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Research Paper

Structure activity relationship, acute toxicity and cytotoxicity of antimycobacterial neolignan analogues

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Abstract

Objectives The study's aims were to evaluate the antimycobacterial activity of 13 synthetic neolignan analogues and to perform structure activity relationship analysis (SAR). The cytotoxicity of the compound 2-phenoxy-1-phenylethanone (LS-2, **1**) in mammalian cells, such as the acute toxicity in mice, was also evaluated.

Methods The extra and intracellular antimycobacterial activity was evaluated on *Mycobacterium tuberculosis* H37Rv. Cytotoxicity studies were performed using V79 cells, J774 macrophages and rat hepatocytes. Additionally, the in-vivo acute toxicity was tested in mice. The SAR analysis was performed by Principal Component Analysis (PCA).

Key findings Among the 13 analogues tested, LS-2 (**1**) was the most effective, showing promising antimycobacterial activity and very low cytotoxicity in V79 cells and in J774 macrophages, while no toxicity was observed in rat hepatocytes. The selectivity index (SI) of LS-2 (**1**) was 91 and the calculated LD50 was 1870 mg/kg, highlighting the very low toxicity in mice. SAR analysis showed that the highest electrophilicity and the lowest molar volume are physical-chemical characteristics important for the antimycobacterial activity of the LS-2 (**1**).

Conclusions LS-2 (**1**) showed promising antimycobacterial activity and very weak cytotoxicity in cell culture, as well as an absence of toxicity in primary culture of hepatocytes. In the acute toxicity study there was an indication of absence of toxicity on murine models, *in vivo*.

Keywords acute toxicity; cytotoxicity; structure activity relationship; tuberculosis

Introduction

In humans, tuberculosis (TB) is caused mainly by *Mycobacterium tuberculosis*, and treatment of patients requires more than six months of chemotherapy. Due to the long duration of the treatment the compliance is low and can result in the development of multidrug resistant strains (MDR-TB) and even extremely resistant strains (XDR-TB).

According to the World Health Organization, 202 countries and territories reported tuberculosis notification data for 2006.[1] These countries comprise 99.6% of the world's population and from 5.1 million of new cases notified, 2.5 million (50%) were smear-positive. The African, South-East Asia and Western Pacific regions accounted for 83% of the number of cases.

Nearly 5% of all new diagnosed cases of tuberculosis were MDR for isoniazid and rifampicin. This represents an increase of 56% since 2000 and 12% since 2004.[2] The successful treatment of MDR-TB requires the use of second-line drugs, which historically have presented an insurmountable cost barrier in resource-poor settings.[3]

The numbers of HIV-positive patients with associated TB increased globally by almost 80% between 2005 and 2006. However, the detected cases represent only 26% of the estimate.[4] Due to the worldwide incidence and mortality rates, in addition to frequent

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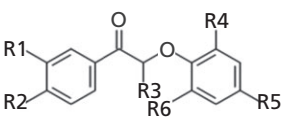

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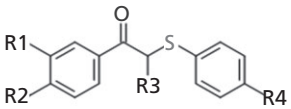

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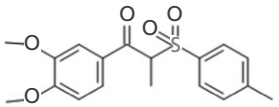

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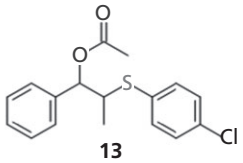

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Compounds	R_1	R_2	R_3	R_4	R_5	R_6	% Inhibition
1	H	H	H	H	H	H	99
2	H	Cl	H	CH_3S	H	H	84
3	CH_3O	CH_3O	H	H	Cl	H	12
4	CH_3O	CH_3O	CH_3	CH_3O	H	CH_3O	4
5	CH_3O	CH_3O	CH_3	CH_3O	$(CH)_2CH_3$	H	7
6	H	H	CH_3	H			40
7	H	H	CH_3	CH_3			10
8	H	H	CH_3	Cl			58
9	CH_3O	CH_3O	CH_3	H			31
10	CH_3O	CH_3O	CH_3	CH_3			15
11	CH_3O	CH_3O	CH_3	Cl			22
12							7
13							45

Inhibition on *M. tuberculosis* H37Rv through BACTEC assay.

