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Indoleamine 2,3-dioxygenase inhibitory activity of derivatives of marine alkaloid tsitsikammamine A

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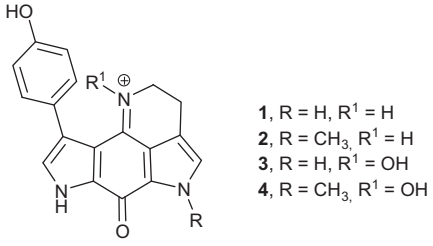
ABSTRACT

Tsitsikammamines are marine alkaloids whose structure is based on the pyrroloiminoquinone scaffold. These and related compounds have attracted attention due to various interesting biological properties, including cytotoxicity, topoisomerase inhibition, antimicrobial, antifungal and antimalarial activity.

Indoleamine 2,3-dioxygenase (IDO1) is a well-established therapeutic target as an important factor in the tumor immune evasion mechanism. In this preliminary communication, we report the inhibitory activity of tsitsikammamine derivatives against IDO1. Tsitsikammamine A analogue **11b** displays submicromolar potency in an enzymatic assay. A number of derivatives are also active in a cellular assay while showing little or no activity towards tryptophan 2,3-dioxygenase (TDO), a functionally related enzyme. This IDO1 inhibitory activity is rationalized by molecular modeling studies. An interest is thus established in this class of compounds as a potential source of lead compounds for the development of new pharmaceutically useful IDO1 inhibitors.

Many alkaloids based on the pyrroloiminoquinone scaffold have been isolated and characterized from marine and other natural sources in recent years. These natural products of unique structures have attracted attention due to various interesting biological properties, such as cytotoxicity, inhibition of topoisomerases, antimicrobial, antifungal and antimalarial activity.[1–8] A subfamily of this class of compounds, the bispyrroloiminoquinones tsitsikammamine A (**1**) and B (**2**, Fig. 1),were first isolated and characterized in 1996 from a sponge of the *Latrunculiidae* family in the Tsitsikamma Marine Reserve, South Africa.[9] Further search for pyrroloiminoquinone metabolites as chemotaxonomic markers yielded two oxime derivatives **3** and **4** isolated from *Triceratium Favus* (Fig. 1).[10] Promising anticancer activities of these compounds were reported because of their abilities to intercalate into DNA and topoisomerase I inhibition.[7,9–13] More recently, the biosynthesis of tsitsikammamines in the latrunculid sponge *T. favus* has been suggested to originate from the microbial community associated with the species.[14]



1, R = H, R^1 = H
2, R = CH_3, R^1 = H
3, R = H, R^1 = OH
4, R = CH_3, R^1 = OH

Figure 1. Structures of tsitsikammamines **1**–**4**.

These results prompted studies on the synthetic accessibility of this class of compounds[15–17] aiming at an improved therapeutic value.[7,18–20] In this context, our group described a cycloaddition-based synthesis of aza-analogues of tsitsikammamines (wherein either of the pyrrole rings was replaced with a pyrazole) and evaluated their topoisomerase I and II inhibition as well as their in vitro

Abbreviations: IDO1, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; NHDF, normal fibroblast cell lines; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; IMAC, immobilized metal ion affinity chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HBSS, Hanks balanced salt solution; SAR, structure–activity relationships.

