Chapter 10

Bioactive Component of Licorice as an Antileishmanial Agent

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Additional information is available at the end of the chapter

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Abstract

The term leishmaniasis encompasses a spectrum of vector-borne protozoan parasitic diseases ranging from self-healing cutaneous to fatal visceral leishmaniasis. The disease affects 12 million people worldwide with 0.5 million new cases per annum. Present antileishmanial chemotherapeutic drugs project limitations because of severe toxicity, lengthy regime and occurrence of resistance, thereby making development of newer, gentler and efficacious therapeutics an urgent need for treatment of leishmaniasis. Application of medicinal plants in treatment of refractory diseases is valued for its clinical efficacy and nontoxicity. The biologically active components derived from them continue to play important roles as chemopreventive agents. Licorice has been known for its medicinal property from ancient times for treatment of various ailments. 18β-Glycyrrhetinic acid, glycyrrhizic acid and licochalcone A are the most extensively studied constituents of licorice in terms of antileishmanial agent. Overall, this chapter is dedicated to highlight the current understanding of the mechanism of these bioactive constituents of licorice, which potentiates them as antileishmanial agents. Furthermore, it also brings to light the importance of folk medicine in curing diseases and thereby gives impetus to explore ancient medicines and thier mode of actions to use them progressively to cure diseases.

Keywords: 18β-glycyrrhetinic acid, glycyrrhizic acid, licochalcone A, antileishmanial, cytokines

1. Introduction

This chapter is dedicated to understand the mode of action of specific constituents of licorice, which have been isolated and characterized as antileishmanial agents. Among over 20 triterpi-

noids and 300 flavanoids present and characterized in licorice, only three, namely 18β-glycyrrhetinic acid, glycyrrhizin and licochalcone A, have been extensively studied for their antileishmanial properties. The structures of the compounds are given in **Figure 1**.

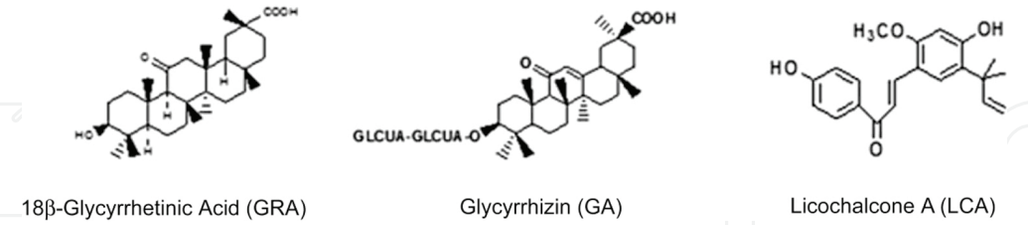

Figure 1. Structure of the characterized antileishmanial constituents of licorice [1].

1.1. Leishmaniasis

Before going into the mechanistic details of these compounds, a brief idea about leishmaniasis will help understand the infection biology and the target of action of these molecules. *Leishmania* is a protozoan parasite that causes a spectrum of diseases ranging from the self-healing cutaneous to fatal visceral leishmaniasis. The visceral leishmaniasis particularly affects the spleen, liver and bone marrow [2]. It is highly endemic in the Indian subcontinent and East Africa. It is second only to malaria in annual worldwide fatalities due to protozoal infections [3–4].

1.2. Life cycle

Leishmania parasites remain in nature by transmission between the mammalian hosts through infected female sandfly bite. *Leishmania* lifecycle is characterized by having three developmental stages: procyclic promastigotes, metacyclic promastigotes and the amastigotes (**Figure 2**).

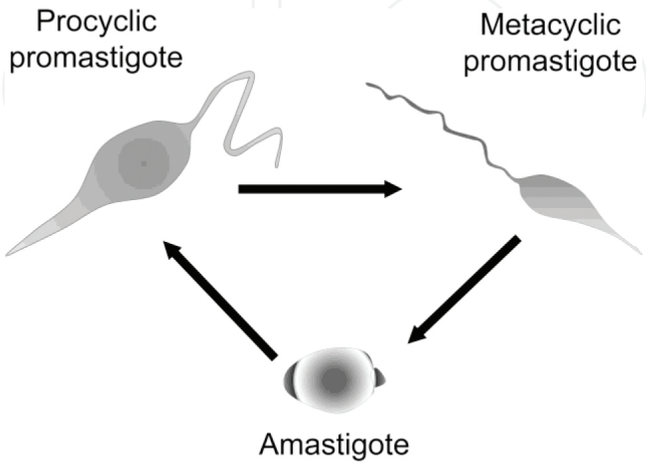

Figure 2. Lifecycle stages of *Leishmania sp* [2].

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1.3. Transmission

In the sandfly (*Phlebotomus* and *Lutzomyia* spp) gut, the promastigote form of the parasite is seen which subsequently transforms to the infective metacyclic form which are regurgitated and injected in the skin of the mammalian host. Infected promastigotes then transforms to the amastigote form in the mammalian host. In a subsequent blood meal of this infected host, the amastigote forms are taken up which then transforms to the promastigotes in the gut and the cycle continues (**Figure 3**).

