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Microscale Thermophoresis and Docking Studies Suggest Lapachol and Auraptene are Ligands of IDO1

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Indoleamine 2,3-dioxygenase 1 (IDO1) is a key target for the development of small molecule immunotherapies in oncology. In this framework, the screening of chemotherapeutic agents to identify compounds binding to IDO1 represents a valuable strategy for the development of multitarget drug candidates that combine synergic immunoregulatory properties to cytotoxic activity. In this study, we report that two natural compounds endowed with anticancer activity, namely lapachol and auraptene, act as IDO1 ligands with dissociation constant (Kd) in the micromolar range of potency. Docking studies provide plausible binding modes of these compounds to the catalytic cleft of IDO1. Our results support the notion that lapachol and auraptene may be considered interesting lead compounds in the immuno-oncology setting.

Keywords: Immuno-oncology, Immunotherapy, IDO1, Microscale thermophoresis, Docking, Coumarin, Quinone.

Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-containing enzyme that accounts for the oxidative degradation of L-Tryptophan (L-Trp, **1**; Figure 1) in the first and rate limiting step of the kynurenine pathway to produce the key intermediate N-formyl-kynurenine (**2**) [1]. Further production of bioactive kynurenine metabolites along the pathway and depletion of L-Trp (**1**), thwart immune responses during host-pathogen interactions, pregnancy, and cancer [2]. IDO1 is ubiquitously expressed in many tissues, including macrophages and dendritic cells [3]. Specifically, its expression is regulated by immunological signals which include inflammatory cytokines, type II interferons (IFN-γ), tumor necrosis factor (TNF), and lipopolysaccharide (LPS) [4]. Observations that IDO1 expression is elevated in several cancer cell lines and in tumor microenvironment of cancer patients [5], have fostered the notion that neoplastic cells exploit enzyme functions to create an immune tolerogenic status to cancer antigens, favoring tumor cell growth and invasiveness.

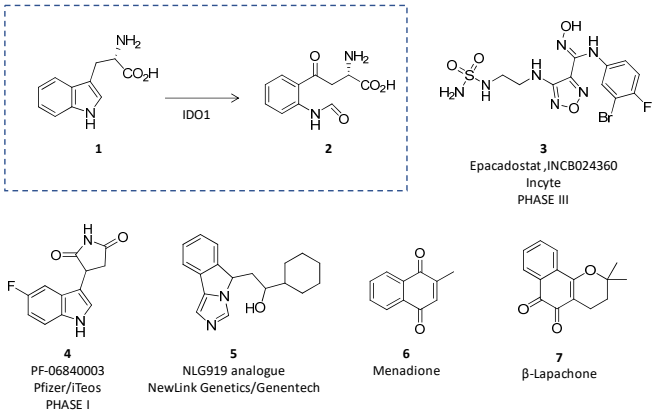

Figure 1: Oxidative degradation of L-Tryptophan by IDO1 (dashed box) and selected inhibitors of the enzyme (**3-7**).

On this basis, academic groups and pharmaceutical companies have considered IDO1 as a valuable anticancer drug target, engaging in the design and development of potent and selective enzyme inhibitors (**3-5**) [6]. Although large part of these inhibitors has been designed leveraging screening campaigns of chemical libraries of synthetic drug-like molecules and/or fragment-like compounds [7], other inhibitors have been identified and/or developed through screening of natural products [8]. At this regard, some researchers have undertaken a peculiar strategy consisting in screening libraries of chemotherapeutic agents, with the goal of identifying compounds that could combine synergic immunoregulatory properties of IDO1 inhibition to existing cytotoxic activity [9]. Embracing this strategy and grounding on previous results about IDO1 inhibition activity of menadione (**6;** IDO1 IC_{50} = 1.1 μM) and β-lapachone (**7;** IDO1 IC_{50} = 0.44 μM) [10], in this study we have investigated IDO1 binding activity of two distinct chemical classes of natural products with reported chemotherapeutic activity (Figure 2).

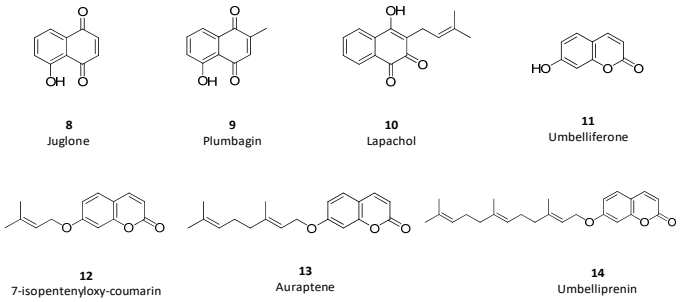

Figure 2: Two selected groups of natural compounds for the study bearing a quinone moiety (**8-11**), and a coumarin scaffold (**12-14**).