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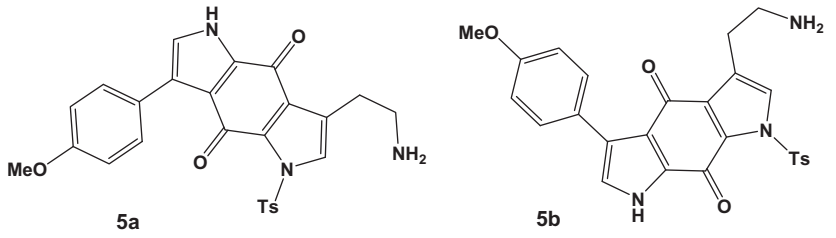
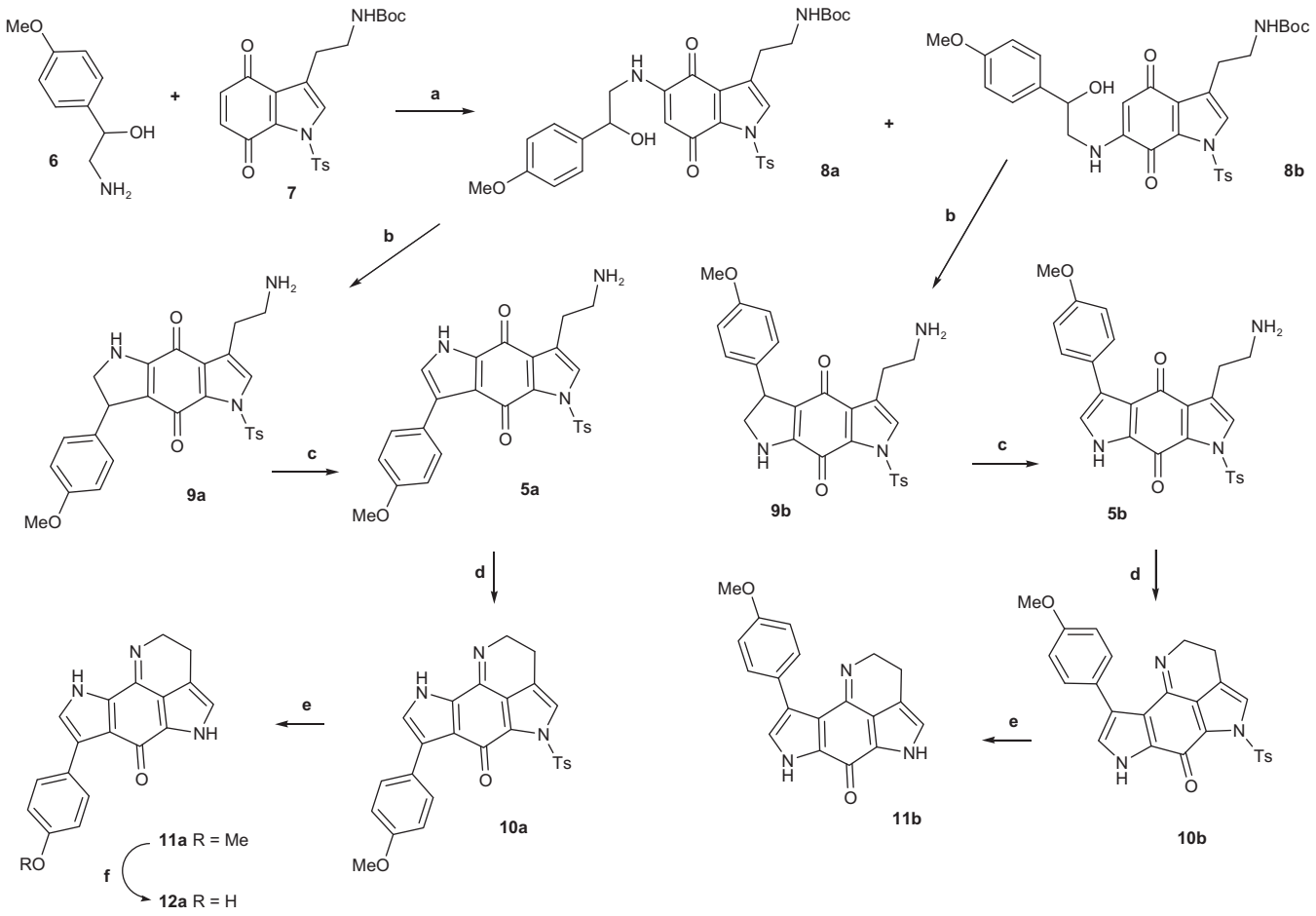


Figure 2. Structures of potential anticancer compounds **5a** and **5b**; Ts = *p*-$CH_3C_6H_4SO_2$–.