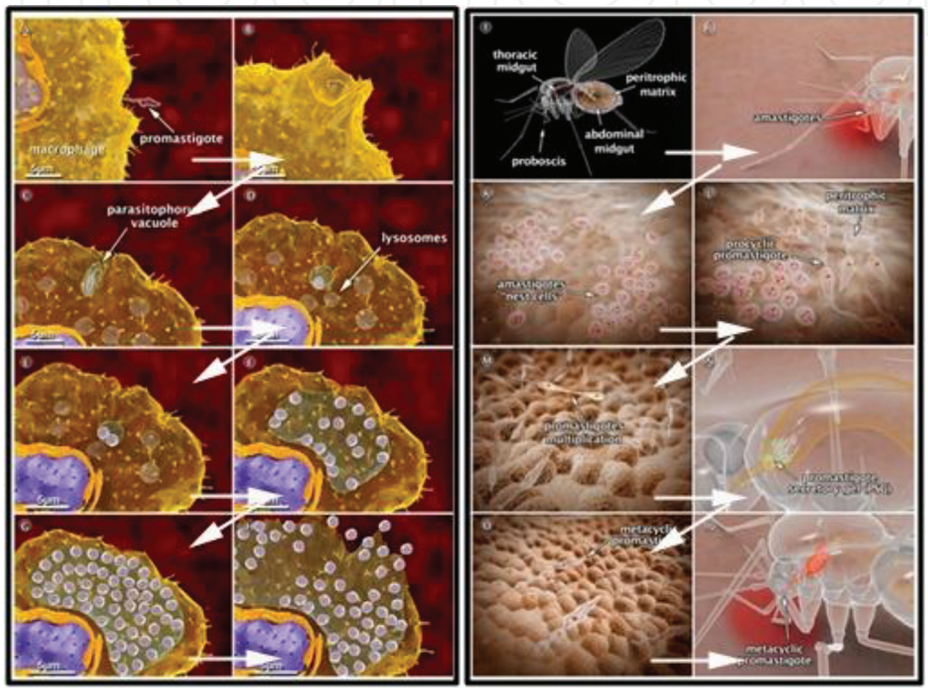

Figure 3. Schematic 3D view of the phases of interaction between the *Leishmania* parasite and sandfly and between the parasite and the vertebrate cells. "(A) Attachment of a promastigote to the macrophage surface. (B) The process of internalization via phagocytosis begins with the formation of pseudopods. (C) Leading to the formation of the parasitophorous vacuole (PV). In the PV, the promastigote transforms into an amastigote. (D) Recruitment and fusion of host cell lysosomes with the PV takes place. (E) In the PV, amastigotes divide several times. (F–G) Intense multiplication generates several hundreds of amastigotes. (H) The host cell bursts, and the parasites reach the extracellular space. (I) Schematic view of female sandfly showing the digestive tract. (J) During a blood meal, a female sandfly ingests infected macrophages with amastigote forms present in the blood of the vertebrate host. (K) Amastigotes form 'nest cells' in the abdominal midgut. (L) Amastigotes transform into procyclic promastigotes. (M) Promastigotes multiply and attach to the midgutepithelium. (N) Parasites migrate toward the anterior midgut, resume replication and start to produce promastigote secretory gel (PSG). (O) Promastigotes transform into infective metacyclic promastigotes. (P) Metacyclic promastigotes infect a new mammalian host via regurgitation during the blood meal. These images are based on micrographs obtained by scanning and transmission electron microscopy and by video microscopy" [5].

1.4. Disease manisfestation

Visceral leishmaniasis patients show signs of systemic infection which include fever, fatigue, weakness, loss of appetite and weight loss. The parasite invades the blood and reticuloendothelial system, such as enlarged lymph nodes, spleen and liver. Darkening of the skin, popularly known as kala azar, which means black fever in Hindi, is infrequent. Anemia which

is worsened by hypersplenism, leucopenia or thrombocytopenia, and hypergammaglobulinemia are characteristic. Untreated disease in any age group in time can produce profound cachexia, multisystem disease, bleeding, susceptibility to secondary infections and death [6].

1.5. Current treatment options

For visceral leishmaniasis, treatment is always recommended due to the fatal nature of the disease. The only treatment currently available for leishmaniasis relies on chemotherapy [7] as no vaccine has been successfully developed till date for humans. The first line of classical drug for treatment of leishmaniasis includes pentavalent antimonials, stibogluconate or meglumine antimonite. Pentavalent antimonials have been the drug of choice for more than 50 years [8–10]. Other common drugs are pentamidine, allopurinol, amphotericin B, imidazoles, miltefosine and paromomycin among others. Even though these drugs are effective other problems associated with these are (1) there lengthy regimens (weeks to months), (2) invasive modes of administration (intramuscular or intravenous) and (3) high drug-related toxicity along with the high cost of therapy which is beyond the capacity of many as this disease is known to be the 'disease of the poor'[10, 11]. All these factors together may lead to the patients discontinuing there therapies in the mid which have resulted in emergence of drug-resistant parasite strains adding to the list of problems associated with antileishmanial therapies [9, 11, 12].

