Dihidro-β-Agarofuran Sesquiterpenes: A New Class of Reversal Agents of the Multidrug Resistance Phenotype Mediated by P-Glycoprotein in the Protozoan Parasite *Leishmania*

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Abstract: Leishmaniasis is the most important emerging and uncontrolled infectious disease and the second cause of death among parasitic diseases, after Malaria. One of the main problems concerning the control of infectious diseases is the increased resistance to usual drugs. Overexpression of P-glycoprotein (Pgp)-like transporters represents a very efficient mechanism to reduce the intracellular accumulation of drugs in cancer cells and parasitic protozoans, thus conferring a multidrug resistance (MDR) phenotype. Pgps are active pumps belonging to the ATP-binding cassette (ABC) superfamily of proteins. The inhibition of the activity of these proteins represents an interesting way to control drug resistance both in cancer and in infectious diseases. Most conventional mammalian Pgp-MDR modulators are ineffective in the modulation of Pgp activity in the protozoan parasite *Leishmania*. Consequently, there is a necessity to find effective modulators of Pgp-MDR for protozoan parasites. In this review we describe a rational strategy developed to find specific Pgp-MDR modulators in *Leishmania*, using natural and semisynthetic dihydro-β-agarofuran sesquiterpenes from Celastraceae plants. A series of these compounds have been tested on a MDR *Leishmania tropica* line overexpressing a Pgp transporter to determine their ability to revert the resistance phenotype and to modulate intracellular drug accumulation. Almost all of these natural compounds showed potent reversal activity with different degrees of selectivity and a significant low toxicity. The three-dimensional quantitative structure-activity relationship using the comparative molecular similarity indices analysis (CoMSIA), was employed to characterize the requirements of these sesquiterpenes as modulators at Pgp-like transporter in *Leishmania*.

1. THE PROBLEM OF DRUG RESISTANCE IN INFECTIOUS DISEASES, NEW DRUGS AND THERAPIES ARE NECESSARY

According to WHO Report 2002, the 10 more important infections diseases are: malaria, leishmaniasis, African and American trypanosomiasis, schistosomiasis, lymphatic filariasis, onchocercosis, dengue, tuberculosis and leprosy. Among them, leishmaniasis has become the most important parasitic disease among tropical diseases research (TDR) category 1 (emerging or uncontrolled diseases). Despite recent scientific and technological advances, infectious diseases continue to affect poor and marginalized people through the world. At least, three key factors contribute to the emergence and re-emergence of such diseases: 1- failure to use properly existing tools effectively; 2- failing or non-existing tools to control the disease, and 3- insufficient knowledge of the disease [1].

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Chemotherapy continues to be the main weapon against infectious diseases. However, there is a significant "loss of interest" in the pharmaceutical companies due to the point of view that current drugs are sufficient for effective disease control. Consequently, there is a "drug gap" because these companies invest almost exclusively in drugs for diseases of the developed world that will be marketable and profitable. All infectious diseases, with the exception of HIV/AIDS, tuberculosis, and malaria, are virtually ignored in terms of drug development although such diseases continue to expand through the developing world [1]. This situation could be changed through different strategies such as: better use of existing drugs, combination therapy using proper dosages-time period and increase basic research on development of new effective and less toxic drugs.

One of the problems concerning control interventions of infectious diseases is the increased resistance of pathogens to drugs. Bacteria, viruses, fungi and protozoa resistant to previously effective chemotherapeutic agents, may well be the most single source of emerging and re-emerging pathogens in the developed and overdeveloped world. Molecular genetic approaches have led to significant progresses in the understanding of mechanisms of resistance. Thus there is

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hope to identify those factors involved in the acquisition of resistance, opening the way of new approaches to anticipatory management of resistance that will maximise the terms of effectiveness of new drugs.

2. LEISHMANIASIS, AN EMERGING DISEASE

As described above, leishmaniasis is the most important emerging-uncontrolled infectious disease. With around 20 *Leishmania* species pathogenic for humans, and 30 sandfly species as proven vectors, leishmaniasis is a worldwide parasitic disease, affecting 88 countries. While the 350 million people living in those countries are the most exposed, others at risk include people travelling through these areas such as adventure vacationers, missionaries, development workers, and soldiers (press release WHO/46 17 June 2002, http://www.who.int/inf/en/pr-2002-46.html).

2 million new cases are estimated to occur annually and about 12 million people throughout the world suffer from any of the four major forms of leishmaniasis: cutaneous (a disabling disease if skin ulcers are multiple), diffuse cutaneous (a disabling disease), mucocutaneous (a mutilating disease) and visceral (affects liver, spleen and bone marrow and is fatal when untreated). Leishmaniasis constitutes a severe public health problem and is spreading in several areas of the world as a result of epidemiological changes that sharply increase the overlapping of AIDS and visceral leishmaniasis [2]. These co-infections are considered to be a real emerging disease particularly in southern Europe (http://www.who.int/emc/diesases/leish/index.html Programme for the surveillance and control of leishmaniasis).

TDR, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (http://www.who.int/tdr), has defined the strategic priorities for research in leishmaniasis consisting in new basic knowledge and better intervention tools. The main control strategy is case finding and treatment plus, when feasible, vector control.

Chemotherapy is the main tool against leishmaniasis, but the limited number of effective drugs, the different disease manifestations, the variation in sensitivity of *Leishmania* species, and the emergence of drug resistance, make difficult the control of the disease through the chemotherapy. Pentavalent antimonials, in the form of Glucantime and Pentostam, are still first-line drugs after more than six decades of use. But the treatment is long, given systemically, toxic and expensive. Second-line treatments for leishmaniasis, like pentamidine, aminosidine or liposomal amphotericin B are also quite toxic or its price is unaffordable for poor countries, and frequently must be used combined with pentavalent antimonials. In the last years two new orally active drugs against leishmaniasis have been developed: miltefosine (hexadecylphosphocholine; HePC) and sitamaquine (WR6026). The most promising new leishmanicidal compounds are alkyllysophospholipids (ALPs) such as HePC and edelfosine (Fig. **1**). ALPs were originally developed as anticancer drugs, and have shown a significant antiproliferative activity against *Leishmania*, *Trypanosoma cruzi*, and *Trypanosoma brucei* parasites *in vitro* and *in vivo*, in experimental models [3].

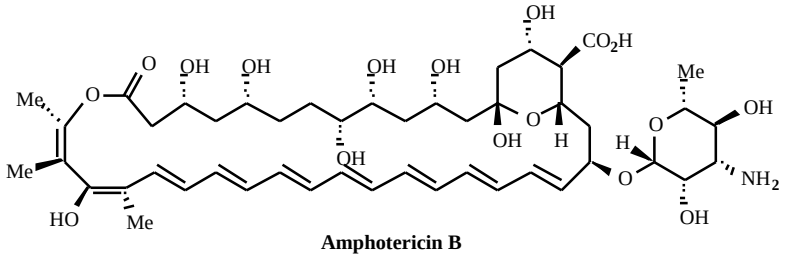
The increasing level of antimonial resistance has changed the pattern of leishmaniasis treatment in the world. Parasite resistance to antileishmanial drugs represents an important problem that may be expected to increase, thus, endemic countries need to consider policies to prevent or control drug resistance. Several resistance mechanisms with different contribution may co-exist in the same parasite and act either additively or synergistically [4]. The understanding of these mechanisms can lead to strategies to avoid or to circumvent the problem.

Resistance to antimonials in most parts of the world is around 1% [5]; however, in India, there has been an epidemic of primary resistance to antimonial drugs with values higher than 60% of resistance in previously untreated patients [6]. This may explain why HePC was recently approved for clinical use in India [7]. Although its leishmanicidal mechanism is not well known yet, HePC has proved to be a highly effective oral drug against visceral and cutaneous leishmaniasis, including antimony resistant cases. However, several mechanisms of *in vitro* resistance to ALPs have been proposed in *Leishmania*, being related to a defective inward of the drug [8, 9] and with the overexpression of Pgp conferring a MDR phenotype [10]. These last findings support the high clinical relevance of understanding the molecular drug-resistance mechanisms that *Leishmania* develops or may develop in the near future and the application of effective strategies to avoid them.