The first class is composed of natural compounds bearing a quinone moiety such as juglone (**8**), plumbagin (**9**) and lapachol (**10**). The second class includes products with a coumarin scaffold such as umbelliferone (**11**), 7-isopentenyloxy-cumarine (**12**), auraptene (**13**) and umbelliprenin (**14**). Results show that lapachol (**10**) and auraptene (**13**) bind to IDO1 with a dissociation constant (K_d) in the micromolar range of potency, supporting the presence of IDO1 mediated immunoregulatory activity that, adding on the reported

cytotoxic property, bestows synergic value on the chemotherapeutic profile of these natural products. Furthermore, docking studies provide a putative binding mode of lapachol (**10**) and auraptene (**13**) to IDO1 structure, casting light on structure-activity relationships of these classes of compounds.

Microscale thermophoresis (MST) is a methodology enabling the determination of dissociation constants (K_d) in ligand/protein interactions [11]. It is based on the movement of a ligand/protein complex in a temperature gradient which is referred to as thermophoresis. This movement depends on size, charge, and hydration shell of the biomolecular complex. Since the interaction of ligand to the target protein affects at least one of these parameters, the method can be used to generate a binding curve as a function of ligand concentration, and to obtain K_d value. MST experiment is run using 16 capillary tubes that are filled with a fluorescent dye-labeled target protein and a serial titration of unlabeled ligand. Capillary tubes are then illuminated with an infrared laser that generates a temperature gradient, and thermophoresis of ligand/protein complex is monitored registering changes in fluorescence from capillary tubes. In our study, K_d values are determined against the oxidized and catalytically inactive form of IDO1, namely the most stable state of our target protein in the adopted experimental conditions. A first single-point MST screening was performed in triplicate for the first group (**8**-**10**) and second group (**10**-**14**) of compounds along with negative and positive controls (NLG919 analogue, **5;** IDO1 IC_{50} = 38 nM) [12] with the aim of identifying ligand binders at a concentration of 50μM. Results show that all quinone derivatives (**8**-**10**) and auraptene (**13**) are able to bind IDO1, inducing thermophoresis to a different extent (Figure 3).

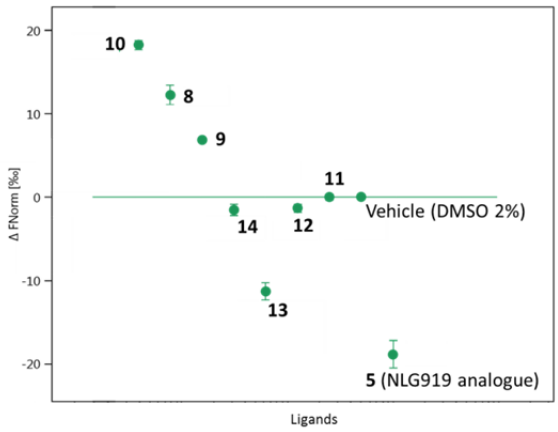

Figure 3: Results of single-point MST screening of compounds **8**-**14**, negative and positive controls (DMSO 2% and **5**).

Germane to quinone derivatives, this finding is in agreement with previous studies reporting 1,4-naphthoquinone and 1,2-naphthoquinone scaffolds as key pharmacophoric elements for IDO1 inhibition activity. Moreover, the same authors reported that IDO1 inhibitory activity of 1,4-naphthoquinones (e.g. **6**) was markedly reduced in cellular assay [10], with other authors questioning whether this class of compounds could act through unspecific redox properties rather than a specific mechanism of target engagement [13]. Conversely, the 1,2-naphthoquinone derivative β-lapachone (**7**) proved nanomolar inhibition potency against IDO1 in both biochemical and cell-based assay, supporting a specific mechanism of action for this natural compound that is currently advancing in clinical trials as anticancer agent [10]. Accordingly, we checked for unspecific effects of tested compounds, inspecting fluorescence perturbation of labelled NT647-rhIDO1 in each capillary tube before generating a temperature gradient (cold conditions) for thermophoresis. As a result, 1,4-naphthoquinone derivatives, namely juglone (**8**) and plumbagin (**9**), induced fluorescence perturbation in cold conditions, suggesting presence of unspecific interactions likely ascribed to the redox property of this class of compounds (Figure 4). Conversely, no fluorescence perturbation was observed for lapachol (**10**) and auraptene (**13**), supporting a specific mechanism of IDO1 engagement for these molecules.

