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An immune response TLR4 agonist adjuvants and their structure activity relationship

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Abstract
In the coming years, a new generation of adjuvants will be available thanks to small molecules' modulation of TLR4. Since many TLR4 modulators, such GLA and MPLA, have been granted clinical acceptance, the potential for TLR4 agonists as adjuvants or medications for cancer immunotherapy has been raised. The use of adjuvants with vaccines can improve immune response and specificity. This article provides a thorough summary of TLR4 agonists that have been created using synthetic or naturally occurring tiny molecules, and it shows how different molecular fragments affect action. Consequently, a discussion of how small molecule modification could alter how they act is presented.

Keywords: TLR 4 agonist, glycolipids and non-glycolipids adjuvant, SAR of adjuvants

Introduction
Vaccines have made an enormous contribution to the reduction of morbidity and mortality across the globe. Therapeutic vaccines aim to reprogram the immune system of the patient in order to better recognize and neutralize specific deleterious molecular targets or immune cells. Hence, the vaccine could target antigens associated with an infectious (Chronic) disease or non-infectious diseases such as cancer, allergy, drug (e.g. nicotine) addiction, or self-molecules associated with autoimmune/auto inflammatory situations (eg, hypertension, neurological disorders, atherosclerosis, and diabetes) [1].

Toll-like receptors
The Toll-like receptors (TLR) are a class of proteins that play a key role in the innate immune system via recognition of a wide variety of pathogens, making them an interesting target to help our body fight disease [2]. Each TLR is specialized in the recognition of a particular pathogen-associated molecular pattern (PAMP) arisen from a bacteria or virus. TLRs 1, 2, 4, 5, and 6 are situated primarily in the plasma membrane, where they recognize bacterial components of microbial cell walls and membranes, such as lipopolysaccharide (LPS) and lipoteichoic acid from the cell wall, lipoproteins from the cell membrane, and flagellin. TLRs 3, 7, 8, and 9 are intracellularly located in the membranes of endosomes and lysosomes, where they bind to microbial nucleic acids, including double- and single-stranded RNA (dsRNA, ssRNA) from RNA viruses, and DNA from most organisms, including self-nucleic acids from the host cell [3, 4].
The MyD88-dependent pathway is activated by all TLRs except TLR3, which only engages TRIF. TLR4 is the only TLR that activates both MyD88- and TRIF-dependent signaling pathways [5]. The inhibition (Antagonism) of TLR4 and TLR3 can prevents cytokine production at a very early stage; this is in principle a more efficient method to block inflammatory diseases compared to cytokines neutralization by antibodies [6]. Therefore TLR4 agonist activity of adjuvant fascinated him to activate both MyD88 and TRIF dependent pathways.
A very few number of appealing small molecule TLR agonists have been described recently [7]. The present article rreview about recent developments in TLR4 regulation by drug-like small compound and recently discovered Glycolipids and non-glycolipids TLR 4 agonist with their mode of action and structure-activity relationship.

Structure –Activity Relationship: Glycolipid base TLR 4 Agonist compounds
The most prevalent class of biomolecules in nature are carbohydrates. They play critical functions in the immune system's operation and in the stimulation of the immunological response, both of which the chemistry community can take advantage [8].

Carbohydrates have a number of advantageous qualities that make them excellent candidates for adjuvants, including great biocompatibility and tolerability, a robust safety profile, and many others [9]. The activity of the carbohydrate moiety as an adjuvant is enhanced by the covalent bonding of the various linear and branched fatty acids to the carbohydrate moiety.

Extensive studies using natural and, especially, synthetic lipid A variants with modifications on the sugar, phosphorylation and acyl chain pattern (Number and length) have provided key SAR insights that distinguish between agonistic and antagonistic activities, as well as disconnecting lipid A adjuvant activity from its pro- inflammatory toxicity [10-13]. Basically Glycolipids can be divide in two parts one is hydrophobic alkyl chain which are essential for T_H2 biased responses. The acyl chains of lipid A interact specifically with the MD2 hydrophobic region, while the disaccharide phosphate groups make electrostatic and hydrogen- bond interactions with charged residues in MD2 and TLR4, promoting dimerization of the lipopolysaccharide/MD2/TLR4 complex [14, 15]. Moreover the different substitution in aliphatic chain can be responsible for increase and decrease activity of Adjuvant. The presence of hydroxyl group at near to head C_2, C_3 and C_4 position of fatty acid will be enhanced activity, the TH2 biased response activity can also be increased by presence of amide and ester as nonlinear fatty acids [16-18]. Additionally the length of aliphatic chain yielded strong TH2-biased cytokine responses. On the deep analysis of different approved adjuvant, it is observed that C12 to C18 fatty acids showing promising activity as adjuvant [19]. Collectively, the 3:1 ratio between acyl chains and phosphate groups seems to be important for agonistic activity [20]. Analogues with phosphate- to- carboxylic- acid substitution retained agonistic activity [21-23], while monosaccharide deletion decreased lipid A potency but also toxicity, yielding simplified variants with encouraging therapeutic profiles. Collectively, the 3:1 ratio between acyl chains and phosphate groups seems to be important for agonistic activity [24, 25].