Scheme 1. Synthesis of compounds **8**–**12**. (a) abs EtOH, rt, 2 h; (b) TFA, CH_2Cl_2, rt, 4 h; (c) MnO_2, CH_2Cl_2, rt, overnight; (d) abs EtOH, 4 Å mol sieves, reflux, 3 h; (e) 1 M NaOH, dioxane, rt, overnight; (f) BBr_3, CH_2Cl_2, N_2, 4h.

antiproliferative activity.[21,22] Interestingly, some of these compounds showed an antiproliferative effect while being devoid of any topoisomerase activity, thus suggesting another mechanism of cell toxicity for this series. More recently, two regioisomeric series of tsitsikammamine analogues along with the corresponding synthetic intermediates were evaluated in an in vitro antiproliferative assay against the U373 glioblastoma, A549 non-small-cell-lung cancer and PC-3 prostate cancer cell lines as well as two human normal fibroblast cell lines (NHDF and Wi38).[23] This work allowed identifying the cytotoxic synthetic intermediate **5b** (Fig. 2) and its regioisomer **5a**. Using the MTT colorimetric assay, compound **5b** showed potent cytotoxicity towards all the cell lines in this study. Isomer **5a** displayed somewhat higher IC_{50} values in this test but also relative selectivity towards some cell lines. It was suggested that this effect could be associated with a specific antiproliferative activity. The conclusion was that compound **5a** and its derivatives represent interesting novel anti-cancer agents with an unknown mechanism of action.

Cancer immunotherapy, a strategy for cancer treatment consisting of stimulating the immune system to attack and destroy tumor cells, has so far shown limited efficacy in vivo, the main reason being the ability of tumors to escape an immune response. Among the various factors accounting for this phenomenon[24–27] are the tryptophan-catabolizing enzymes indoleamine 2,3-dioxygenase (IDO1; EC 1.13.11.52)[28–34] and tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.11).[35] IDO1 is a monomeric extrahepatic cytosolic enzyme while TDO is homotetrameric and normally expressed at a high level in the liver and at low level in the brain.[36,37] Both enzymes are heme dioxygenases catalyzing the oxidation of the indole ring of L-tryptophan (Trp) to produce *N*-formylkynurenine. This reaction is the first and rate-limiting step of the kynurenine pathway of tryptophan catabolism. Another indoleamine 2,3-dioxygenase isoform, IDO2,

has been described.[38–42] However, since its physiological relevance remains unclear,[43] in this work we focused our efforts on IDO1.