Figure 1 Molecular structures of neolignan analogues **1**–**13** and its antimycobacterial activity expressed by inhibition (%).

outbreaks of drug-resistant organisms, there is a pressing need for new therapies.[5] New agents must feature efficacy, be safe and have additional properties required for the specific disease indications. Identifying new drug targets and potential lead therapeutic compounds are also needed to combat MDR-XDR-TB.

Neolignans, which occur in the *Myristicaceae* and other primitive plant families, are usually dimers from oxidative coupling of allyl and propenyl phenols. Natural neolignans were isolated from *Virola surinamensis* and were effective against *Schistosoma mansoni*.[6] Previously, neolignan analogues were synthesized[7,8] and presented antimicrobial activity against *Leishmania*,[9] Schistosoma[10] and *fungi*.[11]

In this study, as part of a screening program searching for antimycobacterial drugs, 13 synthetic neolignan analogues (Figure 1, **1–13**) were evaluated in *M. tuberculosis* H37Rv and a structure activity relationship (SAR) analysis was performed by Principal Component Analysis (PCA). The most effective compound showed promising extra and intracellular antimycobacterial activity in *M. tuberculosis* H37Rv, and its cytotoxicity was evaluated in V79 cells, in J774 macrophages and in rat hepatocytes. Additionally, the acute toxicity was tested in mice.

Neolignan analogues **1–13** are molecules with low molecular weight that already have a defined organic synthetic route. Compound **1**, particularly, can be obtained in two steps, with good yield, that could be easily adapted for an industrial process.[7] Considering that the synthesis process is not expensive, and that the biological activity is remarkable, this could be an interesting molecule for TB drug development.

Materials and Methods

Preparation of neolignan analogues

Neolignan analogues (**1–13**) were synthesized as previously described[7] and the compounds' structures are presented in Figure 1. Stock solutions of the compounds in dimethyl sulfoxide (DMSO; Sigma Chem. Co., St Louis, USA) were sterilized by filtration through a regenerated cellulose membrane of 0.22 μm. The solutions were portioned and stored at –20°C. For the assays, the drugs were diluted in specific culture medium as described below and the final concentration of DMSO never exceeded 1.0%.

Antimycobacterial activity: extracellular and intracellular

The antimycobacterial activity of the neolignan analogues **1–13** was initially tested *in vitro* by the 'Tuberculosis Antimicrobial Acquisition and Coordinating Facility' (TAACF) against *M. tuberculosis* H37Rv (ATCC 27294) in BACTEC 12B medium using a microdilution assay – the Microplate Alamar Blue Assay (MABA) – at 6.25 μg/ml (or molar equivalent of highest molecular weight compound), as previously described.[12]

Continuing the study, compound **1**, 2-phenoxy-1-phenylethanone (LS-2), was also evaluated in *M. tuberculosis* H37Rv (ATCC 27294) by the microdilution assay as previously described[13,14] in concentrations ranging from 1 to 1000 μM. Rifampicin was used as control in concentrations below 64 μM, and the visual minimal inhibitory concentration (MIC) was defined as the lowest that prevented a change in colour from blue to pink due to the inhibition of the mycobacterial growth. LS-2 (**1**) and rifampicin were diluted in Middlebrook 7H9 broth medium and the bioassays were performed in three independent experiments.

Intracellular antimycobacterial susceptibility testing was performed as previously described.[13–15] The suspension of J774 macrophages in RPMI 1640 medium (5×10^5 cells per well) was overlaid with *M. tuberculosis* H37Rv suspension, which was adjusted to yield a ratio of infection of 10 bacteria per macrophage. The *M. tuberculosis* H37Rv-infected cells were re-fed with culture medium containing LS-2 (**1**) in concentrations ranging from 5 to 40 μM. Rifampicin at 12 μM (20 μg/ml) and RPMI 1640 medium were used as controls. After 72 h, cells were lysed and the lysates serially diluted were dispersed onto 7H10 agar plates. The colony forming units (CFU) of *M. tuberculosis* H37Rv were counted 2–4 weeks after incubation at 37°C.