Other carbohydrate hydrophilic part in glycolipid adjuvant is crucial for binding activity. Carbohydrates have traditionally been considered T cell- independent antigens188, typically triggering the innate immune system and inducing weak antibody responses, without affinity maturation and isotype switching. To achieve T cell- dependent B cell responses, carbohydrate- based epitopes have classically been conjugated to immunocarrier proteins that serve both as a scaffold for multivalent epitope presentation and as a source of CD4+ peptide epitopes for TH cell activation [26, 27]. The α-anomeric configuration are essential for adjuvant activity [28]. Moreover the different hydroxyl groups playing different role in activity as 4′-OH and especially the 2′-OH in immunostimulation, while the 6′-OH is dispensable for activity [29]. The 6′-OH is also available for modification and can alter the activity. Compare to all hydroxyl group 3′-OH is less critical for activity but 2′-OH is very much responsible for binding activity and can be used for binding of lipophilic fatty acids chain which can trigger activity [30].

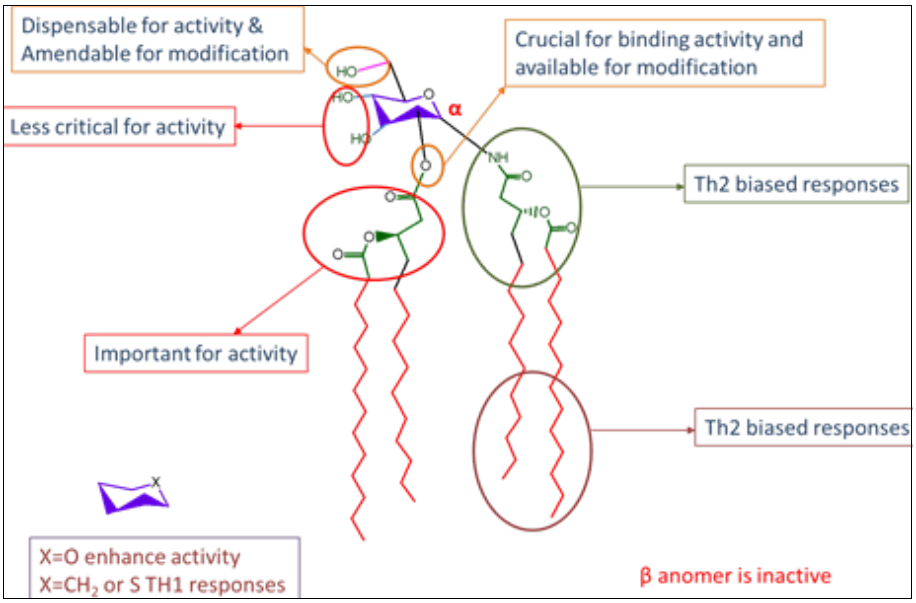

Fig 1: The SAR representation of glycolipid based TLR adjuvant

Carbohydrate-based TLR 4 agonist adjuvants, including both naturally derived as well as chemically synthesized compounds are showing promising effectiveness as adjuvants are discussed below

MPLA

Monophosphoryl Lipid A (MPLA, Fig 2) is a well-characterized TLR4 agonist [31]. MPLA is chemically derived from Salmonella minnesota LPS through treatment with mild acidic conditions, as this achieves the cleavage of the lipid A portion from the oligosaccharide core and the hydrolysis of the 1- phosphate group. TLR4 requirement for MPLA action has been thoroughly validated by numerous studies involving TLR4 −/− mice [32, 33]. MPLA is the only TLR4 agonist to be approved by the FDA for the use as a vaccine adjuvant on human [34, 35].

Glucopyranosyl Lipid Adjuvant (GLA)

The Glucopyranosyl Lipid Adjuvant (GLA, Figure 2) has been developed by Avanti Polar Lipids Inc. as a fully synthetic MPLA analog with TLR4 agonistic activity (Tradename: phosphorylated hexa-acyl disaccharide PHADR) [36, 37]. Being fully synthetic, the main advantage of this compound is its chemical homogeneity, which improves activity and safety with respect to MPLA, a semi-synthetic molecule. Moreover, LPS contamination is avoided and compared to MPLA in terms of activity, it showed an overall better response [38, 39].

GLA-AF was tested as a nasal vaccine adjuvant for HIV immunization *in vivo* on mice and rabbits, resulting in a good immunization profile with strong mucosal immune responses [40, 41]. In 2018, Anderson *et al*. tested HIV immunization in humans following nasal administration of a vaccine containing GLA-AF as adjuvant and the HIV-1 CN54gp140 antigen. Early transcriptional signatures were investigated to identify differentially expressed genes (DEG) and blood transcription modules (BTM) correlated with vaccination and successful immunization [43].