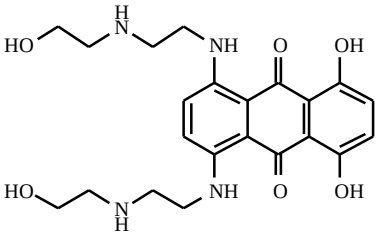
3. MECHANISM OF DRUG RESISTANCE IN *LEISHMANIA* MEDIATED BY ABC TRANSPORTERS AND ITS REVERSION BY CHEMOSENSITIZERS

The ATP-binding cassette (ABC) superfamily of membrane transporters is one of the largest protein classes known from archaeobacterias to higher eukaryotes [11] (see http://nutrigene.4t.com/humanabc.htm). These proteins use the energy of ATP hydrolysis to either import or export a wide range of substrates, ranging from small inorganic ions to large peptides, sugar, lipids or drugs [11]. Many ABC transporters like some permeases that import aminoacids and sugars in bacteria, possess specific substrate specificities and may require a periplasmic binding protein to facilitate the transport. On the contrary, some other transporters are capable of recognizing and removing a large number of chemically unrelated lipids and toxins directly from the cell membrane. Many of these transporters translocate useful cytotoxics as anti-cancer drugs [12].

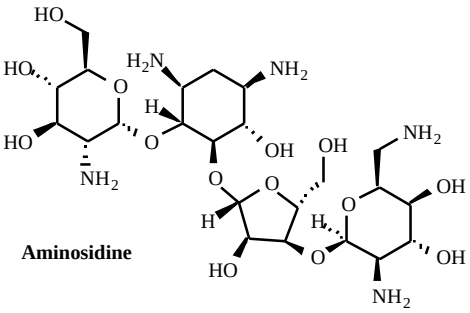
The Pgp, or human MDR1/ABCB1, is probably the best studied ABC drug efflux transporter to date [13]. Its amino acid sequence is arranged in two homologous halves connected by a linker region [12]. Each half is formed by a cytosolic nucleotide-binding domain (NBD) and a transmembrane (TM) domain (TMD) containing six α-helices (Fig. **2**). The NBD, highly conserved among ABC transporters, is the hallmark feature of this family of proteins, and is responsible for ATP binding and hydrolysis. Both TMDs are responsible for binding and transport the substrate, against considerable concentration gradients, across the cell membrane. Biochemical studies support that the two halves are in a "head-to-tail" arrangement [14], with TM4-6 of TMD1 and TM9-12 of TMD2 forming the drug-binding pocket [15, 16]. The 10 Å low-resolution structure of Pgp indicates that large conformational changes occur in the



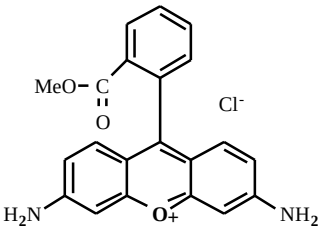
Amphotericin B



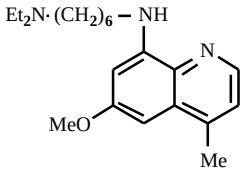
Mitoxantrone



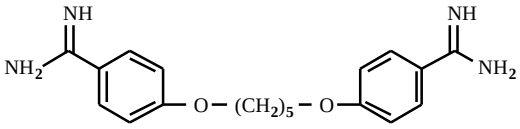
Aminosidine



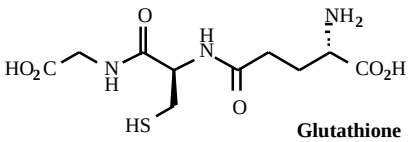
Rhodamine 123



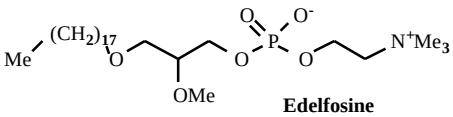
Sitamaquine



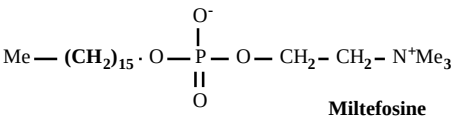
Pentamidine



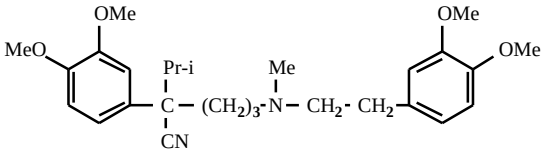
Glutathione



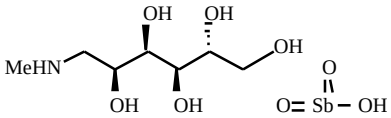
Edelfosine



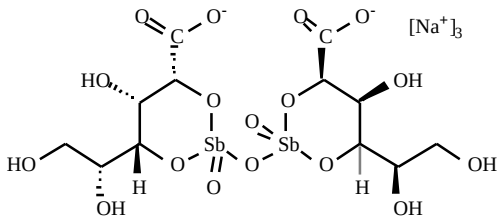
Miltefosine



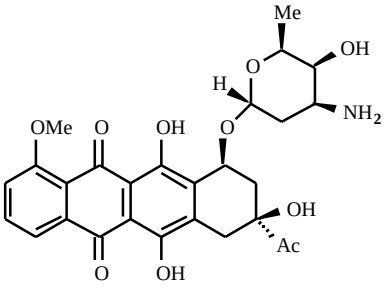
Verapamil



Glucantime



Pentostam



Daunomycin

Fig. (1). Chemical structures of antileishmanial drugs, ABC substrates and reversal agents of ABC transporters.

TMD during substrate transport [17]. The energy of the hydrolysis of two molecules of ATP, which do not occur simultaneously, is necessary for this large conformational change. The hydrolysis of the first ATP molecule is employed in the conformational change required to transport the substrate to the extracellular space, and the second one in effecting conformational changes to reset the pump to the initial state for the next catalytic cycle [18].