To add to these overwhelming situations, emergence of coinfection collaterally with increasing incidences of HIV has decreased the number of options available to patients. As we will see in this chapter that immunosuppression is a key for parasite survival drugs, which can modulate host immune defenses, should be considered for effective treatment of leishmaniasis. Development of vaccines has remained a challenge; however, a canine vaccine has been developed, and it is in use in South America [13]. At present, no immunization options are available against leishmaniasis in humans.

1.6. Subversion of host defense

Like any other successful pathogens, *Leishmania* has also developed strategies to evade host immune mechanisms in order to survive within the host. A significant number of virulence factors discovered in *Leishmania* are directed against circumventing the host immune response. Apart from these, the parasite also has the ability to maintain a chronic infectious state within its host by modulation of regulatory factors, which exemplifies the extent on its immune evasion potential. Indeed, the ongoing battle between the robust immune response mounted by a host and the counter evasion strategies by the parasite ultimately decides the fate of the disease.

All these further establishes the fact that a chemotherapeutic alone is not enough to treat this intelligent parasite, and indeed an immunomodulator which can activate hosts own defense mechanism will be a better adjunct to the current line of treatment, which is relatively better in terms of toxicity, efficacy and mode of administration.

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2. 18β-Glycyrrhetinic acid (GRA)

18β-Glycyrrhetinic acid (GRA), a pentacyclic triterpene derivative of the β-amyrin type, is obtained from the roots of *Glycyrrhizza glabra* L. It is known to exhibit a variety of pharmacological effects like antiulcerative, anti-hepatotoxic, anti-tumorigenic and immunoregulatory activities [14–17]. As evident, GRA has potent immunomodulatory effects, thus its potency as an antileishmanial agent and the underlying mechanism was thoroughly explored.

2.1. Cytotoxicity

The first and foremost criteria for evaluation of a drug for its antiparasitic activity would be to evaluate its cytotoxic effects. The development of new drugs that has immunomodulatory properties requires both pharmacokinetic and toxicity studies to be carried out in conjunction to clinical verification. Our work so far has shown that GRA is relatively a nontoxic compound with up to 1.5 g/day consumption in humans [18].

GRA exhibited potent in vitro activity against intracellular *L. donovani* amastigotes (IC_{50}, 4.3 μg/ml); it was devoid of any obvious cytotoxicity on macrophage host cells, because the cytotoxic concentration causing 50% cell death was ~100 μg/ml [19]. The infected mice treated with GRA were completely cured. Moreover, this therapy was seen to be effective in mice with established disease-progressive Th2 response. After treatment and resolution of parasitism, the cytokine profile indicated a switch to a protective Th1 pattern associated with upregulation of NO [19].

2.2. Mechanism

2.2.1. Macrophage activation by NF-κB-mediated nitric oxide and proinflammatory cytokine production

Once the cytotoxic parameters were found satisfying the mechanistic profile was explored. The disease resolving property of GRA could be attributed to the production of NO and proinflammatory cytokines such as IL-12 [20, 21]. The most diversely studied mechanism that assists the *Leishmania* survival is modulating the macrophage cytokines production to bias the immune response to its benefit [22, 23]. *Leishmania* mainly inhibits the secretion of macrophage proinflammatory or disease resolving cytokines, which include interleukin 12 (IL-12), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and aids the secretion of anti-inflammatory or disease promoting cytokines interleukin 10 (IL-10), transforming growth factor beta (TGF-β) for its own benefit.

Cytokine IL-12 is downregulated by *Leishmania via* ligation with macrophage receptors [22, 23]. One of the most important initial signaling events is the release of IL-12 by the infected macrophage, leading to subsequent priming of the Th1 response and production of IFN-γ [5, 24–26]. *Leishmania* also upregulates the production of anti-inflammatory cytokines such as IL-10. IL-10 is important in suppressing macrophage leishmanicidal activity by opposing IFN-γ [22, 24, 27], nitric oxide (NO) and IL-12 production [28].

The killing of intracellular *Leishmania* parasites by GRA correlated with the induction of the iNOS pathway. Nitric oxide (NO) is known to mediate many of the cytotoxic and immunological effects upon pathogenic challenge. This NO production has been shown to be dependent upon the inducible for of the nitric oxide synthases (iNOS) [29], whereas under normal physiological conditions only the constituitive forms of NOS (cNOS) are functional. Induction of iNOS in turn is under transcriptional regulation of NF-κB, a group of transcription factors that belong to the Rel protein family. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, p65 (RelA) and p50 subunit [30]. In unstimulated cells, NF-κB is found in the cytoplasm bound to an inhibitor, inhibitor of nuclear factor κB (IκB)α and IκBβ [31]. This association prevents nuclear translocation of NF-κB and hence iNOS transcription and NO production. Upon pathogenic challenge, the large multisubunit protein kinase, inhibitor of nuclear factor kappa-B kinase subunit beta(IKK) causes rapid proteasomal degradation of IκBα, thereby releasing NFκB and allowing its nuclear translocation. This event is inhibited in *Leishmania donovani* infection thereby promoting parasite survival [19] (**Figure 4**).