Second-generation lipid adjuvant (SLA)

Carter *et al*. recently developed a second generation lipid adjuvant (SLA), reducing the length of two lipid chains from C14 to C12 (SLA Fig 3). Computational docking studies show that the reduction of the Hydrophobic part make this lipid A derivative better accommodate into the MD-2 hydrophobic pocket, allowing for and stronger interaction with TLR4/MD2 [43].

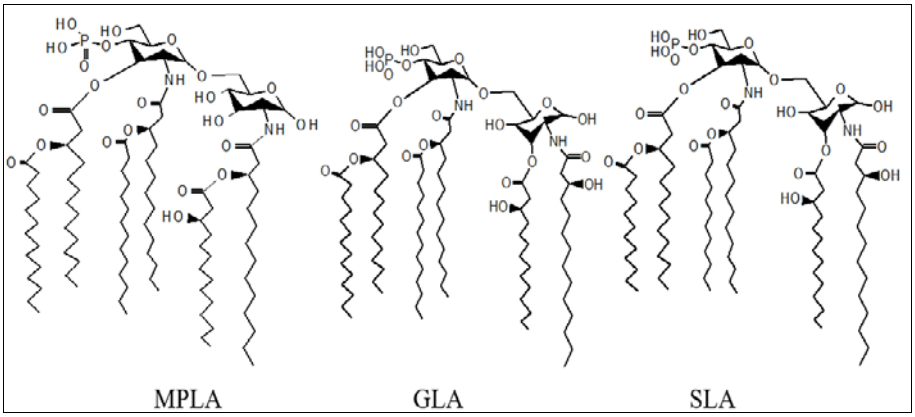

Fig 2: Synthetic Monophosphoryl Lipid A; The Disssacharide-based synthetic Lipid A analogs, GLA is a fully synthetic MPLA. SLA is second generation GLA optimized to be more compatible with MD-2 (by introducing C 12 acyl chains).

Trehalose Derivatives (LAM)

In order to obtain new agonists with increased TLR4/inflammasome selectivity, Zamyatina *et al*. aimed to design molecules capable of activating only the TLR4 pathway without activating NLRP3. a computational structural analysis of the TLR4 dimerization process, two separate hydrophobic clusters are needed in the ligand to optimize the binding with the hydrophobic pocket of MD-2/TLR4, crosslinking the second MD-2∗/TLR4∗ and consequently forming the activated (TLR4/MD-2/ligand)2 complex. Seven different type of trehalose-derived disaccharides were reported based on an α/α -(1-1')-linked diglucosamine scaffold (Lipid A Mimetics, α/α LAMs, Fig 3.) The conformational rigidity of α/α glycosidic bond was exploited by rational design to obtain the two separate hydrophobic clusters for MD-2 binding and TLR4 activation [44, 45].

The activity of α/α LAM was tested on mononuclear cells (MNC), human airway epithelial cells (Calu-3) and human. Interestingly changing the stereochemistry of α/α glycosidic bond into β/α bond, a shift from TLR4 agonist to antagonist was observed [46]. The five novel (α/α) linked diglucosamine LAMs, containing 2-N-, 2'-N-linked β-ketoacyl lipid chains (α/β-LAMs, compounds 8, Figure 3) were tested for their antagonist activity *in vitro*, obtaining full inhibition of LPS-stimulated cytokine production at 1μg/mL concentration. Surprisingly, concentrations higher than 10μg/mL showed reduced antagonist activity, probably because the formation of aggregates.

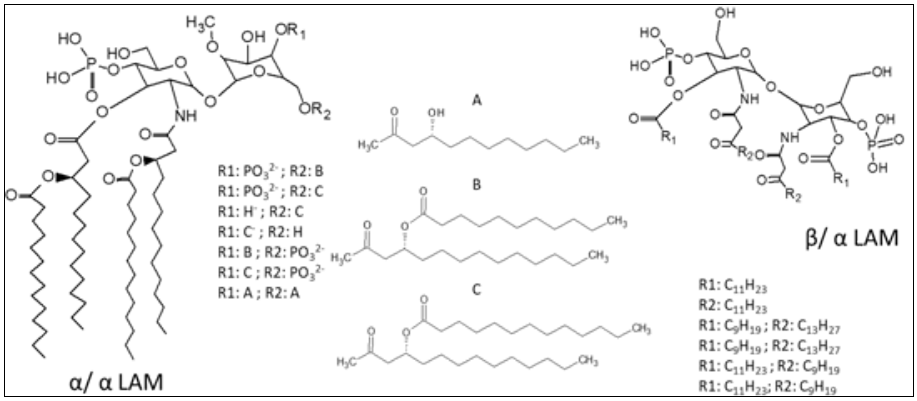

Fig 3: LAMs are Trehalose-derived compound: changing the absolute configuration of the glycoside bond LAM a low the switch from TLR 4 agonism to antagonism.