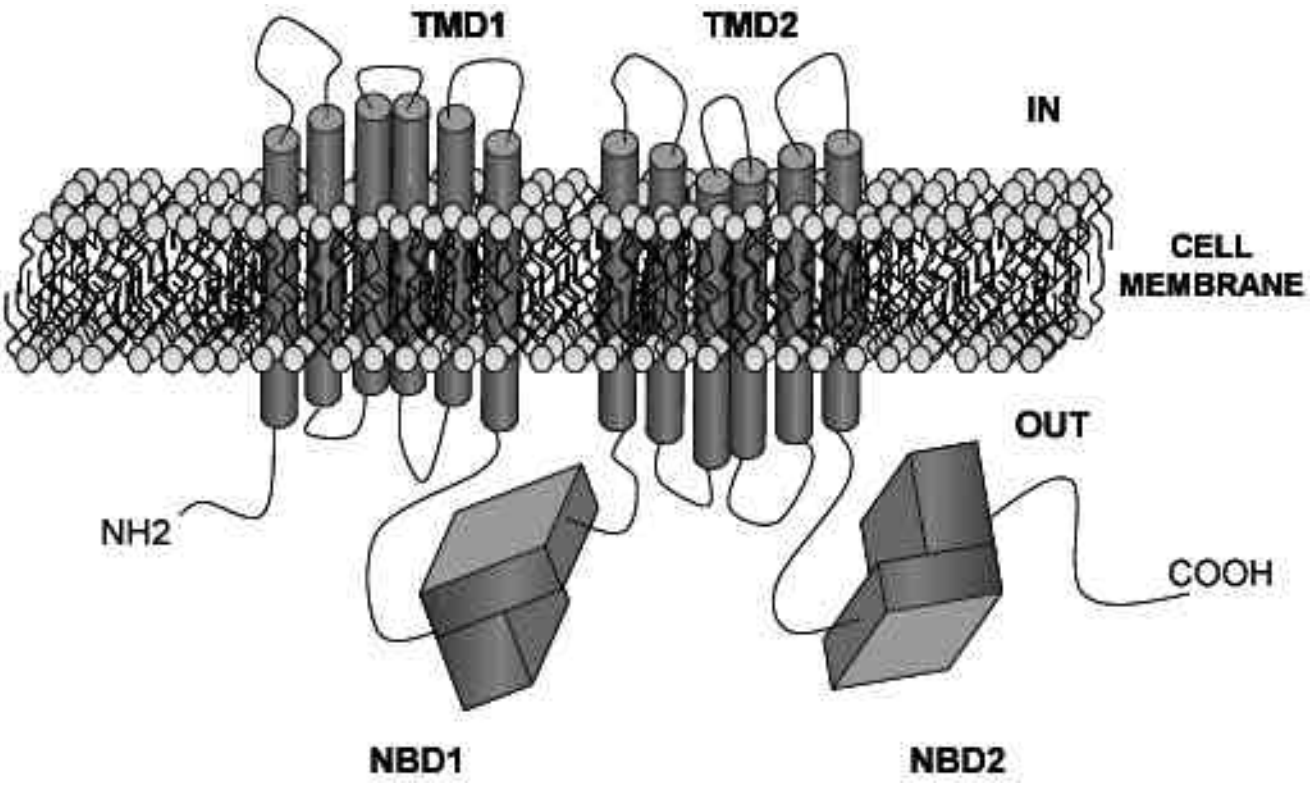


Fig. (2). Membrane topology model of a typical ABC transporter. NBD: nucleotide-binding domain; TMD: transmembrane domain.

Pgp has been proposed to act as a "hydrophobic vacuum cleaner" because of its ability to remove both lipids and drugs as they pass through the cell membrane [19]. Its most striking property is the structural diversity of compounds that this protein is able to transport. Pgp effectively transports many cytotoxic drugs used in cancer chemotherapy and, as a consequence, cells expressing this transporter display *in vitro* cross-resistance to many cytotoxics [20]. Moreover, Pgp express in many human cancers, including leukemias and solid tumours and some studies have demonstrated that expression of Pgp correlates with response to therapy and survival [21, 22]. The only common denominator identified so far in all the Pgp substrates is their amphipathic nature.

The phenomenon of transporters-mediated MDR is not at all restricted exclusively to malignant tumours. Pathogenic microorganisms as well as human cancer cells have developed various biological mechanisms against cytotoxic attack by chemotherapeutics, and some of them are also able to confer MDR phenotype against several unrelated drugs that differ widely with respect to molecular structure and target specificity. The notion that ABC transporters are involved in resistance to antiprotozoal drugs comes mainly from the facts that enhanced drug extrusion has been observed in chloroquine-resistant *P. falciparum* [23], that such chloroquine-resistance in *Plasmodium* may be modulated *in vitro* by the classical human Pgp modulator verapamil [23], and that arsenite efflux in *Leishmania* is also reversed by verapamil [24] (Fig. **1**).

The fact that Pgp is not expressed in all MDR cells let to the identification of the MDR-associated protein 1 (MRP1). MRP1 shares with Pgp both TMDs and NBDs but contains five additional membrane spanning domains at the N-terminal moiety of the protein [25]. MRP1 is able to cotransport hydrophobic drugs together with glutathione, and anionic drugs in conjugation with glutathione [26].

Overexpression of breast cancer resistance protein (BCRP) or mitoxantrone-resistance gene (MXR) was observed in certain mitoxantrone-resistant cell lines and tumors [27]. This transporter is formed by a single NBD and TMD. However, it is thought to form homodimers.

The search for ABC-MDR transporters in parasitic protozoa has revealed the existence of at least 20 ABC drug transporters, and the ongoing genome sequencing projects (see http://www.ebi.ac.uk/parasites/parasite-genome.html for updated information) may discover new ones. Three different classes of ABC transporters have been described in *Leishmania* parasites, but only two of them are related to MDR [28, 29]. The first class is homologous to the human multidrug resistance-associated protein (MRP) subfamily, related with drug resistance to antimony and other compounds [30]. The second group comprises transporters with higher homology with mammalian Pgp that confer a MDR phenotype similar to that observed in cancer cells [20, 21]. These Pgp-like genes have been found in *Entamoeba histolityca* [31], *Trichomonas vaginalis* [32], *Plasmodium falciparum* [33] and in several *Leishmania* species selected for resistance against substrates of the mammalian Pgp: in a step-wise selected vinblastine-resistant *L. donovani* (*LdMDR1*) [34]; in vinblastine-resistant *L. enrietti* (*LeMDR1*)

[35], and in daunomycin-resistant *L. tropica* (*LtrMDR1*) [36] and *L. amazonensis* (*LaMDR1*) [37].

The precise mechanism by which *Leishmania* MDR1 confers drug resistance is still a matter of investigation. A decrease of intracellular accumulation of rhodamine 123 [38] and daunomycin [36] (Fig. **1**) has been observed and, therefore, it is supposed that resistance to these hydrophobic drugs is due to drug efflux, as described for mammalian Pgps. However, recent evidences show that this drug transporter localizes in a number of intracellular secretory and endocytic compartments (mainly endoplasmic reticulum and in the multivesicular tubule named as MVT) rather than in the cell membrane [39], as it occurs with mammalian Pgp. This finding could suggest that *Leishmania* MDR1 may confers MDR phenotype by a mechanism different from the conventional efflux mechanism across the plasma membrane, as observed for mammalian Pgp MDR1. This would imply a combined mechanism of relocation of the drug away from its intracellular targets by transport/sequestration into intracellular vesicles and, finally, exocytosis out of the parasite.

4. REVERSION OF *LEISHMANIA* MDR1-MEDIATED MDR PHENOTYPE

Despite the previous observation suggesting that some Pgp-like protozoan MDR transporters were modulated in some extent by the classic human Pgp modulator verapamil, further research has demonstrated that this and other classic human Pgp inhibitors modulate only poorly the MDR phenotype in protozoan parasites [40]. This fact together with the implication of *Leishmania* Pgps in resistance to the promising last-generation leishmanicidals ALP [10] justify the important of developing inhibitors of these ABC transporters.

In the last years, there has been a great clinical interest in developing inhibitors of Pgp in order to revert the MDR phenotype. The search for inhibitors of human Pgp able to block this transporter in cancer cells has yielded promising third-generation modulators [41], and some of them have entered clinical trials [42-45].

Focusing in *Leishmania* Pgp, we have previously described the use of flavonoids and sesquiterpenes as reversal agents of MDR phenotype in *Leishmania* [40]. We have also shown that flavonoids interact with purified recombinant cytosolic domains displaying a significant correlation between the affinity of *in vitro* binding to the NBD and the efficiency of both *in vivo* modulation of drug efflux and reversion of the MDR phenotype on a *L. tropica* MDR line [46]. Modulation of *Leishmania* Pgp is, therefore, not a naïve pretension.

5. PLANTS OF THE CELASTRACEAE FAMILY AS A SOURCE OF BIOACTIVE METABOLITES

Natural products play a highly significant role in lead discovery for drug development in the treatment of human diseases. This is particularly evident in the areas of cancer and infections diseases, where over 60% and 75% of these drugs, respectively, are of natural origin. Additionally, natural products have been extensively used to elucidate complex cellular mechanisms, including signal transduction and cell cycle regulation leading to the identification of important targets for therapeutic intervention. As a result of recent advances in biology, there is now an increased demand for new natural product-like small molecules. Specifically, the fields of genomics and proteomics promise the rapid identification of a large number of new targets for which small molecule modulators will be of both biological and medicinal interest [47-49].