Cytotoxicity assay in mammalian cells

The cytotoxicity of LS-2 (**1**) expressed as cellular viability was determined, using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, in a permanent lung fibroblast cell line derived from Chinese hamsters (V79), in J774 macrophages and in rat hepatocytes.[16] Additional methods, such as nucleic acid content (NAC)[17] and neutral red uptake (NRU)[18] assays, were used for cell viability evaluations in rat hepatocytes and in macrophages.

The V79 cells and J774 macrophages were maintained, respectively, in DMEM and RPMI 1640 culture media, while hepatocytes were obtained from male Wistar rats as previously described.[19,20] Hepatocyte suspension was prepared in DMEM culture medium supplemented with 10 nM dexamethasone, 1% DMSO, 0.2% bovine serum albumin, 0.1 IU/ml bovine insulin, 50 μg/ml penicillin and 50 IU/ml streptomycin.

Hepatocytes, such as macrophages (2×10^6 cells/ml) and V79 cells (3×10^4 cells/ml), received culture medium containing LS-2 (**1**) at concentrations ranging from 0 to 1000 μM. Cells were exposed for 24 h to specific medium either with or without the LS-2 (**1**) (control). Each drug concentration was tested in six replicates in three independent experiments.

The selectivity index (SI) was calculated considering the concentration of LS-2 (**1**) that reduced the cellular viability of J774 macrophages and V79 cells to 50% (cytotoxic concentration – CC50) by the MTT assay and the MIC for *M. tuberculosis* H37Rv (SI = CC50/MIC).

Acute toxicity

The acute toxicity study was performed in accordance with the 'OECD Guideline for Testing of Chemicals',[21] which suggests an appropriate initial dose to minimize the number of animals used.

Four groups of five Swiss Balb/C male mice (30 g each) were kept in an adaptation period of at least seven days before the experiment. Mice were maintained without any food or water 12 h before the experiment.

Mice of these four groups were treated by gavage with a single dose of LS-2 (**1**) at 100, 1000, 1500 or 2000 mg/kg, respectively. LS-2 (**1**) was dissolved in carboxymethylcellulose (CMC) at 0.5% and used also to treat the control group. For 14 days the mice were observed twice a day, and the LD50 (the concentration at which at least 50% of the mice survived) was calculated by linear regression. Surviving mice were killed 14 days after drug treatment and liver, lung and kidneys were macroscopically and histologically examined for detection of drug toxicity.

The procedures concerning animals were approved by the animal ethics committee of Butantan Institute.

Computational aspects and SAR model

Initial geometry optimization for compounds **1–11** were performed at HF/3-21G level of theory to carry out an adequate exploration of the molecular conformational space of each molecule. This level of theory allows a proper balance between accuracy and computational expenses. The lowest energy conformers for each neolignan analogue were again optimized by doing Density Functional Theory (DFT) calculations at the B3LYP/6-31G* level. All calculations were carried out using GAUSSIAN 03 program package.[22] The calculated descriptors are based on the optimized geometries, electronic densities and orbital energies obtained at the B3LYP/6-31G* level, as this level of theory is of enough accuracy for SAR, and its good performance has been widely demonstrated.

Structure activity relationship analysis was performed by PCA. A total of ten descriptors, theoretical and empirical, were calculated by using the DFT method at the B3LYP/6-31G* level. The classification of neolignan analogues was obtained using auto scaled data. Several trials were performed using different sets of three descriptors until the best set able

to separate the neolignan analogues into active and inactive compounds was found. The theoretical and empirical descriptors used in this study are listed below:

ε_{HOMO} = energy of the highest occupied molecular orbital[23]
ε_{LUMO} = energy of the lowest unoccupied molecular orbital[23]
$\eta = (\varepsilon_{LUMO} - \varepsilon_{HOMO})$ = chemical hardness[24]
$S = 1/\eta$ = softness[24]
α = polarizability[25]
$\mu = (\varepsilon_{HOMO} + \varepsilon_{LUMO})/2$ = electronic chemical potential[24]
$\omega = \mu^2/2\eta$ = electrophilicity index[26]
Log P = octanol–water partition coefficient[27]
SA = surface area[28]
MV = molar volume[28]

Statistical analysis

The cytotoxicity results showing cellular viability are expressed graphically using the Origin-Data Analysis and Technical Graphics, version 6.0 (Copyright Software, Inc.) computer software package. The CC50 concentration that produced a 50% inhibitory effect on the evaluated parameter was obtained by data interpolation.