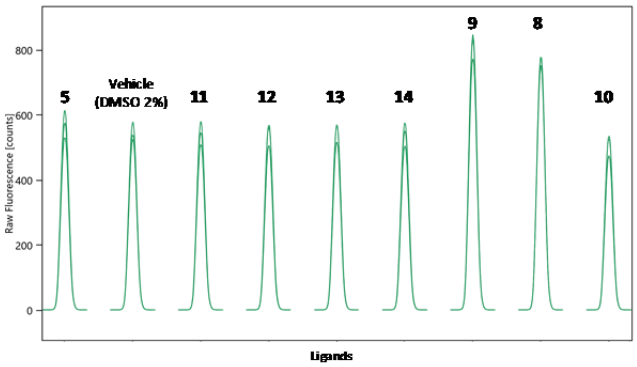

Figure 4: Fluorescence perturbation of labelled NT647-rhIDO1 in capillary tubes at cold condition from three independent analyses (superimposed bell picks). Juglone (**8**) and plumbagin (**9**) increase fluorescence in cold conditions as evidenced by the intensity of relative picks falling outside a ± 10% threshold value.

Dissociation constants (K_d) of these compounds against IDO1 were thus determined using full MST experiments.

Of note, lapachol (**10**) is a 1,2-naphthoquinone derivative, and structurally analogue to β-lapachone (**7**). Both natural compounds are extracted from *Tabebuia impetiginosa* and have been widely studied as potent anticancer agents against various cancer cell lines [14]. Recently, anti-mycobacterial activity and immune-modulatory effects of lapachol (**10**) have also been reported in literature [15]. Alike compound **7**, lapachol (**10**) is able to bind to IDO1 with a K_d value of 16.0±1.3 μM (Figure 5), supporting this interaction as part of the mechanism of action that accounts for reported biological properties of this natural product.

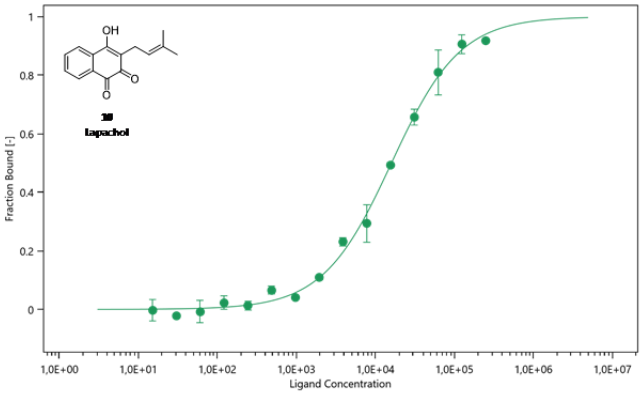

Figure 5: MST binding curve of lapachol (**10**) to IDO1.

Docking studies of lapachol (**10**) into the catalytic site of IDO1 reveal a top scored binding pose wherein the quinone oxygen is involved in a coordinative interaction with the iron of the heme group, the hydroxyl group in position C4 makes a hydrogen bond with the carbonyl group of Gly262, and the 3-isopentenyl moiety is packed with Leu230 and Leu234 through hydrophobic interactions (Figure 6).

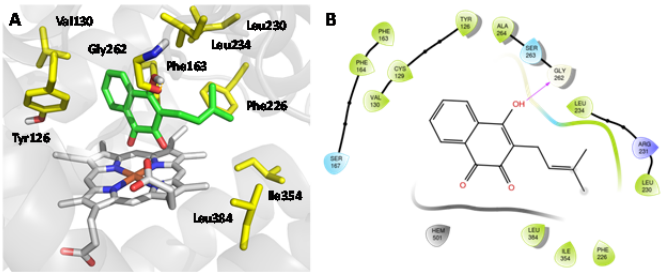

Figure 6: A) Binding mode of lapachol (**10**) to IDO1 according to the top scored solution of docking study. B) Plot of interactions between lapachol (**10**) and residues of the catalytic pocket of IDO1.