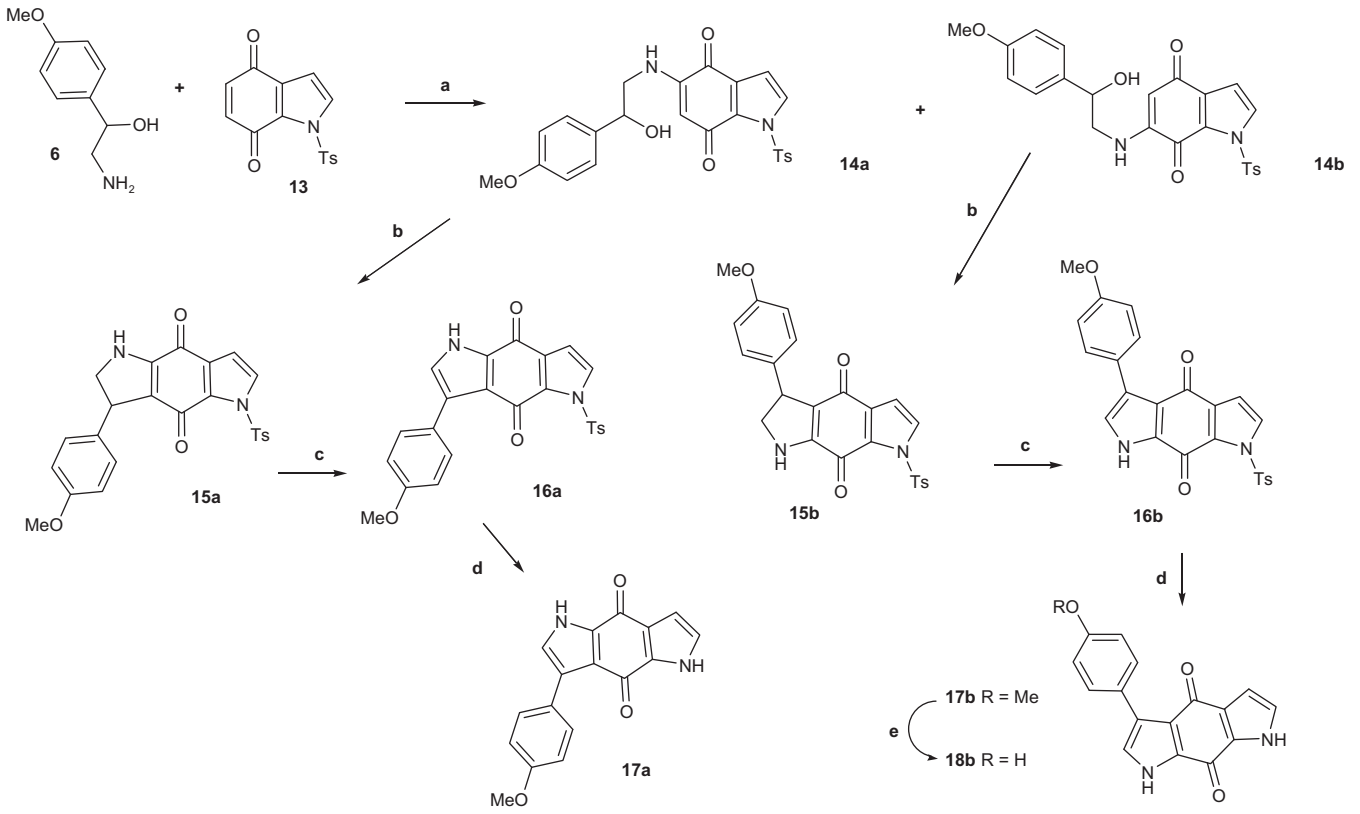
Our group showed that many human tumors express IDO1 in a constitutive manner.[44] It has been suggested that IDO1 exerts its immunosuppressive effects on T-lymphocytes in a twofold manner: by depleting Trp locally and by action of toxic Trp catabolites.[24,27,31,43,45,46] TDO has also recently been shown to be expressed constitutively in human glioblastomas where it promotes tumor progression by action of a downstream tryptophan catabolite kynurenine which acts as endogenous ligand of the aryl hydrocarbon receptor.[47] We showed that TDO inhibition reverses tumoral immune resistance in vivo.[48] Tryptophan catabolites have also been connected to other pathological conditions, particularly in the central nervous system (CNS).[49–52]

Not surprisingly then, the recent years witnessed many attempts to discover and develop IDO1[53–57] and TDO[58,59] inhibitors. However, with only two IDO1 inhibitors to have entered clinical trials so far, D-1-methyltryptophan[32] and INCB024360,[60] there is still an urgent need for the discovery and development of new anticancer compounds with this mode of action.