The intracellular antimycobacterial activity (CFU) statistical analysis was done with GraphPad Prism 5.0 (GraphPad Software, Inc.). Viable cell counts were converted to logarithms, which were then evaluated by one or two-variable analyses of variance. After that the carry-forward data from those groups were analysed by paired *t*-test compared with control cells containing bacilli and treated only with RPMI 1640. Significance was defined as $P \leq 0.05$.

Results

Antimycobacterial activity: extracellular and intracellular

By the microdilution assay using BACTEC, the MICs of the neolignan analogues **2–13** were attributed as higher than 6.25 μg/ml and LS-2 (**1**) was the most effective showing a MIC below 6.25 μg/ml (29.45 μM) inhibiting 99% of the mycobacterial growth (Figure 1). In a second assay, LS-2 (**1**) was evaluated in a wide range of concentrations and the MIC was confirmed as 6 μM. As control, the antimycobacterial drugs ethambutol and rifampicin showed, respectively, MIC of 14 μM and lower than 0.03 μM for *M. tuberculosis* H37Rv, in the same experimental conditions.

The LS-2 killed the intracellular bacillus at, and above, 10 μM. Although not statistically different ($P > 0.05$), this effect was similar to that of rifampicin at 12 μM (20 μg/ml) (Figure 2).

Cytotoxicity to mammalian cells

The viability assays in cell cultures to measure the cytotoxicity of the LS-2 were performed from 5 to 1000 μM by the NAC, MTT and NRU techniques. The LS-2 (**1**) concentration at which at least 50% of the cells were viable (CC50) was 700 μM for V79 cells by the MTT assay. The CC50 values in J774 macrophages were 545, 587 and 587 μM, using, respectively, MTT, NRU and NAC assays (Figure 3). Hepatocytes viability was higher than 70% for LS-2 (**1**) at concentrations up to 1000 μM by the three endpoints evaluated (Figure 4).

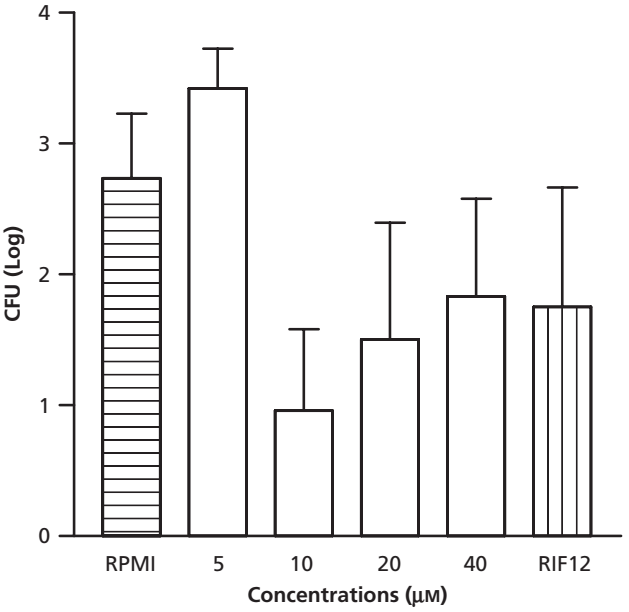

Figure 2 CFU in J774 macrophages infected with *M tuberculosis* H37Rv and treated with LS-2 at 5, 10, 20 and 40 μM. Controls received only RPMI1640 medium or were treated with rifampicin at 12 μM. Bars represent mean ± SEM. For RPMI 1640 vs LS-2 at 5, 10, 20 and 40 μM and RIF at 12 μM, $P = 0.4421$, 0.0428, 0.2160, 0.3142 and 0.3395, respectively.