The Celastraceae family is indigenous to tropical and subtropical regions of the world, including North Africa, South America, and many parts of East Asia, particularly China. The family constitutes approximately 88 genera and 1300 species of plants. These plants generally grow as small trees, bushes, or lianas and have resinous stems and leaves. This family contains several species with claims that they are useful in medicine and folk agriculture; indeed, the variety of properties attributed to crude plant extracts of the Celastraceae is astonishing. Thus, extracts of the bark of the thunder-god vine *Tripterygium wilfordii* have been used by the Chinese for centuries to combat life-threatening illness, and have recently found application in the treatment of leukemia and rheumatoid arthritis. Similarly, the leaves of the "khat" bush *Catha edulis*, which grows in certain parts of East Africa and Southern Arabia, have as principal physiological effects the appetite suppression and stimulant action, with dependence being psychological rather than physical. *Euonymus europaeus* the sole representative of this genus in central and western Europe have been used as a cardiotonic, emetic and purgative and also against insects and vermin. In several areas of the subandean rain forest in the Amazonian river basin a plant known as "chuchuhuasha" or "chuchuaso" is used by several tribes for the treatment of rheumatism, as anti-arthritic, anti-inflammatory and as an aphrodisiac; for topical use it is employed in skin cancer and also for the treatment of sores. *Maytenus* species considered to be used as "chuchuhuasha" are *M. chuchuhuasha, M. krukonovii, M. colasii* and *M. laevis*. All these properties have catapulted the natural product chemistry of the Celastraceae into the limelight [50].

Over the last 30 years or so, a large number of secondary metabolites exhibiting a wide range of bioactivity have been extracted from the Celastraceae. In addition to numerous terpenoids, including a diverse array of sesquiterpenoids which constitute the focus of this article, various bioactive maytansinoids, phenylalkylamines, and flavonoids have also been isolated. The Celastraceae family took on a new lease of life in the seventies when the maytansinoids, compounds with exceptional antitumoral properties, were discovered; nonetheless, they were found to cause serious gastro-intestinal damage in rats, but recents advances in drug targeting offer new opportunities for clinical application. The phenylalkylamine (-)-cathinone has now been established to be the constituent responsible for the effects of the fresh "khat" leaves. On the other hand, flavonoids as quercetin, displays a wide spectrum of anti-oxidant and anti-inflammatory activity [51].

The bulk of the bioactive constituents of the Celastraceae, are terpenoids. Terpenoids are a structurally diverse class of secondary metabolites, which may be regarded as

derivatives of oligomers of the C_5 hydrocarbon isoprene (2-methylbuta-1,3-diene). Monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}) comprise two, three, four, and six isoprene units, respectively. All these types of terpenoids are found in extracts of the Celastraceae.

Monoterpenes such as β-thujone are volatile compounds responsible for the characteristic odour of *e.g.* freshy picked "khat" leaves. The tri-epoxide diterpenoid triptolide was isolated from the roots of *Tripterygium wilfordii,* and has been shown to be responsible for some of the significant anti-leukemic and anti-tumour activity of this plant. Triptolide also displays interesting male anti-fertility properties and is one of a number of diterpene epoxides contained in a commercially available "total multi-glycoside extract" of *T. wilfordii* that has been used clinically for male fertility control in China. The triterpenes described for the Celastraceae almost invariably belonged to the friedo-oleane, lupane, oleane, glutinane, taraxerane, ursane, and dammarane series. The widespread reports in recent years on their useful biological activities and indeed some practical applications of triterpenes, have made these metabolites more relevant and interesting. The triterpenoid quinonemethides constitute a relatively small group of unsaturated and oxygenated D:A-friedo-*nor*-oleananes, which are considered to be indicators of the family and coined the general name "celastroloids", and they have showed antileukemic, antitumor and antimicrobial properties [50, 51].

5.1. Structure Diversity of Sesquiterpenes from Celastraceae

The sesquiterpenes are the most widespread and characteristic metabolites of this family. Generally, they occur as polyesters of variously poly-oxygenated tricyclic scaffolds, all based on a core C_{15} skeleton known as dihydro-β-agarofuran [5,11-epoxy-5β,10α-eusdesm-4(14)-ene], and they are considered to be chemotaxonomic indicators of the family [52]. X-Ray data and conformational studies using molecular mechanics procedures showed that the *trans*-fused A and B rings formed a rigid *trans* chair-chair decalin system, slightly distorted by the presence of the 1,3-diaxial bond responsible for the tetrahydrofuran C ring, practically perpendicular to the plain formed by carbons C-5, C-7, C-8 and C-10. These core structures can have as few as two additional ester groups, as in the case of boariol, or as many as nine, as in the case of euonyminol. The esterifying residues vary widely from acetic and benzoic acids through more complex aliphatic and heterocyclic acids to stereochemically elaborate pyridine-containing dicarboxylic acids which form macrodilactone bridges between C-3 and C-12 and/or C-8 and C-15. The relative position, number, and configuration of these residues, distinguishes each individual sesquiterpene [51].

5.2. Biological Activities of Sesquiterpenes from Celastraceae

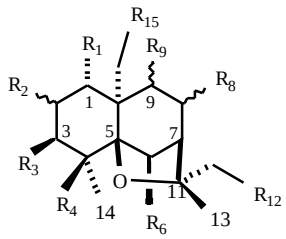
The interest generated by sesquiterpenes from Celastraceae has increased in line with the complexity of the substances isolated and, more importantly, with their wide range of biological actions, suggesting that derivatives of this sesquiterpene motif may be capable of interacting with a variety of cellular targets. They have attracted a great deal of interest because of their immunosuppressive [53], cytotoxic [54], insect-antifeedant [55] and insecticidal [56] and more recently, they have shown anti-HIV [57], reversal of MDR phenotype [58], and antitumor-promoting activities [59]. In addition, the fact that many of the compounds are active in cell-based assays suggested that products with a dihydro-β-agarofuran unit remain sufficiently lipophilic to cross cell membranes, a key feature of any biologically relevant compound. On the basis of the biological and structural properties, we propose the selection of sesquiterpene polyesters with a dihydro-β-agarofuran skeleton as a "privileged structure" [60]. This term describes selected structural types, like polycyclic heteroatomic systems capable of orienting varied substituent patterns in a well defined three-dimensional space and these structures represent a class of molecules capable of binding to multiple receptors with high affinity. The exploitation of these molecules should allow medicinal chemists to rapidly discover biologically active compounds across a broad range of therapeutic areas on a reasonable time scale [61]. The range of biological activities and their highly oxygenated tricyclic frameworks, comprising a number of contiguous stereocenters, associated with this intriguing class of natural products, pose a formidable synthetic challenge and have attracted the interest of synthetic chemist. Three synthetic approaches to obtain Celastraceae sesquiterpenes exist; first, early synthetic work on the agarofuran skeleton synthesis; second, synthetic approaches to polyoxygenated agarofuran skeletons; and third, enantioselective approaches. In addition to the synthesis of the core structure, the elaboration of the different patterns remains a challenge. To date there has been limited synthetic work carried out on the preparation of this sesquiterpenes [51, 62].

6. CHEMOSENSITIZATION OF A PGP-MDR1 *LEISHMANIA* LINE BY SESQUITERPENE*S*

In a search for MDR-reversing compounds from natural sources, diterpenoids [63, 64], flavonoids [65], naphthoquinones [66], steroids [67], and saponins [68], have being recently identified for their ability to inhibit MDR pumps. In addition, some analogues and/or derivatives of natural products have shown relevant reversal MDR effects [69, 70]. Interestingly, dihydro-β-agarofuran sesquiterpenes isolated from the root of *Celastrus orbiculatus*, were reported to partially or completely reverse MDR phenotype in human oral-epidermal (KB-V1) and breast (MCF7/ADR) cancer cell lines [58].