Auraptene (**13**) is the most abundant prenyloxycoumarin found in nature. Citrus fruits including lemon, grapefruit and orange contain high levels of auraptene (**13**) [16]. Several therapeutic functions have been ascribed to auraptene (**13**), including antioxidant, cardioprotective, and anticancer properties as reviewed elsewhere [17]. More recent studies have highlighted specific functions for auraptene (**13**) in reducing proliferation and migration of breast cancer cells [18], inducing hepatoprotective effects in vitro and in animal model of cholestatic liver disease through the activation of FXR receptor [19], and stimulating glucose metabolism by activating GLUT4 translocation and glucose influx into skeletal muscle cells [20]. Present results suggest that auraptene (**13**) is able to bind to IDO1 with a K_d value of 48.6±3.7 μM (Figure 7).

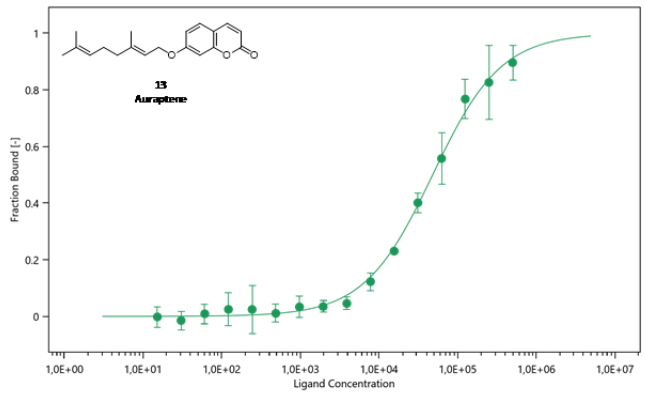

Figure 7: MST binding curve of auraptene (**13**) to IDO1.

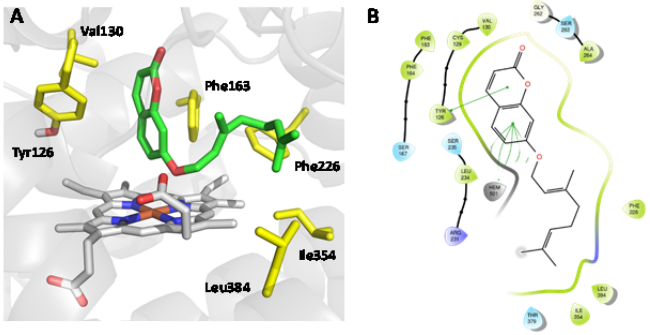

Figure 8: A) Binding mode of auraptene (**13**) to IDO1 according to the top scored solution of docking study. B) Plot of interactions between auraptene (**13**) and residues of the catalytic pocket of IDO1.

Accordingly, auraptene (**13**) may be endowed with IDO1 mediated immunomodulatory properties that combine with its aforementioned functions. Docking studies of auraptene (**13**) into the catalytic cleft of IDO1 suggest a binding mode wherein the coumarin scaffold interacts with the heme cofactor and Tyr126 through π-π interactions, while the geranyl moiety engages Phe226, Ile354 and Ile384 with hydrophobic contacts (Figure 8).

In conclusion, IDO1 is considered a valuable drug target for the development of small molecule immunotherapies aimed at awaking the immune system to seek and destroy tumor cells. To achieve this goal, adopted strategies on part of academic groups and pharmaceutical companies include the design of enzyme inhibitors as well as screening of chemotherapeutic compounds from natural sources to identify molecules that could synergistically combine cytotoxic activity with IDO1 mediated immunoregulatory properties. In this framework, we have herein reported that lapachol (**10**) and auraptene (**13**), namely two natural compounds with reported anticancer activity, bind to IDO1 in the micromolar range of potency. This molecular interaction may thus provide a new level of mechanistic interpretation to therapeutic functions of these compounds, as well as paves the way to design and develop more potent semisynthetic analogues of **10** and **13** as IDO1 ligands for novel small molecule immunotherapeutic agents.

Experimental

Binding Assays: Juglone (**8**), plumbagin (**9**) and lapachol (**10**) were purchased from Merck Chemical Company and used without any further purification. Natural products **12-14** were synthesized from commercially available umbelliferone (**11**). The general procedure of the alkylation was carried out dissolving 7-hydroxycoumarin (1.0 mmol) and the alkylating agents (1.1 mmol), 3,3-dimethylallyl bromide, geranyl bromide and *trans,trans*-farnesyl bromide respectively to obtained compounds **12**,**13**, and **14**, in dry acetone (5 mL) and in the presence of K_2CO_3 as the base (1.1 mmol). Their purity (> 98 %) was assessed by GC/MS and ^{1}H NMR.