Some of the best IDO1 inhibitors are marine and other natural products that contain an oligocyclic quinone scaffold.[61] Indoloquinone exiguamine A, isolated from the marine sponge *Neopetrosia exigua*, was shown to possess a K_i of 210 nM for IDO1 inhibition in vitro[62] (K_i = 41 nM in Ref. 63), establishing exiguamine A as one of the most potent IDO1 inhibitors known. Synthetic analogues of simplified structures retaining nanomolar potency were described in the following study.[63] The structures of these compounds can be regarded as being partially derived from a tryptamine and partially from a quinone moiety; this also applies to the tsitsikammamine derivatives described herein. A naphthoquinone pharmacophore-containing annulins from the marine hydroid *Garveia annulata* are submicromolar IDO1 inhibitors in vitro.[64] Their skeleton was pharmacomodulated to afford novel pyranonaphthoquinones possessing nanomolar potencies in vitro.[65] Mitomycin C, an indoloquinone from *Streptomyces*, is an uncompetitive IDO1 inhibitor with a K_i of approx. 25 μM. The mechanism by which such compounds inhibit the enzyme is debated. For instance, binding into an inhibitory substrate binding site (S_i site) has been suggested, although mitomycin C was the only quinone-type compound for which such type of binding has been proposed.[66] Also, a recent study by Pearson and colleagues on the IDO1 inhibitory potency of menadione, another quinone-type compound, suggested the possibility that its redox potential could interfere with the reductant cofactor in vitro (methylene blue) and thus lead to irrelevant IDO1 inhibition data, although other mechanisms were also suggested in the original study to account for this phenomenon.[67] In any case, some of these naphthoquinones have also been shown to possess some IDO1 inhibitory potency in a cellular assay that does not involve methylene blue, thus adding confidence to the potential of this type of compounds.[65]

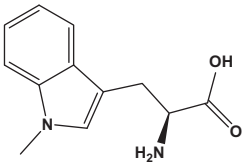
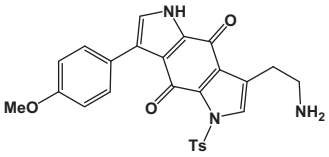
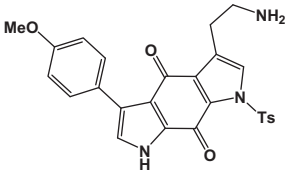
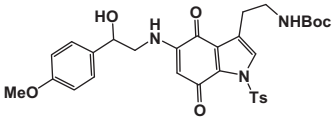
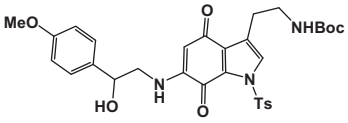
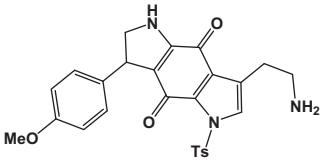
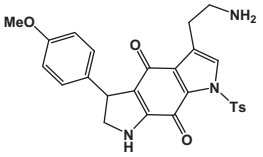
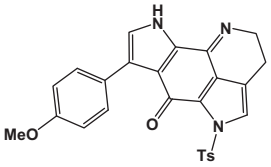
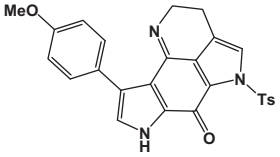
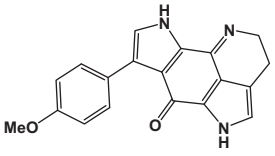
Prompted by the tsitsikammamine derivatives' anticancer activity and their relative structural likeness to quinone-containing IDO1 inhibitors, we decided to undertake an inhibitory study of tsitsikammamine derivatives and synthetic intermediates on IDO1 while also performing a counter screen on TDO.