Lipoaminoglycosides

Aminoglycosides, a family of amino-modified sugars produced by fungi, are capable of stimulating acute renal cell signaling[47] by allosterically activating phosphatidylinositol phospholipase C (PLC) [48]. This results in an increase in intracellular calcium levels and activation of the extracellular signal-regulated kinase (ERK) pathway in a dose- and time dependent manner [49]. Leading ultimately to a proinflammatory response [50]. Hence lipidic derivatives of naturally occurring aminoglycosides (Paromomycine, neomycin, kanamycin, tobramycin) could activate both NFkB dependent and -independent pathways (e.g., PLC–ERK),

paving the way for breakthrough mechanisms of innate stimulation.

The two synthetic lipoaminoglycoside molecules (namely, tobramycin (DOST) and kanamycin (DOSK) shown in Figure 4), which are able to strongly stimulate the cell immunity *in vitro* in a wide panel of cells, including 3D-organized human cells.

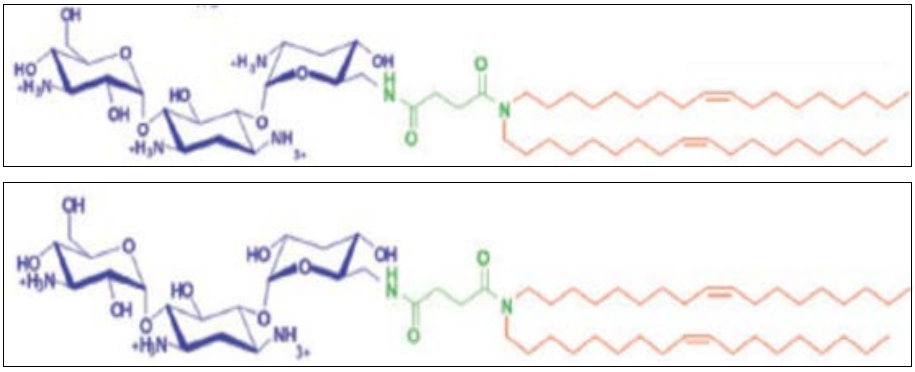

Fig 4: Tobramycin (DOST) and Kanamycin (DOSK)

Nonglycolipid base TLR 4 Agonist compounds

Structure –Activity Relationship: Nonglycolipid base TLR 4 Agonist compounds

Currently, the discovery of many small molecule adjuvants has demonstrated immense potential and opened up the possibility for new therapies. However discovery of adjuvants has been empirical, but with synthetic small molecule adjuvants, modern drug discovery techniques permitted optimized adjuvantcity.

When all of the TLR4 agonist small molecule adjuvants' structures are examined, it becomes apparent that most of the compounds have hydrophilic and lipophilic parts. The design of the molecule Lipid is the basis of E6020. The sole structural variation is the replacement of the carbohydrate component with substituted urea functional groups and substituted phosphate groups, which can aid to maintain the compound's lipophilic nature. The hydrophobic nature of the molecule is due to the long, branched aliphatic chain with ester and amide linkage. So activity of functional group is as similar to MPLA structure.

Other small molecules with various functional groups can balance a compound's lipophilicity. The polar functional groups amide, which enhance hydrophilic character and are essential for activity, are shared by the molecules 1Z105, AZ617, Neoseptin, Compound LS, and VS1 (Figure 7, 8, 9, 10). However, in chalcone compounds, -unsaturated ketone is present in place of the amide function group, and it is crucial for the inhibition of NF-kB activity. Other common characteristics of molecules include the existence of substituted aromatic rings, which give compounds their hydrophobic properties. One, two, or more aromatic rings make a compound more lipophilic. Examples include the LS-like adjuvant, which has a single aromatic (Indole) ring with a log P value of 0.83, and the VS1-like substance, which has two aromatic rings and a log P value of 1.66. In the same way, the lipophilic properties of 1Z105 with three aromatic rings and AZ617 with four aromatic rings rise by 4.26 and 7.49, respectively. When the structure of 1Z105 thoroughly studied that it is observed that the different type of substitution at 5-position of indole ring can trigger relative human TLR4 activity of compound for example 5-position is substituted by thiophene, furan or benzene ring than compound's TLR4 activity is high compare to substitution with methoxy, carboxylic acid, hydroxyl or amino compounds.

Chemical modification of Neoseptin-1 combined with structure-activity relationship (SAR) research resulted in structurally simpler, substantially stronger, and roughly equipotent agonists, Neoseptin-3 and Neoseptin-4 (Figure 8). Further SAR analysis showed that only a few chemical changes were consistent with biological activity retention. Even minor changes, such as the replacement of fluorine for hydrogen at the para position of the phenyl ring or the transfer of the aniline ring's amino group to an adjacent location, resulted in a drastic decrease in activity. However, some of the changed chemicals may work against Neoseptin-3.