Recombinant human IDO1 (rhIDO1) was purchased from Proteros. Experiments to assess dissociation constants (K_d) against rhIDO1 were conducted as previously reported [21], using microscale thermophoresis (MST) [22]. Fluorescence labeling of rhIDO1 was performed following the protocol for N-hydroxysuccinimide (NHS) coupling of the dye NT647 (NanoTemper Technologies, Munich) to lysine residues. Accordingly, 100 μL of a 20 μM solution of rhIDO1 protein in labeling buffer (130mM $NaHCO_3$, 50mM NaCl, pH 8.2) was mixed with 100 μL of 60 μM NT647-NHS fluorophore (NanoTemper Technologies) and incubated for 30 minutes at room temperature (RT) in the dark. Unbounded fluorophores were removed by size-exclusion chromatography with MST buffer (50 mM TRIS, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.4, 0.05% Tween20) as running buffer. The real concentration of each element of the sample, such as protein, heme group and RED dye, and the degree of labeling (DOL) were determined using extinction coefficient ε280 = 51,380 M^{-1} cm^{-1} for rhIDO1, ε405 = 159,000 M^{-1} cm^{-1} for rhIDO1 heme group and ε650 = 250,000 M^{-1} cm^{-1} for NT647 fluorophore, with a correction factor (F_{corr}) of 0.028 at 280 nm, using C_{prot} = [A280 – (A280 x Fcorr) / ε280 x l] and DOL resulted between 0.6 and 0.8 throughout all labeling reactions. The stability of NT647-rhIDO1 and unmodified rhIDO1 protein was checked using circular dichroism [21]. Natural compounds (**8**-**14**) were dissolved in DMSO to obtain 50 mM stock solutions, then they were diluted to 100 μM in buffer solution (Tris-base buffer), maintaining DMSO at 4%. A similar solution was also prepared for IDO1 inhibitor NLG919 analogue (**5**), which was used as positive control. A sample solution of 4% DMSO was also used as negative control. NT647-rhIDO1 solution 10 μL was then added to each compound solution, including positive and negative controls, and mixed to reach a final NT647-rhIDO1 concentration of 50 μM, including 2% DMSO, 2mM DTT and a reaction volume of 20 μL. After 10 minutes incubation, single-point MST screening was performed in triplicate for each compound along with positive and

negative controls, loading ligand/protein solutions into premium-coated capillaries of the MST instrument (Monolith NT.115, NanoTemper Technologies) for thermophoresis analysis. MST signals were recorded at 40% MST and 20% LED power. Capillaries were scanned for constant emission of fluorescence at cold condition, to check tested compounds for presence of false positives due to intrinsic fluorescence properties. Compounds not providing a binding signal with respect to positive control were discarded. Conversely, compounds showing binding signal were further analyzed by 16-fold 1:1 serial dilutions against NT647-rhIDO1 in full MST experiments. K_d values were obtained from compound concentration-dependent changes in normalized fluorescence (F_{norm}) of NT647-rhIDO1 after 21 seconds of thermophoresis. MO Affinity Analysis software (NanoTemper Technologies) was used to generate binding curves (protein fraction bound vs ligand concentration) and calculate confidence values. Confidence values define the range where the K_d falls with a 68% of certainty.

Docking studies: Compounds (**10**, **13**) were designed in Maestro v10.6 (Schrödinger Inc.) and then prepared with LigPrep v3.8 (Schrödinger Inc.) in order to generate all tautomeric states at pH 7±2 [23]. Among available human IDO1 crystallographic structures, 5EK3 was selected from the Protein Data Bank (PDB) in view of its best resolution factor (2.21 Å) [24]. After deleting chain B, the Protein Preparation Wizard (Schrödinger Inc.) tool was used to prepare chain A for docking studies, adding hydrogen atoms, setting ionization states of charged residues at pH 7.0, and refining the structure by energy minimization. The oxidation state of the iron atom of the heme group was set to Fe^{3+}. A docking grid was generated using the Receptor Grid Generation module of Glide v7.1 (Schrödinger Inc.). Specifically, it was centered on the co-crystallized ligand in 5EK3, with a size of 12x12x12 Å for the inner box and 20x20x20 Å for the outer box. Compounds were docked with Glide v7.1 standard precision (SP) mode, using enhanced sampling and keeping 50.000 poses for each ligand within a scoring window of 500 kcal/mol during the initial phase of docking [25]. A number of 1.000 binding poses for each ligand was stored and submitted to energy minimization. The top five scored poses from energy minimization were saved for each molecule, and visually inspected in Maestro v10.6. The best scored pose in terms of interaction energy (*G-score*, kcal/mol) was selected as putative binding mode of compounds **10** and **13** to IDO1.

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