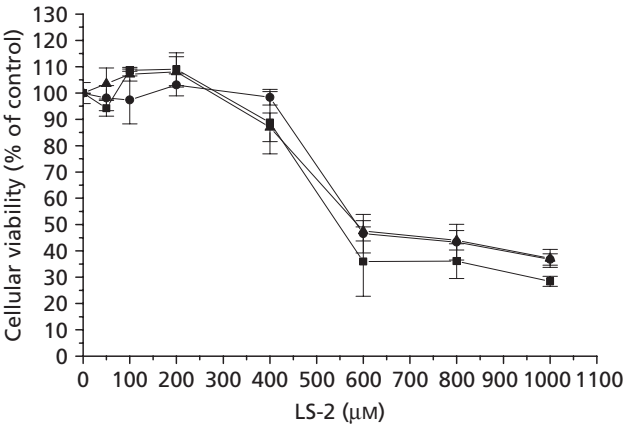

Figure 3 Cytotoxicity of the neolignan analogue LS-2 (**1**) in J774 macrophages through the MTT (●), NAC (▲) and NRU (■) assays.

The selectivity indexes of LS-2 (SI = CC50/MIC) were 117 and 91 calculated using, respectively, the CC50 values obtained in V79 cells (CC50 = 700 μM) and in J774 macrophages (CC50 = 545 μM). Due to the low cytotoxicity, the CC50 was not found in the hepatocytes MTT assay, but a theoretical extrapolation of the data suggests an SI higher than 167 (SI ≥ 1000/6).

The low cytotoxicity of LS-2 in the mammalian cells detected by the three endpoints assayed is indicative of the integrity of the organelles involved in these assays. MTT reduction occurs through the action of the mitochondrial enzyme succinate dehydrogenase, the content of nucleic acid is

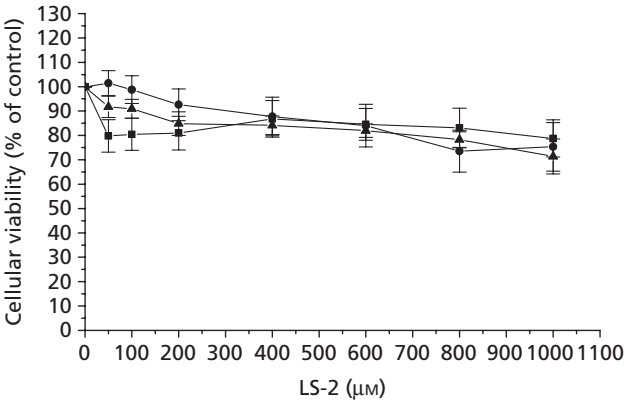

Figure 4 Cytotoxicity of the neolignan analogue – LS-2 (**1**) in hepatocytes through the MTT (●), NAC (▲) and NRU (■) assays.

related to cell death or proliferation and the dye neutral red is taken up by lysosomes. When organelles remained intact after treatment, the results are expressed by high cellular viability.

In-vivo acute toxicity

The in-vivo acute toxicity assay was performed in mice starting with LS-2 (**1**) at a low initial dose of 100 mg/kg and increased up to 2000 mg/kg. After treatment, mice were observed daily for 14 days. All mice from the groups treated with LS-2 (**1**) at 100 and 1000 mg/kg survived. However, one mouse died in the group that received LS-2 (**1**) at 1500 mg/kg, while three died in the 2000 mg/kg group.

After the first hours of treatment, mice treated with LS-2 (**1**) at 1500 or 2000 mg/kg showed signs of toxicity such as behaviour change, reduced vitality with decrease of physical activity and loss of the postural reflexes.

After 14 days of daily observation, from the four groups all 16 surviving mice were in good health, which was confirmed by the necropsy analysis that showed no macroscopic alteration in the organs such as liver, kidneys and lungs (data not shown). The calculated LD50 was 1870 mg/kg, indicating low acute toxicity of LS-2 (**1**).