Therefore, as part of an ongoing project to identify plant natural products from Celastraceae, which modulate parasites MDR, we decided to analyze the *Leishmania* MDR reversal effect of fifty-eight agarofuran sesquiterpenes from the medicinal plants *Crossopetalum tonduzii*, *Maytenus macrocarpa*, *Maytenus magellanica*, *Maytenus chubutensis*, *Maytenus canariensis*, *Maytenus chiapensis*, and *Maytenus cuzcoina* (Table **1**) [10, 71-74]. Most of the analysed compounds showed low intrinsic toxicity (Fig. **3**) and many of them were able to efficiently overcome the MDR phenotype in the resistant line by modulating drug accumulation (Fig. **4**), being MAMA 7, MAMA10, MACHU 4, MACU 5, MACU 7 and MACU 8 the compounds that showed the highest reversal activity (Fig. **5**).

Table 1. Structure of the Studied Sesquiterpenes



Sesquiterpene	NP[a]	R1	R2	R3	R4	R6	R8	R9	R12	R15
CROTO 1	1[b]	OH	αONic	H	OH	OH	βOAc	αOBz	H	OMeBut
CROTO 2	2[b]	OAc	αOAc	H	OH	OAc	βOAc	αOBz	H	OMeBut
CROTO 3	3[b]	OAc	αOAc	H	OH	OH	βOAc	αOBz	H	OMeBut
CROTO 4	4[b]	OAc	αOAc	H	OH	OAc	αONic	βOBz	H	OMeBut
CROTO 5	5[b]	OAc	H	H	OH	OAc	αONic	βOBz	H	OMeBut
CROTO 6	6[b]	OBz	αOH	H	OH	OAc	βOAc	αOBz	H	OMeBut
CROTO 7	7[b]	OBz	αOH	H	OH	OH	βOAc	αOBz	H	OMeBut
CROTO 8	8[b]	OAc	αOH	H	OH	OH	βOMeBut	αOBz	H	OMeBut
MACRO 9	9[b]	OAc	H	H	OH	OAc	βOAc	αOBz	H	OAc
MAMA 1	6[c]	OBz	βOAc	ONic	OH	OAc	H	βOCin	H	H
MAMA 2	7[c]	OBz	βOAc	ONic	OH	OAc	H	βOBz	H	H
MAMA 3	8[c]	ONic	βOAc	OAc	OH	H	βOBz	βOBz	H	H
MAMA 4	9[c]	ONic	βOAc	OBz	OH	H	βOAc	βOBz	H	H
MAMA 5	10[c]	OBz	βOAc	OAc	OH	H	βOBz	βOBz	H	H
MAMA 6	11[c]	OBz	βOAc	OAc	OH	H	βOAc	βOBz	H	H
MAMA 7	3[c]	OCin	βOAc	ONic	OH	H	H	βOBz	H	H
MAMA 10	1[c]	OBz	βOAc	H	OH	H	H	βOCin	H	H
MAMA 11	2[c]	OBz	βOAc	H	OH	H	H	βOBz	H	H
MAMA 12	14[c]	OAc	αONic	H	OH	OBz	H	βOBz	H	OAc
MAMA 13	12[c]	OBz	αONic	H	H	OAc	βOAc	βOBz	H	H
MAMA 14	13[c]	OBz	αOBz	H	OH	OAc	βOAc	βOBz	H	H
MACHU 1	16[c]	OBz	βOAc	OH	H	OAc	H	βOBz	H	H
MACHU 4	15[c]	OAc	αONic	H	H	OBz	H	βOBz	H	OAc
MACA 3	C3[d]	OAc	H	H	OH	OAc	αOMeBut	αOBz	H	OAc
MACHI 1	1[e]	OAc	αOAc	H	OH	OAc	C=O	αOAc	H	OAc
MACHI 2	5[e]	OAc	αOAc	OAc	OH	OAc	αOAc	αOAc	OAc	OAc
MACHI 3	2[e]	OAc	αOAc	H	OH	OH	C=O	αOAc	H	OAc
MACHI 4	3[e]	OAc	αOH	H	OH	OH	C=O	αOAc	H	OAc
MACHI 5	4[e]	OAc	αOAc	OAc	OH	OAc	C=O	αOAc	OAc	OAc
MACU 1	4[f]	OAc	αOBz	H	OH	OFu	H	βOFu	H	H
MACU 2	1[f]	OAc	αOFu	H	OH	OFu	H	βOFu	H	H
MACU 2H	11[f]	OAc	αOFu	H	OH	OH	H	βOFu	H	H
MACU 2Ac	12[f]	OAc	αOFu	H	OH	OAc	H	βOFu	H	H
MACU 3	9[f]	OAc	H	H	OH	OFu	H	βOFu	H	H
MACU 4	6[f]	OAc	αOMeBut	H	OH	OFu	H	βOFu	H	H
MACU 5	2[f]	OAc	αOAc	H	OH	OFu	H	βOFu	H	H
MACU 5H	13[f]	OAc	αOAc	H	OH	OH	H	βOFu	H	H

(Table 1) contd….

Sesquiterpene	NP[a]	R1	R2	R3	R4	R6	R8	R9	R12	R15
MACU 5Ac	14[f]	OAc	αOAc	H	OH	OAc	H	βOFu	H	H
MACU 6	5[f]	OAc	αOPr	H	OH	OFu	H	βOFu	H	H
MACU 7	7[f]	OAc	αOAc	H	OH	OFu	H	βOFu	H	OAc
MACU 8	8[f]	OAc	αOAc	H	OH	OBz	H	βOBz	H	OAc
MACU 9	3[f]	OAc	αOH	H	OH	OFu	H	βOFu	H	H
MACU 15	17[f]	OAc	αOAc	H	OH	ONap	H	βOFu	H	H
MACU 16	18[f]	OAc	αOAc	H	OH	OPiv	H	βOFu	H	H
MACU 17	16[f]	OAc	αOAc	H	OH	OLau	H	βOFu	H	H
MACU 17Ac	15[f]	OAc	αOAc	H	OH	OMeBut	H	βOFu	H	H
MACU 18	19[f]	OAc	αOAc	H	OH	OTFAc	H	βOFu	H	H
MACU 19	20[f]	OAc	αOAc	H	OH	4-MeO-Bz	H	βOFu	H	H
MACU 20	21[f]	OAc	αOAc	H	OH	4-NO2-Bz	H	βOFu	H	H
MACU 21	22[f]	OAc	αOBz	H	OH	OH	H	βOFu	H	H
MACU 22	23[f]	OAc	αOBz	H	OH	OAc	H	βOFu	H	H
MACU 23	26[f]	OAc	αOBz	H	OH	ONap	H	βOFu	H	H
MACU 24	27[f]	OAc	αOBz	H	OH	OPiv	H	βOFu	H	H
MACU 25	25[f]	OAc	αOBz	H	OH	OLau	H	βOFu	H	H
MACU 25Ac	24[f]	OAc	αOBz	H	OH	OMeBut	H	βOFu	H	H
MACU 26	28[f]	OAc	αOBz	H	OH	OTFAc	H	βOFu	H	H
MACU 27	29[f]	OAc	αOBz	H	OH	4-MeO-Bz	H	βOFu	H	H
MACU 28	30[f]	OAc	αOBz	H	OH	4-NO2-Bz	H	βOFu	H	H

[a]Number assigned in the corresponding publication: [b][71], [c][72], [d][10], [e][73], [f][74].