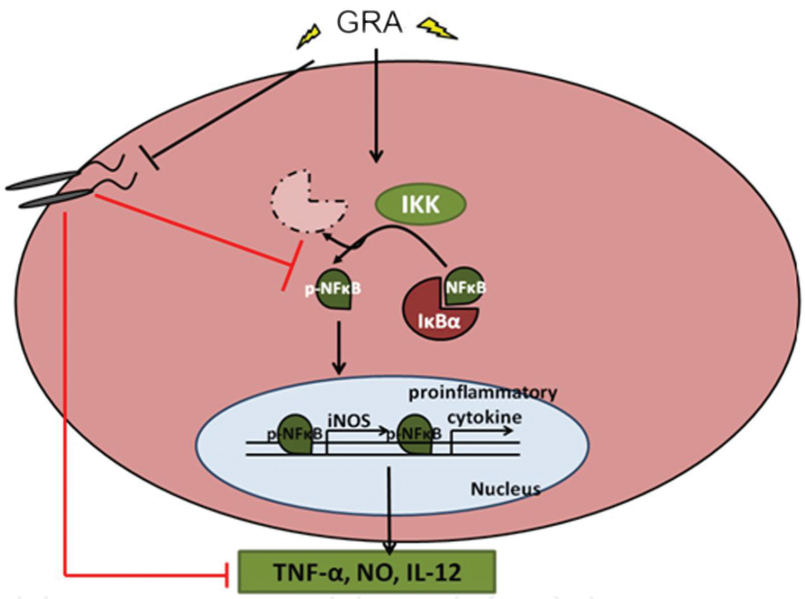

Figure 4. GRA promotes NF-κB-mediated parasite killing.

GRA, on the other hand, activates NF-κB through the regulation of genes essentially involved in encoding proinflammatory cytokines and inflammatory mediators such as NO. *L. donovani* infection suppressed NF-κB activation and translocation which was restored upon GRA administration via induction of IκBα phosphorylation. This in turn was achieved by modulating the upstream signal leading to IKK (inhibitor of nuclear factor kappa-B kinase subunit beta) activation, that is, without directly interfering with IKK (inhibitor of nuclear factor kappa-B kinase subunit beta). Overall this signaling activation by GRA led to degradation of IκBα leading to the translocation of NF-κB in the nucleus and transcriptional activation of iNOS and proinflammatory cytokines [19] (**Figure 4**). The antileishmanial activity of GRA was dependent on the iNOS activity and NO production was further justified by addition of a specific NOS

inhibitor, N^G-monomethyl-L-arginine (NMMA). Upon administration of NMMA along with GRA, a reduction in the parasite killing capability of GRA was seen and withdrawal of NMMA led to decreased parasite survival indicating a role of GRA-induced NO-mediated parasite killing.

2.2.2. MAPK and phosphatases

Mitogen-activated protein kinase(MAPK) plays an important role in activation of NF-κB. MAPK signaling cascades are rather complex events which ultimately results in manifold increase in stimulus-mediated responses. However, if this response remains unchecked, it may be detrimental for the cells and thus a balance between the activity of the kinases and phosphatases play important roles in physiological scenario. In macrophages, the MAPK (mitogen-activated protein kinase) cascade and the NF-κB pathway play important roles in the regulation of functions involved in inflammation and host defense. *L. donovani* successfully sabotage these pathways creating an anti-inflammatory milieu by inhibiting production of NO and proinflammatory cytokines thereby strengthening their existence within the macrophages [32]. Thus, the agents that can activate NF-κB pathway creating a proinflammtory milieu potent enough to kill the parasites might prove attractive candidates to control *Leishmania* infection. Our studies also revealed that the Mitogen-activated protein kinase kinase - extracellular signal regulated kinases(MEK-ERK) pathway.