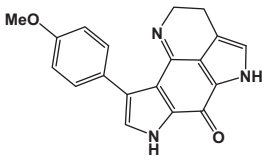
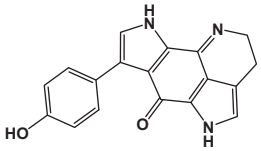
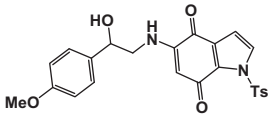
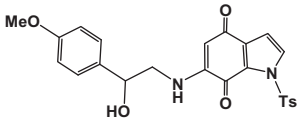
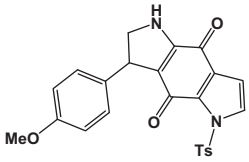
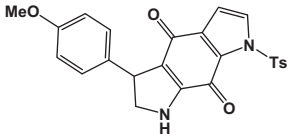
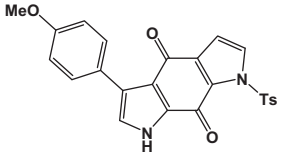
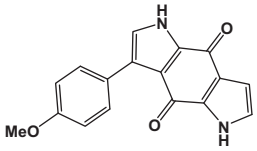
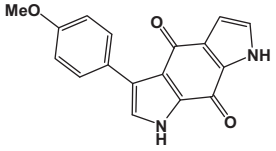
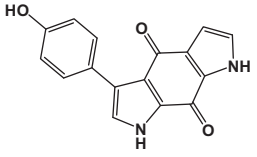
The compounds were synthesized as described[23] in Schemes 1 and 2. A Michael addition of amino alcohol **6** to the indolequinone **7** gave two isomeric products, which were further elaborated to the final tsitsikammamine derivatives by acid-mediated cyclization/deprotection, oxidative aromatization, a second cyclization followed by the removal of the protecting tosyl group and the final aromatic methyl ether demethylation effected by BBr_3 (Scheme 1).



Scheme 2. Synthesis of compounds **14**–**18**. (a) abs EtOH, rt, 2 h; (b) TFA, CH_2Cl_2, rt, 4 h; (c) MnO_2, CH_2Cl_2, rt, overnight; (d) 1 M NaOH, dioxane, rt, overnight; (e) BBr_3, CH_2Cl_2, N_2, 4h.

Table 1
Biological activity and cell viability data for the tsitsikammamine derivatives **5a**–**18b**

Compound	Structure	Mean IC_{50} (μM) in the enzymatic test on hIDO1[a]	mIDO1 cell. Assay inhib. @ 10 μM (%)	mTDO cell. assayinhib. @ 10 μM (%)	Cell viability @ 10 μM (%)
L-1-MT		138.5	14.5 @ 22 μM	NI[b] @ 22 μM	>90
5a		11.9	26.0	NI	>90
5b		5.4	15.6	NI	80
8a		10.1	17.4	NI	>90
8b		26.9	31.3	NI	>90
9a		5.6	40.2	4.7	>90
9b		2.8	26.1	NI	83
10a		4.2	ND[c]	ND	<50
10b		2.2	32.0	3.1	>90
11a		7.1	ND	ND	<50

11b		0.9	19.4	NI	>90
12a		7.0	5.3	NI	>90
14a		8.8	37.3	1.1	>90
14b		2.5	24.3	NI	>90
15a		5.6	54.2	3.2	>90
15b		1.8	40.2	2.5	>90
16b		[d]	19.6	NI	>90
17a		22.4	ND	ND	<50
17b		12.6	ND	ND	<50
18b		[d]	NI	NI	>90

[a] Result = mean of two independent determinations. Error between experiments is no more than 10%.
[b] NI = no inhibition.
[c] ND = not determined because of cellular toxicity of the compound.
[d] <50% Inhibition at maximal concentration.

Analogous chemistry was applied for the synthesis of the derivatives **14**–**18** lacking the iminoquinone motif (Scheme 2).

Protein expression and purification, cell line constructions and enzymatic and cellular assays were carried out as described previously.[54,56,58] Inhibitory activities of synthetic derivatives of tsitsikammamines on purified recombinant hIDO1 as well as on murine cancer cell lines overexpressing mIDO1 and mTDO and cell viabilities are presented in Table 1.