SAR model for antimycobacterial activity

SAR model for antimycobacterial activity was performed taking into account the MIC bellow 6.25 μg/ml for the neolignan analogue LS-2 (**1**), while for the others analogues (**2**–**11**) the MICs were attributed as higher than 6.25 μg/ml. Table 1 displays the values of 10 chemical descriptors used in this study. Compounds **1** and **2**–**11** were classified as active and inactive compounds, respectively. The classification process was performed carrying out successive PCA on different sets of three variables. The best classification was obtained by a set of descriptors that include molar volume (MV), electrophilicity index (ω) and energy of the lowest unoccupied molecular orbital (ε_{LUMO}), whose two principal components are defined by Equations 1 and 2.

$$PC1 = 0.648\ MV - 0.572\ \omega + 0.502\ \varepsilon_{LUMO} \quad (1)$$

$$PC2 = -0.092\ MV + 0.595\ \omega + 0.798\ \varepsilon_{LUMO} \quad (2)$$

In Figure 5 it can be observed that PCA was able to perform the separation between active and inactive compounds. The active compounds have PC1 values lower than −1.0 and PC2 values higher than 1.0. PC2 is associated with the clustering of inactive compounds, and only one analogue, compound **3**, was incorrectly classified. The incorrect classification of compound **3** can be associated to a slight overestimate in the calculated electrophilicity index. The most active compound, LS-2 (**1**), was classified as featuring the highest electrophilicity and the lowest molar volume, which suggests that these are important parameters contributing to the antimycobacterial activity of this molecule.

Discussion

LS-2 (**1**) can be considered as a promising antimycobacterial candidate drug, with an MIC of 6.25 μg/ml (6 μM) and 99% inhibition in *M. tuberculosis* H37Rv. It is more effective than the antimycobacterial drug ethambutol, which has a MIC of 14 μM. Additionally, it is important to emphasize that there was a considerable reduction of the intracellular burden of mycobacteria on J774 macrophages treated with LS-2 at, and above, 10 μM. However, above 10 μM the effect was not higher. This unexpected result could be explained by insufficient phagocytosis of LS-2 (**1**) by the infected macrophages and, consequently, the intracellular antimycobacterial effect was not proportional to the LS-2 (**1**) concentrations increase.

During rational drug discovery, in-vitro and/or in-vivo assays are carried out. In-vitro assays are performed to determine the activity of the candidate molecules before toxicity studies in the real organism. In-vitro tests are generally more practical, faster and less expensive than in-vivo assays. Furthermore, in-vivo studies require significantly larger quantities of the potential therapeutic agent when compared with in-vitro assays.

Simple, well established in-vitro assays, such as primary hepatocytes and cell lines, are increasingly in demand for identifying potential hepatotoxicity in the early stages of investigative toxicology and for decreasing the numbers of drugs used during lead optimization. However, extrapolation of in-vitro results to the in-vivo situation remains a scientific challenge.[19] In this context, the cytotoxic effects of LS-2 (**1**) were investigated using different cell cultures.

The SI values are indicative of the specificity of LS-2 (**1**) for *M. tuberculosis* H37Rv bacillus and very low cytotoxicity on mammalian cells suggesting LS-2 (**1**) as potential drug for in-vivo studies in animals. Analysing the in-vitro cytotoxicity results and the acute toxicity of LS-2 in mice, the data suggest very low toxicity for LS-2 (**1**) both in in-vitro and in-vivo assays.

Experimental and theoretical studies are in accordance. The antimycobacterial activity of compound **1** (LS-2) can be due to the physical-chemical properties of this molecule. Through the SAR model for antimycobacterial activity, LS-2 (**1**) was classified as featuring the highest electrophilicity and the lowest molar volume, which suggests they are important parameters contributing to the antimycobacterial activity of this molecule.