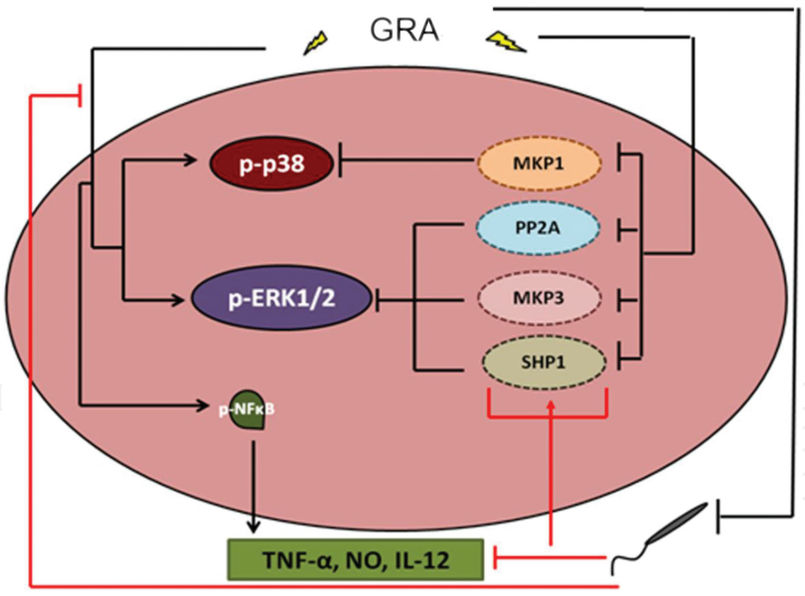

Figure 5. The balance between kinases and phosphatases is restored upon GRA treatment.

pathway is compensated in infected cells [33]. GRA, the triterpenoid is an ideal candidate both because of its pharmacologically safe parameters and immunomodulatory properties [19]. The switching of the immunological response was found to be dependent upon the MAPK (mitogen-activated protein kinase) activation among which only ERK and p38 were found to be regulated by GRA [34]. In *Leishmania* infection, these MAPK (mitogen-activated protein

kinase) activation was severely compensated which was in turn is attributed to the fact that activity of MAPK (mitogen-activated protein kinase) phosphatases, which dephosphorylate and thereby inactivate MAPK (mitogen-activated protein kinase), significantly increased. This corroborated with the fact that inhibition of SHP-1, a MAPK (mitogen-activated protein kinase) phosphatase led to stronger proinflammatory responses against infection [35]. However, GRA treatment could potentially inhibit the activity of the phosphatases, thereby shifting the total kinase to phosphatase balance in favor of creating an antileishmanial milieu [34] (**Figure 5**).

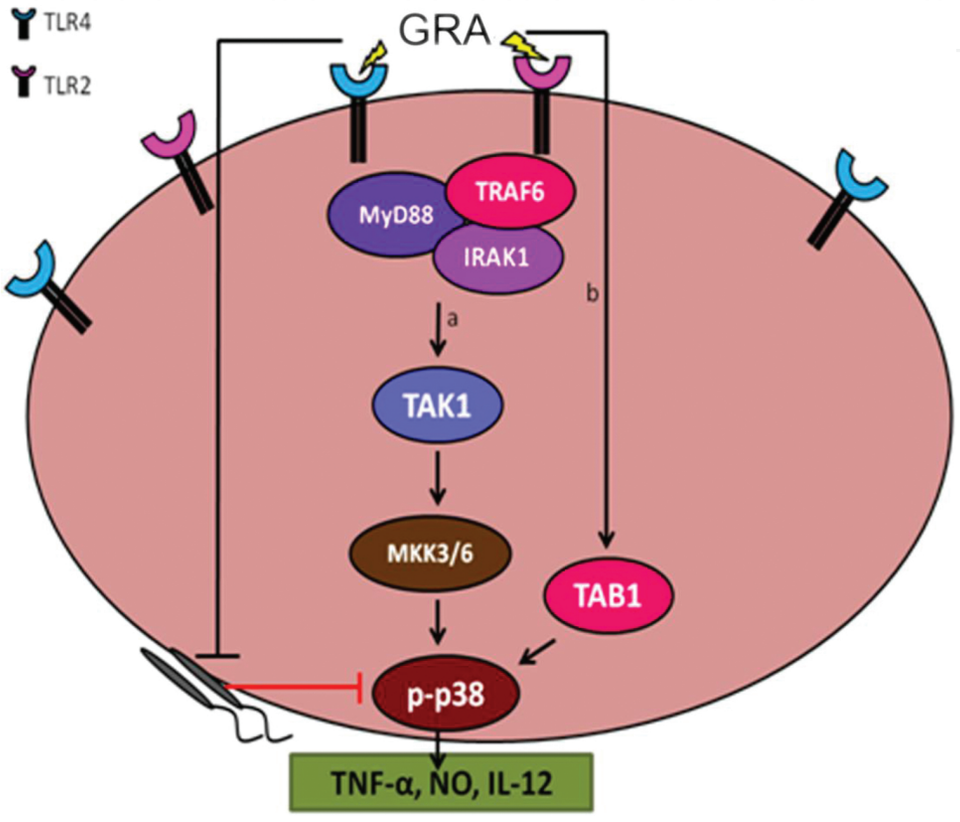

Figure 6. GRA activates p38 via the TLR2/4 pathway.