In all cases except for the intermediates **8**, the b regioisomer (leading to the 'natural geometry' of the final tetracyclic product) is at least twice as active on IDO1 in the enzymatic assay as the corresponding **a** regioisomer. Similar tendencies were reported

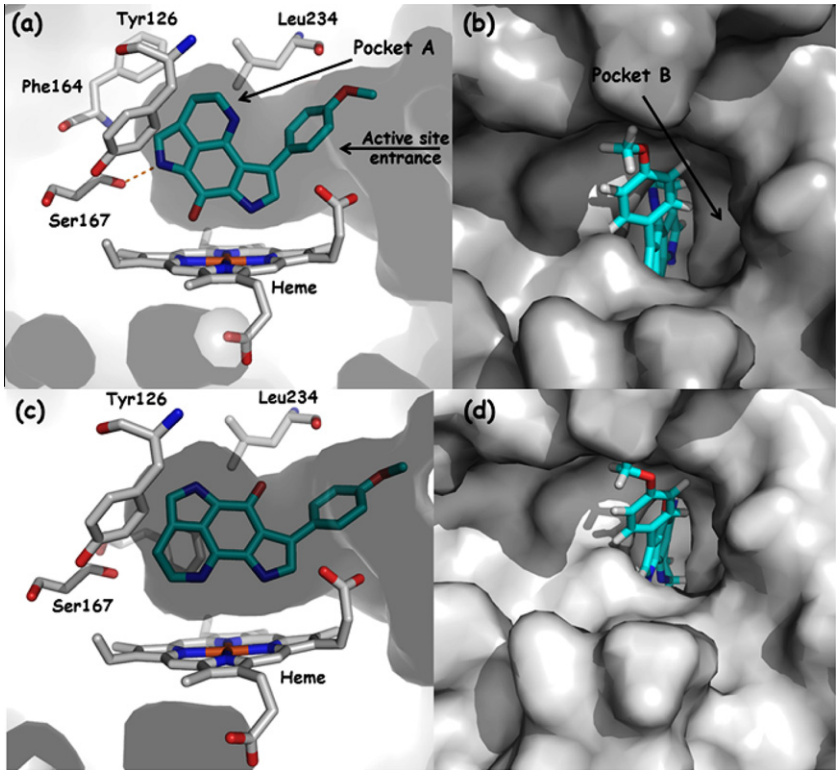


Figure 3. View of (a) **11b** and (c) **11a** docked within the IDO1 active site (PDB code 2D0T) showing Pocket A, and (b) **11b** and (d) **11a** showing Pocket B. Pictures made with Pymol (Delano Scientific).

earlier[23] regarding their in vitro activity in an MTT colorimetric assay. On the contrary, a reverse pattern is seen in the cellular test.

Deletion of the conjugated double bond bearing the anisyl moiety in the tricyclic series improves the activity in the enzymatic assay (compd **5**–**9** and **15b–16b**). This effect persists in the cellular assay, where three out of the four compounds possessing this structural feature (**9a**, **15a** and **15b**) are the most potent compounds with 40% or more IDO1 inhibition at 10 μM.

Interestingly, deleting the aminoethyl side chain, N-protected or not, does not seem to have a significant effect on the potency in the enzymatic assay (compd **8a–14a**, **9a–15a**, **9b–15b** and **5b–16b**), except in one case (**8b** and **14b**). This is in good agreement with the results for exiguamine A derivatives published in 2008 by Carr et al.[63] The compounds described in Carr's work share some structural features with the tsitsikammamine-related compounds studied here; both classes can be seen as being partially derived from a tryptamine and from a quinone moiety. The presence of a side chain in the compounds described in this work does, however, in most cases (and certainly for the free primary amines) cause a drop in potency in the cellular assay. This could be a consequence of lower cell membrane permeability due to the presence of a protonated amino group.

The tetracyclic iminoquinone motif, as present in the natural scaffold, enhances the potencies in both tests (compd **5–10** and **11–17**) but also provokes enhanced cellular toxicity, especially in the a series (Table 1).