Firstly, we studied the *Leishmania* MDR reversal effect produced by agarofuran sesquiterpenes isolated from *C. tonduzzi* [71]. Compounds CROTO 5-8, at 15 μM, produced in the presence of daunomycin (DNM) more than 75% growth inhibition (GI) in the MDR line, while compounds CROTO 1-3, and MACRO 9 required two-fold higher concentrations for similar effect. In addition, compound CROTO 5 efficiently modulated DNM accumulation in the *Leishmania* resistant line (unpublished results). From these results, we deduced some preliminary information concerning structure-activity relationships:

a) The presence of the substituent at the C-2 position plays an important role in reversal activity. Thus, compound CROTO 5 that contains a proton on C-2 produced three-fold higher chemosensitization relative to compound CROTO 4 that contains an acetate group at this position. Interestingly, the also active compounds CROTO 6-8 contain hydroxyl groups at this C-2 position whereas the less active compounds CROTO 1-3, and MACRO 9 contain an acetate or a nicotinate group. This supports the importance of the substituent at this position. b) The esterification of C-1 increased the activity, as deduced from the lower reversal effect of compound CROTO 1, (please note, however, the additional substitution at the C-2 position of this compound). c) The presence of a benzoate group at the C-1 position seemed to increase the cytotoxic effects as deduced for the effects of compounds CROTO 6 and CROTO 7 on the wild-type line.

These findings led us to engage a study with new dihydro-β-agarofuran sesquiterpenes isolated from *M. magallenica* and *M. chubutensis* as modulators of MDR phenotype mediated by Pgp-like transporter in *Leishmania* [72]. These compounds have the C-2 position esterified, at alpha or beta configuration and, no substituent at the C-15 position with the exception of compounds MAMA 12 and MACHU 4. We observed a good correlation between the ability of these sesquiterpenes to modulate Calcein-Acetoxy methyl esther (Cal-AM) accumulation in the resistant line and their reversal effect on drug resistance phenotype. Thus, Cal-AM was an excellent dye to monitor the pump modulation as described for mammalian Pgp. The following structure-activity relationships were also obtained:

a) The substituent at the C-6 position is essential for reversal activity, since the presence of a hydrogen in compound MAMA 7 produced a 10-fold higher chemosensitization than an ester group in compound MAMA 1. b) The type of substituent at C-1 also modified the reversal activity, since compound MAMA 5 was two-fold more active than compound MAMA 3. However, contrary to that observed in compounds from *C. tonduzzi*, the benzoate ester at this position did not contribute to the intrinsic cytotoxicity of the compound in the parental wild-type line. c) Finally, the frequent tertiary hydroxyl group at C-4 did not seems to be important for the optimal reversal activity, since its presence or absence did not produce any significant modification of activity (MAMA 12 versus MACHU 4).

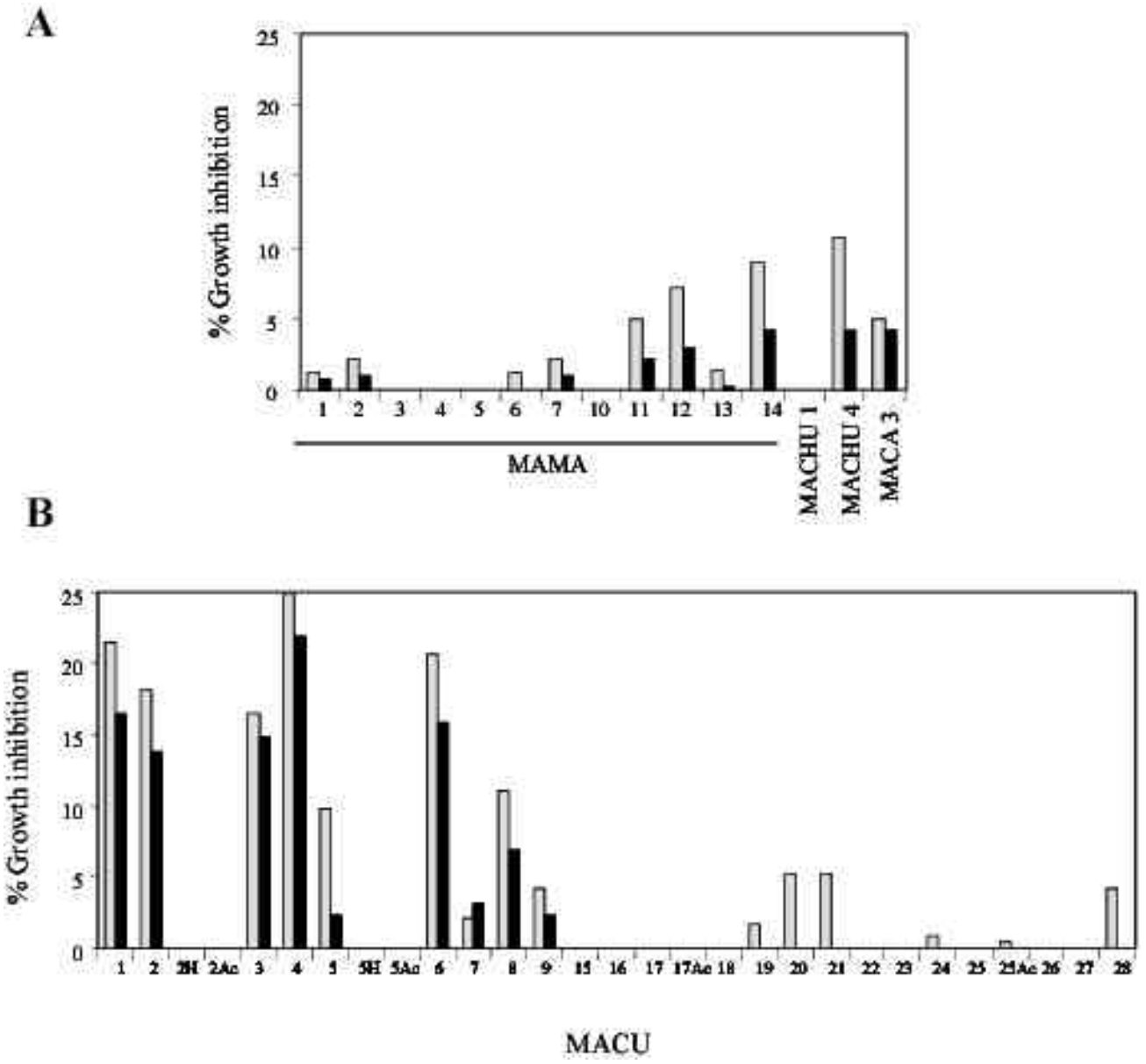


Fig. (3). Effect of sesquiterpenes on the growth of wild-type *L. tropica* line (intrinsic toxicity). The results are expressed as percentage of growth inhibition (%GI) observed after incubation at 28ºC for 72h with different sesquiterpenes, at 7μM (grey bars) and 3μM (black bars), compared to control growth in the absence of sesquiterpene. A: effect of sesquiterpenes MAMA, MACHU and MACA. B: effect of sesquiterpenes MACU.

Sesquiterpene MACA 3, isolated from *M. canariensis*, was also assayed as a reversal agent of MDR phenotype in *Leishmania* [10]. This compound was able:

a) to efficiently overcome the MDR phenotype in the *L. tropica* resistant line, including ALPs resistance; b) to modulate DNM accumulation in this line reaching similar levels as the wild-type line; c) to increase in a similar way the accumulation of bodipy-C5-PC, a fluorescent analogue of phosphatidylcholine.

In more recent studies, compounds MACHI 1-5, isolated from *M. chiapiensis*, were tested against MDR *L. tropica* line to determine their ability to revert the resistance phenotype [73]. From this series, only compound MACHI 5 showed weak activity (with a 28% GI at 60 μM). Thus, any of the following factors might impede their activity: a) the compounds are too bulky to bind at the active site, b) they are only acetate-substituted, c) the substituent in compounds MACHI 1-4 at the C-8 position modifies the pucker of the B ring and the position of the other substituents, or/and d) these compounds possess the lowest solubility in lipids, among the tested ligands, as estimated by their logP (unpublished results), impeding the access to the active site.