2.2.3. Downregulation of toll-like receptor (TLR) pathways

Toll-like receptor (TLR) expressed on the cells of the innate immune system are critical for recognition of pathogen-associated molecular patterns (PAMP). Upon ligand binding, the TLR gets activated leading to downstream signaling cascade activation leading to NF-κB- and MAPK (mitogen-activated protein kinase)-mediated proinflammatory cytokine production. The TLR2 agonists used to activate TLR2 signaling pathway showed host protective immune response resulting in parasite clearance from *L. donovani* infected macrophages [36]. To subvert this inflammatory response *Leishmania* either recruits suppressors of the cytokine signaling (SOCS) family proteins, SOCS-1/3 [37], or activates host de-ubiquitinating enzyme A20 to negatively regulate TLR2/4-induced host protective response [38, 39]. *Leishmania* can alter TLR4 signaling to favor its establishment within the macrophages. TLR4-mediated macro-

phage activation was shown to be suppressed in *Leishmania* infection through the release of TGF-β [40]. *L. mexicana* capitalizes on TLR4 signaling to inhibit the production of IL-12 by infected macrophages and promotes parasite establishment [41]. Other TLRs involved in infection with *Leishmania* include TLR3 and TLR9.

The effect of GRA is mediated by means of MAPK (mitogen-activated protein kinase) activation and phosphatase downregulation. Deeper analysis revealed that the MAPK (mitogen-activated protein kinase) p38 activation was dependent on GRA-mediated canonical and noncanonical activation where numerous upstream molecules play important roles [42]. This study further highlighted the importance of p38 MAPK (mitogen-activated protein kinase) in GRA-mediated parasite elimination and its activation by upstream kinases like MKK3/6 which itself is dependent upon upstream signaling molecules (**Figure 6**).

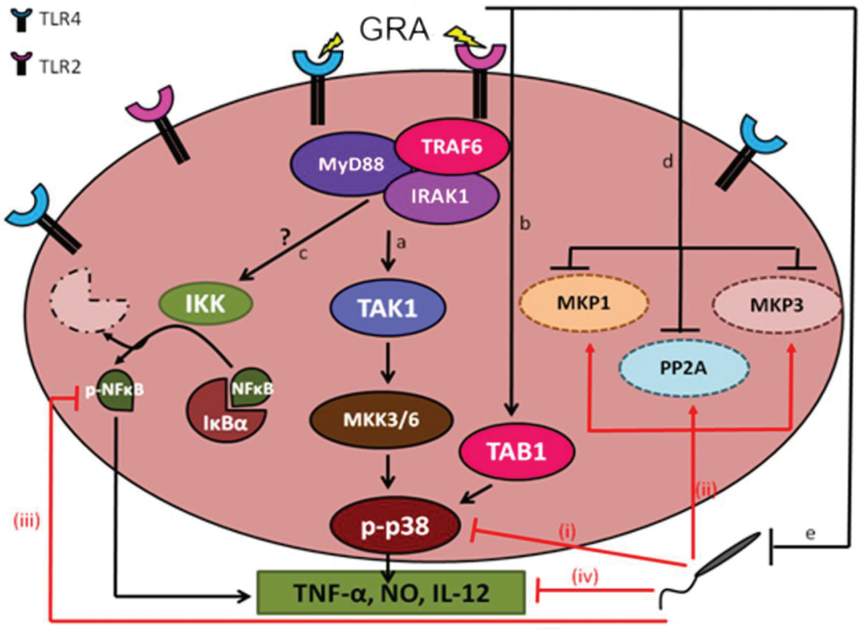

Figure 7. Mode of Action of 18β-Glycyrrhetinic Acid-potent antileishmanial immunomodulator. *Leishmania* (i) inhibits p38 phosphorylation, (ii) activates phosphatases, (iii) inhibit activation of NFκB thereby leading to (iv) inhibition of proinflammatory cytokine reponses. 18β-Glycyrrhetinic Acid acts as an immunomodulator which in infection activates (a) canonical and (b) non canonical pathways leading to phosphorylation and activation of p38, (c) activates IκB Kinase which in turn results in IκBα phosphorylation and degradation leading to NFκB phosphorylation and activation, (d) inhibit phosphatases which would otherwise lead to dephosphorylation and inhibition of major kinases therby promoting proinflammatory cytokine response generation and (e) parasite killing.

Thus, the effective macrophage activation via NO and proinflammatory cytokines production in response to GRA treatment justifies the candidature of this potent immunomodulator as an antileishmanial. Furthermore, this compound may prove to be effective in terms of generating immunity not only in nonhealing leishmaniasis but also for the treatment of other chronic infectious diseases. A comprehensive model giving the overall mechanistic insight into the mode of action of GRA as antileishmanial agent will help in deducing its function in other

intramacrophage pathogens which assume similar immunoevasive mechanisms to escape host defenses (**Figure 7**).