Removing the tosyl group from the 'eastern' pyrrole ring (compd **10–11** and **16b–17b**) or the methyl group from the anisole moiety (compd **11a–12a** or **17b–18b**) does not seem to exert a significant effect on the potency in the enzymatic assay. Nevertheless, in contrast to the phenolic compounds, their O-methylated counterparts are cytotoxic. Despite being the most potent compound in the enzymatic assay (IC_{50} = 0.9 μM), pyrroloiminoquinone **11b**'s cellular activity is almost halved compared to that of its N-tosylated analogue **10b** (which is somewhat less potent in the enzymatic assay). Again, compound **11b** might be too polar to display good membrane permeability. This could also be the reason for the even poorer cellular activity of the yet more polar tsitsikammamine unnatural regioisomer **12a**.

It should be noted that, in contrast to the enzymatic test, the cell-based assay does not involve the reductant cofactor methylene blue to activate IDO1, thus strengthening the hypothesis of IDO1 inhibition not based on a redox mechanism.

Finally, hardly any TDO inhibitory activity is detected in the cellular assay on this series, showing its selectivity for IDO1.

In order to understand the affinity observed, we studied the binding mode of compounds **11a** and **11b** within IDO1 by means of docking using the automated GOLD program.[68] For each compound, 20 docking solutions were generated and ranked according to the the GoldScore scoring function. The key interactions stabilizing the compounds within the IDO binding cleft are depicted in Figure 3. First, regarding the more potent compound **11b,** the 20 solutions obtained are found in the same orientation and a very high score was obtained (GoldScore of the best pose = 55.80). In this orientation, the pyrroloiminoquinone moiety is deeply inserted inside pocket A of the IDO1 active site (Fig. 3a), interacting via an H-bond to the Ser167 residue and complexing the heme iron by its carbonyl function (distance C═O···Fe = 2.89 Å, angle ~120°). The compound is also well stabilized by hydrophobic interactions with the Tyr126, Phe164 and Phe163 (omitted for clarity) residues. Interestingly, a nice shape complementarity occurs between the pyrroloiminoquinone scaffold and pocket A of IDO1. On the contrary pocket B (Fig. 3b) is only partially filled by the methoxyphenyl side chain. This suggests that the introduction of larger side chains in this position could possibly enhance IDO1 inhibition. Then, regarding the less potent compound **11a**, one preferential orientation was obtained (Fig. 3c,d; 10 solutions out of the 20 generated) with a GoldScore for the best pose of 47.27. Two other

orientations with an even lower stabilization were also suggested. In the preferred orientation (Fig. 3c,d) the compound seems to adopt a reverse binding compared to the compounds of the 'b-series' (Fig. 3c), probably to allow a better accommodation of the methoxyphenyl sidechain. In this orientation, the pyrroloiminoquinone interacts with the heme via its nitrogen (distance N|···Fe = 4.0 Å) rather than the carbonyl function and the hydrogen bonding to the Ser167 residue is lost. Indeed the nice fit with high stabilization (high GoldScore) of the pyrroloiminoquinone of the 'b-series' and the IDO1 active site can certainly account for their usually higher IDO1 inhibition compared to compounds of the 'a-series' (for instance compare compounds **10a** vs **10b**, **11a** vs **11b**, **14a** vs **14b**, **15a** vs **15b** and **17a** vs **17b**).

In conclusion, derivatives of natural products tsitsikammamines have previously been shown to be potent anticancer agents. Here we show for the first time that these compounds exert inhibitory activity on indoleamine 2,3-dioxygenase, an enzyme involved in tumoral immune resistance. Although the previously demonstrated anticancer activity cannot probably be attributed to their IDO1 inhibitory potency, the present work sheds light on one possible mechanism of anticancer activity in this series. Some of these compounds were also shown to possess reasonable activities on IDO1 in a cell-based assay, thus strengthening their potential for future development in the field of anticancer immunotherapy. They also display a selectivity for IDO1 versus tryptophan 2,3-dioxygenase. A molecular modeling study of compounds **11a–b** in the IDO1 active site suggested that the pyrroloiminoquinone was deeply inserted in the cavity A, leaving pocket B only partially filled. In the more potent 'b-series', introduction of larger side chains in order to fill this other pocket can thus be proposed as a potential means of enhancing the IDO1 inhibitory potency.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.036.

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