Finally, twenty-nine dihydro-β-agarofuran sesquiterpenes isolated from the leaves of *M. cuzcoina* or semisynthetic

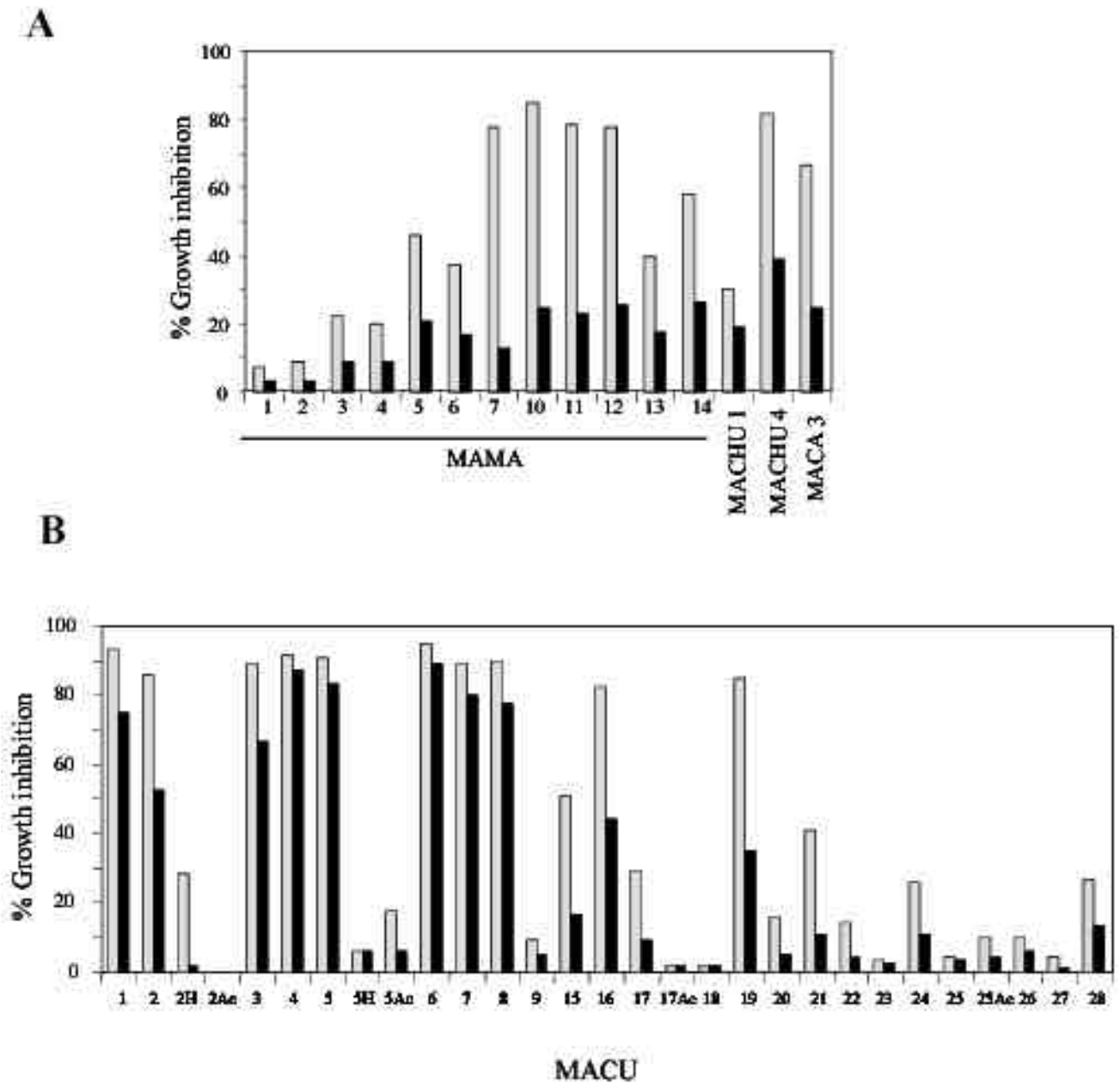
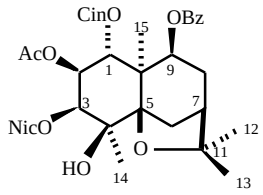


Fig. (4). Chemosensitization to DNM of MDR *L. tropica* line by sesquiterpenes. The results are expressed as percentage of growth inhibition (%GI) observed after incubation at 28ºC for 72h with different sesquiterpenes at 7μM (grey bars) and 3μM (black bars) in the presence of 150 μM DNM, compared to control growth in the absence of sesquiterpene. A: effect of sesquiterpenes MAMA, MACHU and MACA. B: effect of sesquiterpenes MACU.

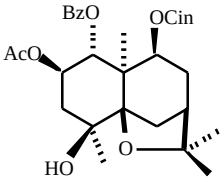
derivatives have been tested on a MDR *Leishmania tropica* line overexpressing a Pgp transporter to determine their ability to revert the resistance phenotype and to modulate intracellular drug accumulation [74]. Almost all natural compounds showed potent reversal activity with different degrees of selectivity. Sesquiterpenes MACU 5, MACU 7, and MACU 8 showed a high reversal activity on the DNM-resistant phenotype in *Leishmania* with values of GI at 3 μM higher than 75%, and very low values of intrinsic toxicities. These compounds are, at present, the most effective reversal agents tested against the MDR phenotype of *Leishmania*. The structure-activity relationships from these sesquiterpenes revealed the following trends:

a) The substitution at the C-2 position is essential for reversal activity. Ester- substituted compounds at this position exhibited high potent activity (MACU 1-2, and MACU 4-6); the hydrogen-substituted compound leads to potent activity (MACU 3); and the hydroxyl-substituted compound very low activity (MACU 9). Among the ester -substituted acetate is the most potent. b) The presence of a hydroxyl group (MACU 2H, MACU 5H, and MACU 21) or acetate

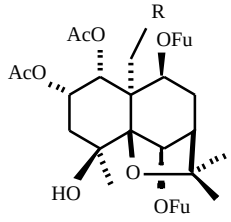
group (MACU 2Ac, MACU 5Ac, and MACU 22) at the C-6 position has a detrimental effect in the activity with respect to the presence of a furoate group (MACU 1, MACU 2, and MACU 5). Any other tested substituent resulted in a substantial loss in activity. c) Replacement of the methyl group at the C-15 position compound (MACU 5) by acetate (MACU 7) does not modify the activity. d) Compounds MACU 7 and MACU 8, which contain furoate or benzoate group at the C-6 and C-9 positions respectively, showed similar reversal activity. e) Replacement of the acetate group at the C-2 position (compound MACU 8) by nicotinate (compound MAMA 12), showed a slight decrease in the activity. f) The regiosubstitution is an important element for activity, since MACU 2Ac is complete inactive and MACU 5 shows strong activity, having the same molecular formula, and being, the only difference the presence of acetate or furoate at the C-2 and C-6 positions.



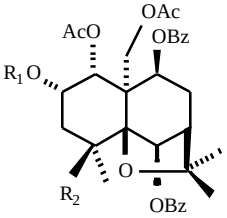
MAMA 7



MAMA 10



MACU 5 R =H
MACU 7 R=OAc



MACHU 4 R_1 =Nic R_2 =H
MACU 8 R_1 =Ac R_2 =OH

Fig. (5). The most active sesquiterpenes as reversal agents of the MDR phenotype of *Leishmania*.