It has been noted that the alkyl chain plays a critical role in activity for all TLR4 agonist adjuvants, whether they are glycolipids or not. The length of the alkyl chain of small molecules, such as chalcone derivatives, Neoseptin, LS, and VS1-like substances, can change how adjuvant they are. Long alkyl chains will typically allow for increased activity For instance, the 5-position of the indole ring in the 1Z105 molecule can be substituted with a C10 alkyl chain to boost TLR4 activity, or the aromatic ring's meta position in the chalcone can be substituted with a long O-alkyl chain to increase activity.

TLR 4 agonist small molecules are showing promising effectiveness as adjuvants are discussed below

Linear Lipid A Analogs (E6020)

E6020 (Figure 5) is a synthetic agonist patented by Eisai Inc., which has been previously been experimented on as a vaccine adjuvant *in vivo*, and it turned out to be a viable alternative to traditional alum adjuvant both on boosting mucosal and systemic antibodies responses and in enhancing vaccine efficacy on a toxic shock syndrome model [51, 52, 53]. It has been recently assessed on the central nervous system (CNS) to test its activity in enhancing remyelination in spinal cord white matter following lysolecithin-induced demyelination. This novel study opens the possibility to use TLR4 agonists to repair damages caused by aging or injury, and this prevents a series of CNS pathologies, including dementia [54].

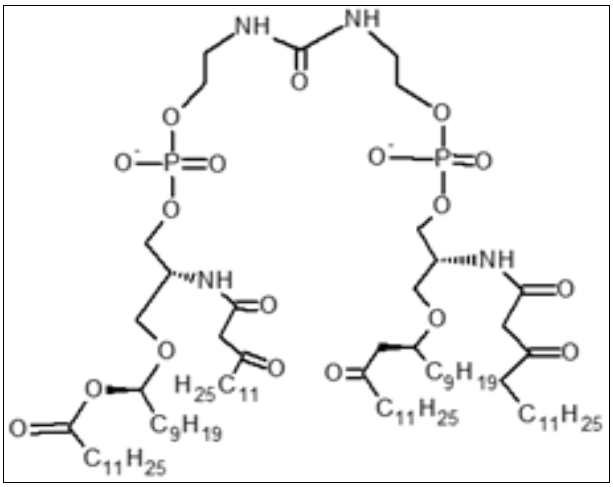

Fig 5: E6020 is a linear Lipid A, synthetic agonist patented by Eisai Inc

Pyrimidoindoles

Pyrimido [5,4-b] indoles are a class of synthetic TLR4 agonists Subsequently, a structure- ctivity relationship (SAR) study allowed to select 1Z105 (Figure 6) as the best agonist compound. 1Z105 has been tested as a vaccine adjuvant in combination with 1V270, a TLR7 agonist [55, 56]. As a follow-up of these studies, an influenza vaccine formulated with both 1Z105 and 1V270 was shown to function *in vivo* through TLR4 and TLR7 activation without any significant off-target effect, and it succeed in inducing protective immunity.

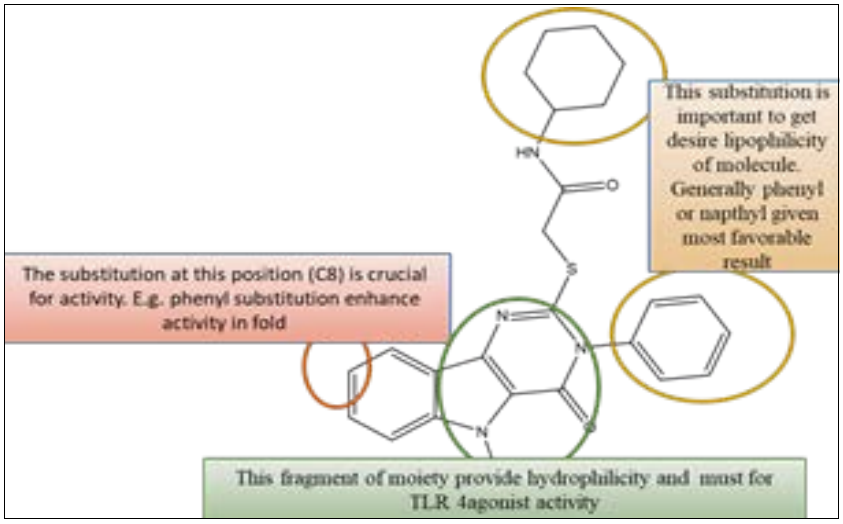

Fig 6: IZ106 Pyrimidoindoles, is currently being tested *in vivo* as vaccine adjuvant.