3. Glycyrrhizic acid

Another constituent of licorice, which serves as an antileishmanial compound, is glycyrrhizic acid (GA) [43]. The studies using this compound showed an increase in NO production with restoration of Th1 cytokine balance and inhibition of immunosuppressive prostaglandin E2(PGE2) production. This is in line with recent evidences, which suggests that the parasite is able to induce PGE2 production via promoting inducible COX2 expression. This in turn resulted in activation of EP receptors (PGE2 receptors) on the host cell surface thereby causing Cyclic adenosine monophosphate(cAMP) induction and cytokine production [44] (**Figure 8**).

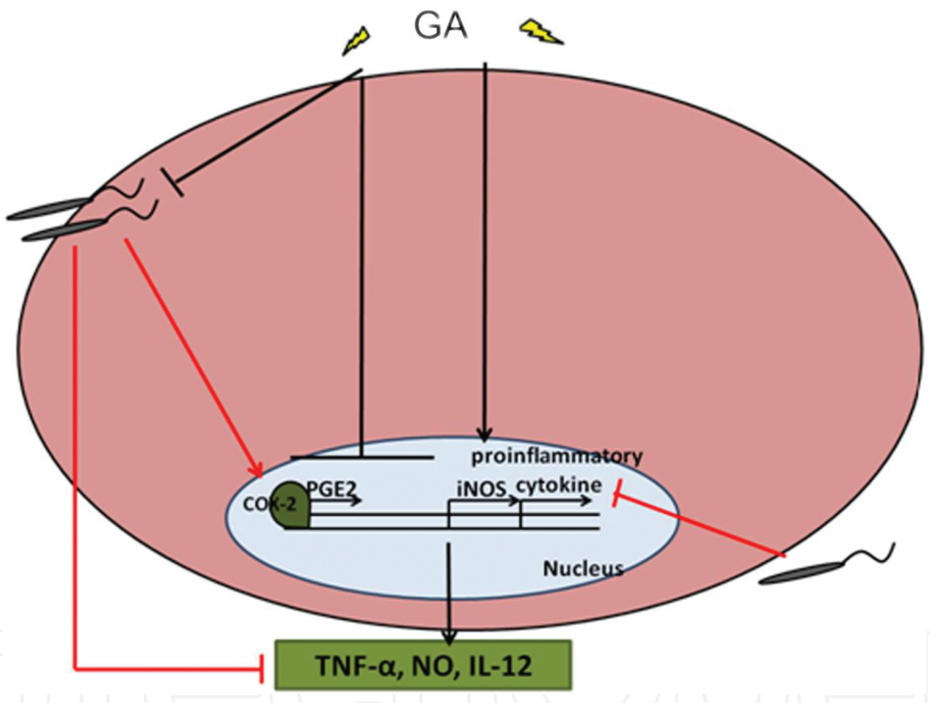

Figure 8. Glycyrrhizic acid mediated PGE2 inhibition and NO and proinflammatory cytokine production helps parasite suppression.

This is also anticipated as glycyrrhizic acid is readily hydrolyzed to glycyrrhetinic acid in human body [45] which is already discussed in details to have antileishmanial activities. Recent studies further shows that this compound when used in conjunction with antimonials can help in overcoming the resistance seen in the antimony resistant parasites. Extensive use of the antimonials and low follow-up of cases had led to emergence of antimonial resistant strains of the parasite [46]. The main criteria for this resistance is attributed to the overexpression and efflux activity of a class of transporters on the surface of the host cells namely P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP-1) thereby leading to efflux of the

antimonials and parasite persistence. However, administration of glycyrrhizin led to suppression of these transporters and simultaneously shifted the overall anti-inflammatory milieu to the proinflammatory one [46] (**Figure 9**). Thus, this herbal compound can serve as an economical effective counterpart to its expensive counterparts like miltefosine and amphotericin B.

Further studies demonstrated the role of glycyrrhizin in mediating proliferation of T cells, thereby promoting disease resolving IFN-γ production in *Leishmania* infected cells. This was in turn an effect of suppression of COX-2 by glycyrrhizin on the myeloid-derived suppressor cells, a heterogeneous population of precursor cells, which promotes parasite survival by suppressing T cell functions [47].