7. PHARMACOPHORE MODEL OF SESQUITERPENES AS REVERSAL AGENTS

Three-dimensional quantitative structure-activity relationships using the comparative molecular similarity indices analysis (CoMSIA) were employed to characterize the steric, electrostatic, lipophilic, and hydrogen bond donor and acceptor requirements of the MACU series of sesquiterpenes as modulators of the Pgp transporter [74]. Fig. **6** shows the obtained structural elements of the ligands that are key for high reversion of the MDR phenotype of *Leishmania*. The carbonyl group of either the OFu, OAc, OBz, OPr, and OMeBut substituents at the C-2 position acts as a H-bond acceptor in the H-bond with an area of the receptor depicted as a green sphere. The oxygen of the furan ring at the C-6 position seems to form a hydrogen bond with the receptor (see green sphere). The C-H moieties of the furan ring at the C-6 position are also involved in the interaction with the receptor (grey sphere). Moreover, the CH_2OAc group at the C-15 position in compound MACU 7 is engaged in the H-bond interaction with an area of the receptor depicted as a green sphere.

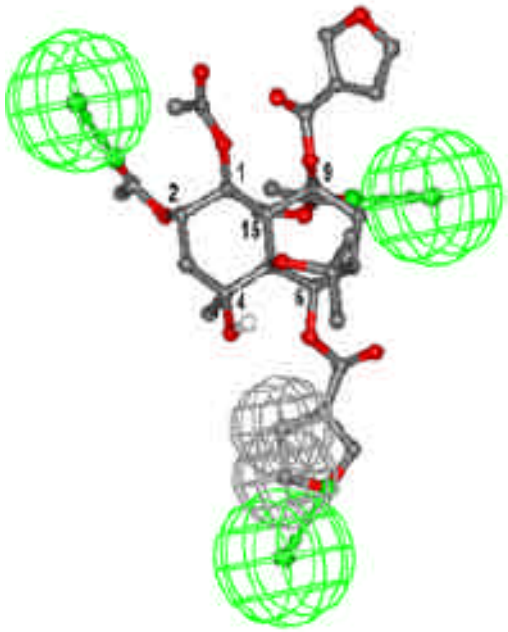


Fig. (6). Structural elements of sesquiterpenes that are key for their specific function as active modulators of the *Leishmania* Pgp transporter. Green spheres represent areas of the receptor that interact with H-bond-acceptor moieties of the ligand. Grey spheres represent areas of the receptor that interact with hydrophobic moieties of the ligand.

Several other pharmacophore models have been reported for MDR related drugs [75-77]. These models were obtained for Pgp inhibitors of very different chemical classes and, thus, there are significant differences in the defined structural properties. This is reasonable because of the extremely broad diversity of MDR reversal agents that suggests several binding sites and binding modes. However, these models share the importance of both hydrogen bond donors and acceptors, hydrophobic, and aromatic interactions in inhibitory binding to a Pgp-like transporter.

The data obtained in our structure-activity relationship study among sesquiterpenes show tendencies in agreement with the above pharmacophore models. We observe some trends that seem important for high reversal activity: a) the overall esterification level of the compounds; b) the presence of two aromatic ester moieties, such as benzoate-nicotinate, benzoate-cinnamate, benzoate-benzoate, and furoate-furoate; c) the size of the molecule, being sesquiterpenes tetra, penta or hexa substituted the most potent, and additional esters in the molecule leads to inactive compounds. In contrast to other models [78] the presence of a basic tertiary nitrogen atom is not essencial for MDR reversal activity.

Weak polar and hydrophobic interactions are believed to play an important role in stabilizing protein structures and ligand-receptor complexes binding. It has already been noted that the transmembrane segments of Pgp are rich in aromatic

residues, which are thought to play a functional role in Pgp. We, thus, believe that these weak polar interactions are the key for the binding of sesquiterpenes to Pgp.

8. TRANSMEMBRANE DOMAINS OF PGP-LIKE TRANSPORTERS AS NEW TARGET FOR SESQUITERPENES

In order to understand the nature of the reversal activity of dihydro-β-agarofuran sesquiterpenes, our group has also undertaken the elucidation of the molecular mechanism of action of these natural compounds at molecular level. We have shown that sesquiterpenes interact specifically to Pgp MDR1 within its TMDs [79], modifying the substrates drug binding sites but not binding of the ATP fluorescent analogue TNP-ATP to the NBDs. As a result of such molecular interaction, sesquiterpenes potently block Pgp-mediated drug transport in living intact cells, plasma membrane from Pgp-overexpressing cells and proteoliposomes containing Pgp. Sesquiterpenes stimulates Pgp ATPase activity at very low concentrations and inhibits at higher concentrations. Thus, it seems reasonable to suggest that sesquiterpenes act as allosteric modulators of the ATPase activity through the existing coupling between the TMD and NBD [73].

The fact that these compounds exhibit similar MDR reversal effects in both mammalian and *Leishmania* cells overexpressing Pgp, and that these two proteins share a significant homology (37% of aminoacid sequence identity), suggest that sesquiterpenes may interact with both Pgps in a similar way. Molecular evidences that support this conclusion come from the finding that in fluorescence quenching experiments with the purified recombinant cytosolic domains NBD2 [46] and NBD1 (unpublished results) of LtrMDR1, sesquiterpenes showed no significant interaction with none of the NBDs. Meanwhile, some flavonoids interacted with high affinity to both purified NBDs from mammalian an *Leishmania* Pgp.

It is worth noting that our studies with sesquiterpenes in *L. tropica* MDR line did not show any hint of cross-resistance to these natural compounds [74], which suggests that LtrMDR1 is unable to transport sesquiterpenes. In fact, our work with a fluorescent analog of a natural sesquiterpene (MANT-CROTO 8) has demonstrated that, indeed, human Pgp did not transport this fluorescent sesquiterpene (unpublished results). This claims in favour of these natural compounds as efficient inhibitors of Pgp, since it is well known that good substrates of human Pgp are poor inhibitor of this transporter and viceversa [80], given that transported compounds behave as mere pseudo-substrates. In this way, to get an effective reversion, higher concentrations of such compounds would be needed and, therefore, undesirable side-effects would be produced, precluding their clinical use.

Despite the similar mechanism of sesquiterpenes in both Pgps, further research is still in progress in our laboratory to explain some differences between human and *Leishmania* Pgp inhibitory efficiencies found for some sesquiterpenes. Understanding these palpable differences will aid to the rational design of sesquiterpenes specific for each type of Pgp.

CONCLUDING REMARKS

Drug resistance represents the major impediment for the successful treatment of diseases produced by protozoan parasites. ABC transporters are involved, at least *in vitro*, in the resistance to many antiparasitic drugs. Therefore, the development of inhibitors of these transporters is of high clinical relevance. We described that natural and semisynthetic dihydro-β-agarofuran sesquiterpenes isolated from Celastraceae plants represent highly effective and specific modulators of the MDR phenotype in *Leishmania*, some of them could be considered as lead compounds for further reversal drug development. Due to lack of detailed structural information at the discrete atomic level about the tertiary structure of Pgp, 3D-QSAR/CoMSIA models have been employed to characterize the structural elements of sesquiterpenes that are key for their specific function as active modulators of the *Leishmania* Pgp transporter. We observe that the esterification level of the compounds, the presence of two aromatic ester moieties and the size of the molecule are important factors for the reversal activity. The recently determined structures [17, 81, 82] and homology models [83, 84] of ABC-transporters will contribute in the near future to our understanding of the mechanisms of MDR. Future studies using this information will be used for the design and hemisynthesis of more potent and less toxic reversal agents of the MDR phenotype of *Leishmania*.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Pgp = P-glycoprotein

MDR = Multidrug resistance

ABC = ATP-binding cassette

3D-QSAR = Three-dimensional quantitative structure-activity relationship

CoMSIA = Comparative molecular similarity indices analysis

TDR = Tropical Disease Research

HePC = Hexadecylphosphocholine

ALPs = Alkyl-lysophospholipids

NBDs = Nucleotide-binding domains

TM = Transmembrane

TMDs = Transmembrane domains

MRP = Multidrug resistance-associated protein

DNM = Daunomycin

GI = Growth inhibition

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