New Rationally Designed TLR4 Agonists Pyrimindoles

A N-(2-(1Hindol-3-yl) ethyl) benzamide and a anthracene-succinimide hybrid (LS1 like and VS1-like, Figure 7). Both compounds were synthetized and chemically modified for SAR studies. While LS and LS-derived molecules didn't achieve a good activity profile (10% of MPLA activity), VS1 and VS1-derived molecules showed a much more promising efficacy when tested *in vitro* and ex vivo, scoring 50% of MPLA activation [57, 58]. VS like (logP 6.80) structure identified by virtual screening to less lipophilic lead structure LS (logP 3.16)

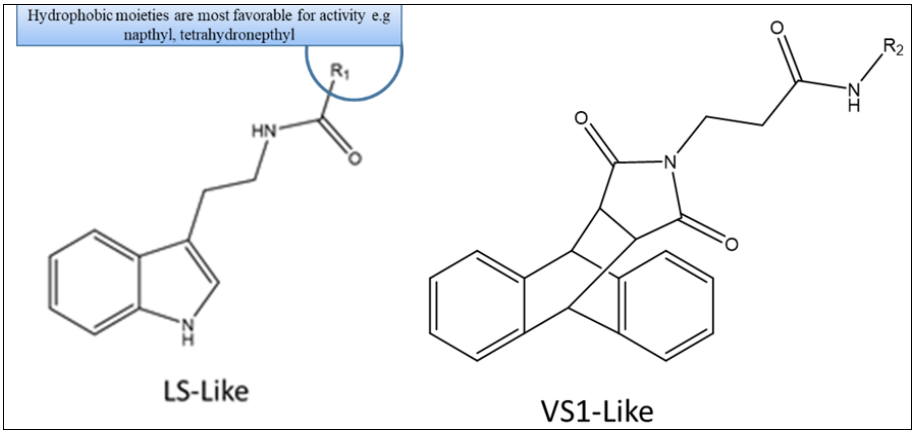

Fig 7: LS- and VS-like compounds are novel TLR4 agonist structures obtained by computational approach.

Neoseptin

Neoseptins the first structurally characterized class of murine TLR4 agonists that bear no structural similarity to bacterial lipopolysaccharide (LPS) or its active core Lipid a (LPA). Neoseptin-3 activates mTLR4/MD-2 and triggers myeloid differentiation primary response gene 88- and Toll-interleukin 1 receptor domain containing adaptor inducing IFN-beta-dependent signaling. Neoseptin-3 binds as a dimer within the hydrophobic pocket of MD-2, contacting residues distinct from those contacted by LPS or lipid A, yet triggering a conformational change very similar to that elicited by LPS or lipid A. Natural peptides might conceivably produce similar effects [59].

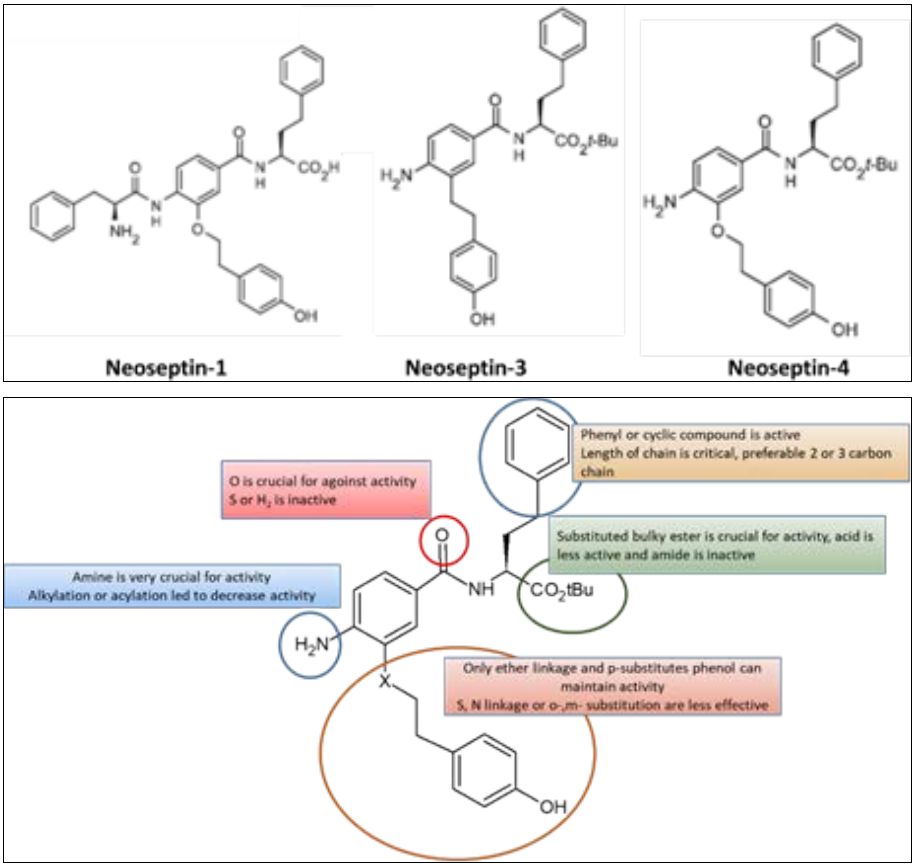

Fig 8: Neoseptins

Compound AZ617

The compounds were created using a four-component Ugi condensation procedure (the more potent molecule being AZ617 in Figure 9) [60]. They were TLR4 agonists in HEK-293 cells transfected with hTLR4/hMD-2/hCD14 while were almost inactive in stimulating HEK cells transfected with mTLR4/mMD-2/mCD14.