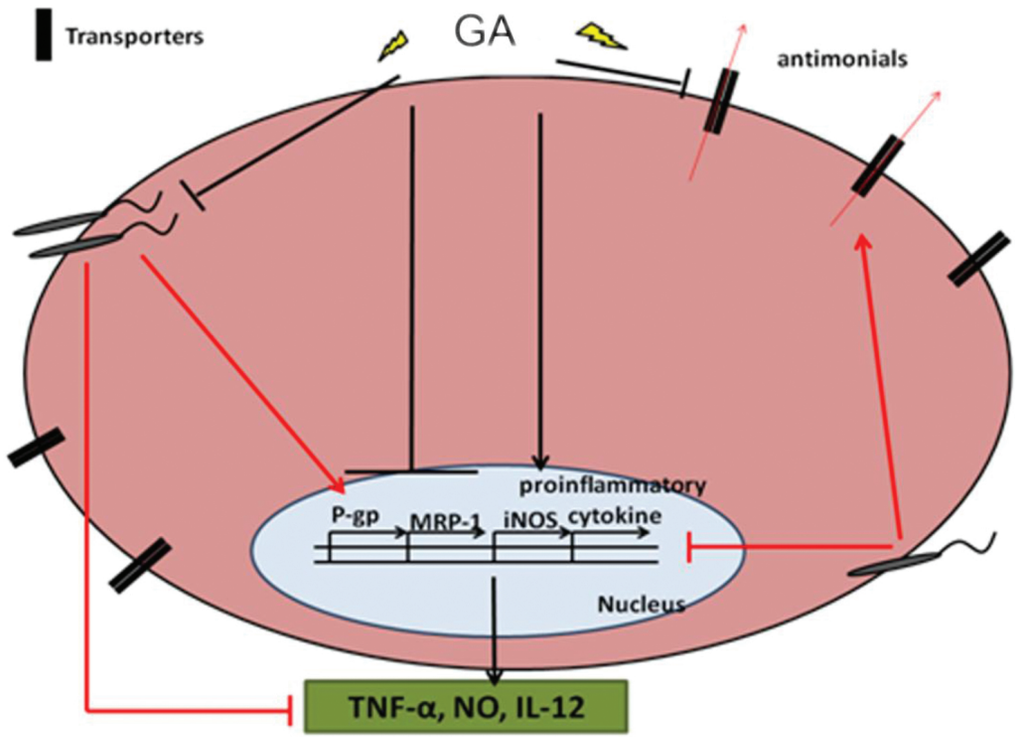

Figure 9. Glycyyrhizic acid (GA) suppresses P-gp and MRP-1 activation leading to antimonial retention and parasite inhibition.

4. Licochalcone A

The oxygenated chalcone, licochalcone A, has also been extensively studied to understand the mechanism of this compound as an antileishmanial agent [48–50]. Previous studies have shown its effect as antioxidant, antimicrobial and anti-tumor promoting properties [51, 52]. The use of this compound in infection particularly affected the amastigote forms of the parasite and to a lesser extent the promastigote form having practically no effect on the host monocytes even at higher concentrations. The main target of licochalcone A was the mitochondrion of the parasite, whereas the host mitochondrion remained unaffected [48]. The reason for the same

is still unknown but further studies suggested that the parasite respiratory rate was affected resulting in an overall decrease in parasite O_2 consumption and CO_2 production with a decrease in the activity of the mitochondrial dehydrogenases [50]. In vivo studies also showed high parasite elimination in in vivo studies upon intraperitoneal administration of licochalcone A as opposed to intralesional or oral administration [49]. All these findings suggests potential role of licochalcone A as an antileishmanial agent although studies understanding the mechanism of this compound is still underway (**Figure 10**).

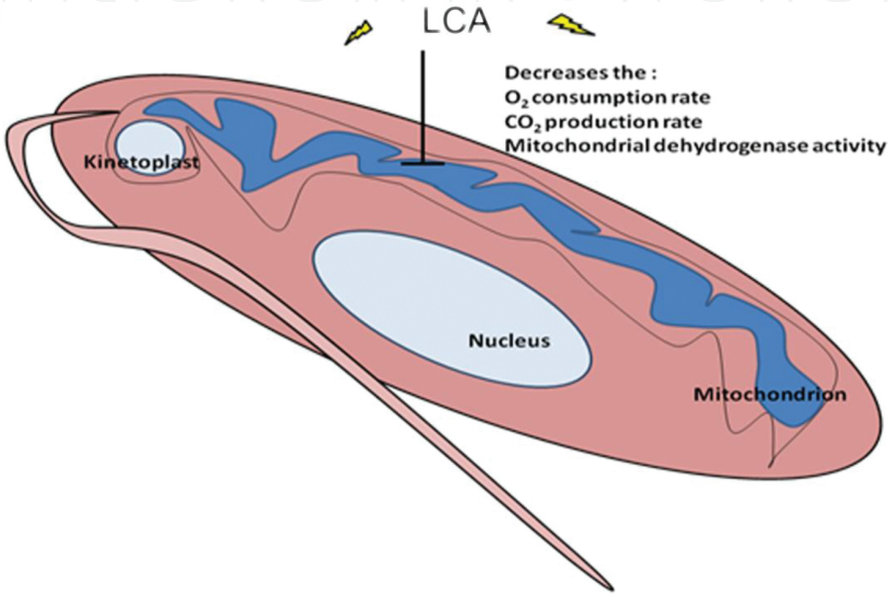

Figure 10. The effect of licochalcone A on the mitochondrion of the *Leishmania* parasite.

5. Conclusion

All these compounds have effectively showed their potential as antileishmanial agents. Future works leading to clinical trials of these compounds alone or in conjunction with present chemotherapeutics may highly benefit the *Leishmania* distressed people. Overall the immunomodulators of the herbal origin like licorice and its constituents open up avenues for formulation of cost-effective and low-toxicity drugs for diseases with limited treatment.

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