ml Erlenmeyers and kept in a water bath. Samples were dissolved in a mixture of Tween and DMSO (1 g l^{-1}) and diluted in the culture medium with *E. coli*. All the samples were tested in duplicate from 50 to 500 mmol l^{-1} and the final concentrations of DMSO and Tween in the test medium were 0.45 and 0.2%, respectively. The parameter used to define toxicity was IC_{50} (concentration that inhibit 50% of the cellular respiration). The culture that received no drugs was regarded as a negative control (0% inhibition) and Tetracycline 0.5 mg l^{-1} was used as a positive control (concentration that inhibits bacterial proliferation by about 40%). Calibration curves in the range of 0.25 to 3.00 mmol l^{-1} of CO_2 were determined using dried Na_2CO_3 on the day of each experiment (data not shown).

3.3.3. Cytotoxicity assay

The cytotoxic effect of BPM derivatives (XX = −H, −Cl, −Br, −I, $-CH_3$, $-OCH_3$, $-OCOCH_3$, $-SO_2CH_3$, $-NO_2$), expressed in terms of cell viability, was assayed on a permanent lung fibroblast cell line derived from chinese hamsters (V79) [10]. V79 fibroblasts were grown as monolayers in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% heat- inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were plated at a density of 3×10^4 cell/ml in 96-well plates. The medium was removed 48 hours after cell seeding and replaced with medium containing BPM derivatives at concentrations ranging from 0.002 to 0.350 mmol l^{-1}. The samples were first dissolved in DMSO and then diluted in DMEM. The final concentration of DMSO in the test medium and controls was 0.4%. Cells were exposed for 24 hours to the test medium with or without BPM derivatives (control). Each drug concentration was tested in eight replicates, and repeated three times in separate experiments. At the end of incubation, three independent endpoints for cytotoxicity were evaluated: MTT, NAC and NRU.

3.3.3.1. NAC

Cell number in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. [25]. Cells were washed twice with cold phosphate buffered saline (PBS) and a soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then dissolved in 0.5 mol l^{-1} NaOH and incubated at 37 °C for 1h and the absorbance read at 260 nm. Results are expressed by a comparison of absorbance between cells treated with drugs and controls (no drugs).

3.3.3.2. MTT

The tetrazolium reduction assay was performed according to the method of Denizot and Lang [5]. Briefly, cells were washed once with phosphate buffered saline (PBS) and 0.1 ml of serum-free medium containing MTT (1 mg ml^{-1}) was added to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 0.1 ml ethanol with stirring for 15 min on a microtitre plate shaker and the absorbance was read at 570 nm.

3.3.3.3. NRU

The neutral red uptake assay was performed according to the method of Borenfreund and Puerner [26]. After 4 h of incubation with serum-free medium containing neutral red (50 µg ml^{-1}), the cells were washed quickly with PBS and then 0.1 ml of a solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After rapid agitation on a microtitre plate shaker the absorbance was read at 540 nm.

3.3.4. Principal Component Analysis (PCA)

The theoretical descriptors were calculated by using the AM1 semi-empirical molecular orbital method. Of several parameters studied the nucleophilic superdelocalizability of carbon atom and π-hydrophobic constant were useful for the classification of BPM derivatives [11].

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