As Ugi compounds have a TLR4 agonist preference toward human species, and a better water solubility compared with other lipid a analogs, making them a good starting point for the development of adjuvants that can activate TLR4 in cells with physiological low levels of CD14, such as DCs.

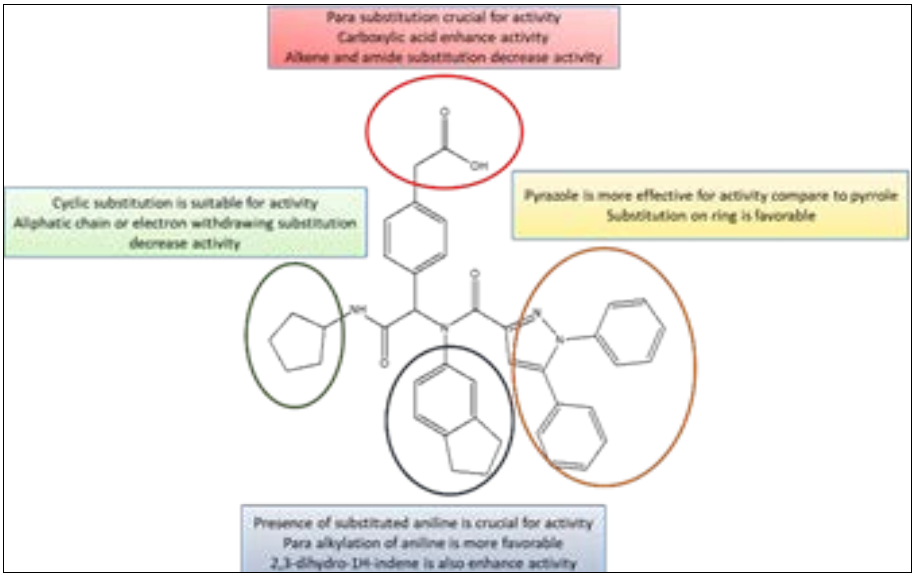

Fig 9: Compound AZ617

Chalcone derivatives

Several chalcone derivatives that contain the moiety of (E)-4-phenylbut-3-en-2-one, considered the core structure of currently known MD-2 inhibitors of natural origin such as curcumin, caffeic acid phenethyl ester and 1- dehydro-10-gingerdione. They have been designed and synthesized by Ying S *et al*. [61] and among all the synthesized chalcone compounds, compound in Figure 10 turned out to be the more potent in antagonizing the TLR4 pathway both *in vitro* and *in vivo*. This finding was experimentally confirmed by using MD-2 mutants [62]. The compound 11 is also able to attenuate LPS-induced lung injuries, in particular by diminishing pulmonary inflammation and by preventing the interaction between MD-2 and TLR4 in lung tissue. Because of this, the chalcone derivative might be regarded as a promising therapeutic candidate for the treatment of ALI and other TLR4-dependent disorders brought on by pathogen infections.

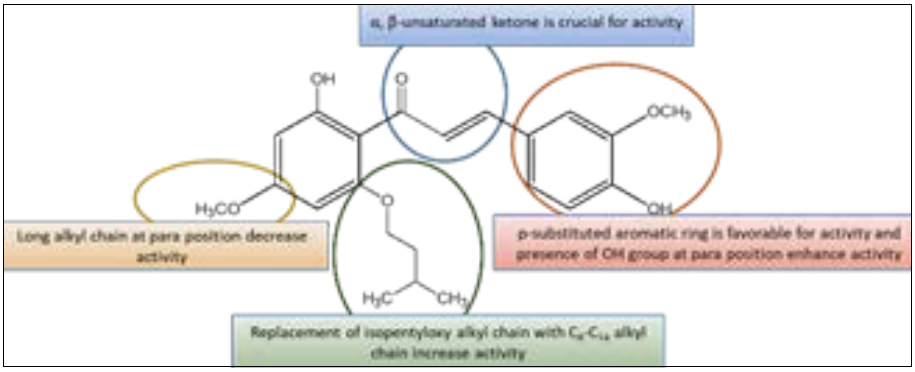

Fig 10: Chalcone compound

4, 4'-Diisothiocyanostilbene-2, 2'-disulfonic acid

The anti-inflammatory effects of 4, 4'-Diisothiocyanostilbene-2, 2'-disulfonic acid in Figure 11, a chloride channel blocker, were investigated in a recent study [63]. 4, 4'-Diisothiocyanostilbene-2, 2'-disulfonic acid significantly inhibits LPS-induced release of pro-inflammatory cytokines *in vitro* and *in vivo* studies, down regulating the inflammatory cytokines via inhibition of the TLR4/NF-κB pathway, with a clear indication that ClC-3 (a volume-activated chloride channel protein) is involved in the inhibitory effect.