In a parallel study we observed that LS-2 (**1**) is metabolized to two compounds through the action of hepatocytes in

Table 1 B3LYP calculated and empirical values of descriptors used in the SAR study

Compound	ε_{HOMO} (a.u)	ε_{LUMO} (a.u)	η (a.u)	S (a.u)	α (bhor^3)	μ (a.u)	ω (a.u)	log P	SA (Å^2)	MV (cm^3/mol)
1	–0.238	–0.0584	0.1796	5.569	144.4	–0.1482	0.0020	1.44	431.3	130.0
2	–0.210	–0.0658	0.1443	6.932	190.7	–0.1379	0.0014	0.57	509.8	185.8
3	–0.237	–0.0551	0.1818	5.502	180.0	–0.1460	0.0019	1.01	514.9	181.4
4	–0.200	–0.0497	0.1498	6.676	227.2	–0.1246	0.0012	1.20	611.2	262.4
5	–0.212	–0.0512	0.1609	6.216	207.8	–0.1316	0.0014	2.34	567.6	258.0
6	–0.222	–0.0615	0.1601	6.245	172.7	–0.1415	0.0016	2.32	460.1	201.5
7	–0.219	–0.0604	0.1587	6.302	187.1	–0.1397	0.0015	2.94	489.4	179.3
8	–0.228	–0.0654	0.1625	6.153	187.2	–0.1467	0.0017	2.10	488.4	200.1
9	–0.219	–0.0558	0.1632	6.129	211.5	–0.1374	0.0015	1.89	542.3	231.3
10	–0.217	–0.0548	0.1622	6.166	225.9	–0.1359	0.0015	2.51	572.3	215.8
11	–0.217	–0.0541	0.1634	6.121	227.9	–0.1357	0.0015	0.0085	3.75	572.6

ε_{HOMO}, energy of the highest occupied molecular orbital; ε_{LUMO}, energy of the lowest unoccupied molecular orbital; η, ($\varepsilon_{LUMO} - \varepsilon_{HOMO}$) = chemical hardness; S, $1/\eta$ = softness; α, polarizability; μ, ($\varepsilon_{HOMO} + \varepsilon_{LUMO}$)/2 = electronic chemical potential; ω, $\mu^2/2\eta$ = electrophilicity index; Log P, octanol–water partition coefficient; SA, surface area; MV, molar volume.

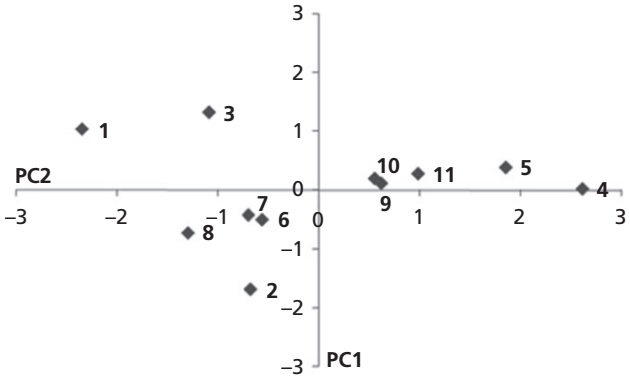

Figure 5 Plot of the object scores from Principal Component Analysis (PCA) of neolignan analogues. First vs second principal component.

an in-vitro assay (unpublished data). This data, taken together with the cytotoxic results, allow us to conclude that LS-2 (**1**) or its metabolites were not toxic on rat hepatocytes.

Literature studies showed that the antimycobacterial drug 1,2-ethylenediamine (SQ109) has an SI of 16.6 and a maximum tolerated dose of 600 mg/kg.[29] The nitroimidazopyran PA-824 presents a maximum toxic dose higher than 1000 and 500 mg/kg by acute toxicity and chronic toxicity study, respectively.[30]

Conclusions

The neolignan analogue 2-phenoxy-1-phenylethanone (LS-2, **1**) showed promising antimycobacterial activity and very low cytotoxicity in cell culture, as well as absence of toxicity in primary culture of hepatocytes. Results from acute toxicity studies in murine models are indicative of the absence of toxicity *in vivo*. LS-2 (**1**) can be considered as a promising antimycobacterial candidate drug and should be evaluated through in-vivo studies on experimental infection with *M. tuberculosis* H37Rv and by chronic toxicity studies.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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