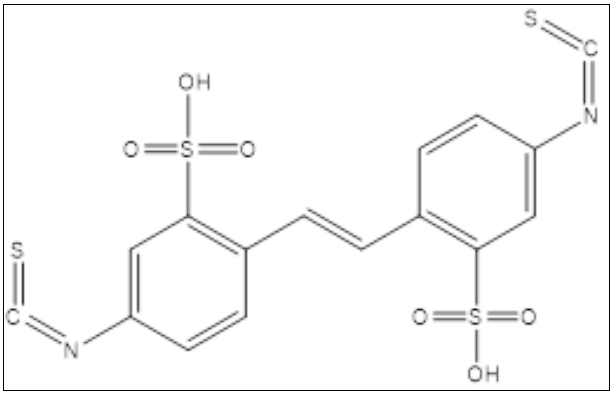

Fig 11: 4, 4'-Diisothiocyanostilbene-2, 2'-disulfonic acid

Calcineurin inhibitors

Calcineurin inhibitors (CNIs) cyclosporine A and tacrolimus are active in increasing the production of pro-inflammatory cytokines and endothelial activation markers through TLR4 activation in cultured murine endothelial and vascular smooth muscle cells as well as in ex vivo cultures of murine aortas [64]. The studies data showed that CNIs were unable to induce inflammation in aortas from Tlr4−/− mice and after pharmacological inhibition of TLR4 in endothelial cells. However, further research is required to clarify the exact mechanisms of action by which CNIs are able to activate the TLR4 pathway.

Conclusions: In this review, we presented here last advancements in the field of TLR4 modulators, focusing on TLR4 agonist small molecules of both synthetic and natural origin. All TLR4 agonist described in this review have been validated or at least evaluated for their capacity to interact specifically with TLR4. TLR4 is the only TLR that initiates two different signal pathways: the MyD88 and the TRAM/TRIF, ending up with the production of inflammatory cytokines or type-I interferons. Interestingly, TLR4 modulators with different chemical structures can activate differentially the two different pathways. Glycolipid TLR4 agonists, such as MPLA (and its synthetic form GLA)- or BECC-derived compounds, were found to preferentially activate the TRIF way, and this skews lymphocytes toward a TH1 response, which is better suited to pathogens and pathogen-infected cells opsonization and elimination. On the other hand, the pyrimidoindole derivative 1Z105 was found to activate TLR4 in a MyD88-biased fashion, leading to a TH2 response, which is better in fighting parasites and extracellular pathogens infections. However, some peptides or other molecules of endogenous origin might act as agonists for the TLR4/MD-2 complex and helping to initiate sterile inflammation to contribute to the repair of damaged tissues [65].

The different chemical structures and sizes of Neoseptin-3 and LPS (Or lipid A) translate to distinct modes of receptor binding. The t-butyl ester group and the benzene ring of both Neoseptin-3 molecules reside within the hydrophobic pocket of MD-2, occupying less than half the total volume of the pocket. Generally agonists activate TLR4 by direct binding to MD-2, antagonists have much more possibilities to impact on TLR4 activity besides competitive inhibition.

The important thing is that treatment with MPLA alone has no effect on MTAL HCO_3 absorption and prevents ERK-mediated inhibition by LPS. Which does not adversely affect the basic transport function of the MTAL and that the ability of MPLA to prevent inhibition by LPS is not due to a nonspecific cytotoxic or metabolic effect on the MTAL cells 13. Therefore TLR4 immune response adjuvant which follow TRIF-Akt pathway are safer or has less toxicity compare to adjuvants which follow MyD88 protein pathway.

Discussed how several TLR4 agonists differ structurally and

how structural fragments have a significant effect in activation. All TLR4 agonists are often categorized in two categories: glycolipid and non-glycolipid. When all TLR4 agonist molecules are examined closely, the structure of each molecule reveals two distinct parts: hydrophilic and hydrophobic. For instance, in MPLA, the carbohydrate moiety enhanced the compound's hydrophilic property, while other lengthy fatty acid chains contributed to its hydrophobic nature. Similarly, hydrophilic properties are provided by amide bonds, heterocyclic compounds like indole and imidazole, and hydrophobic properties are given by aromatic rings or long alkyl chains in other non-glycolipid adjuvant molecules. Therefore, it is crucial that the moiety should be balanced with regard to lipophilicity in order to give efficacy as an adjuvant.

The structural diversity of TLR4 modulators leads open new area of design and development of a novel adjuvant which are less adverse effect and enhance immune response in fold.

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