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Antitumour agents as inhibitors of tryptophan 2,3-dioxygenase

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ABSTRACT

The involvement of tryptophan 2,3-dioxygenase (TDO) in cancer biology has recently been described, with the enzyme playing an immunomodulatory role, suppressing antitumour immune responses and promoting tumour cell survival and proliferation. This finding reinforces the need for specific inhibitors of TDO that may potentially be developed for therapeutic use. In this work we have screened ~2800 compounds from the library of the National Cancer Institute USA and identified seven potent inhibitors of TDO with inhibition constants in the nanomolar or low micromolar range. All seven have antitumour properties, killing various cancer cell lines. For comparison, the inhibition potencies of these compounds were tested against IDO and their inhibition constants are reported. Interestingly, this work reveals that *NSC 36398* (dihydroquercetin, taxifolin), with an *in vitro* inhibition constant of ~16 μM, is the first TDO-selective inhibitor reported.

1. Introduction

Tryptophan 2,3-dioxygenase (TDO) is a homotetrameric enzyme, which along with indoleamine 2,3-dioxygenase (IDO) and indoleamine 2,3-dioxygenase-2 (IDO2) catalyses L-tryptophan dioxygenation in the first step of the kynurenine pathway. First discovered in the 1930s [1], TDO is primarily expressed in the liver in humans [2], but after stimuli it can be also detected in other tissues including the placenta [3], pregnant uterus [4], epididymis, testis [5] and brain [6]. In contrast, IDO was first identified in 1967 [7] and is expressed ubiquitously throughout the human body, except for the liver. A groundbreaking discovery in 1998 [8] revealed for the first time the immunosuppressive role of IDO and suggested a potential role for the enzyme in tumour cell survival. In the years following, a number of new pieces of evidence came to show that IDO plays a key role in regulating immune evasion by tumours [9]. The proven role of IDO in cancer biology has resulted in the extensive study of the enzyme and the identification of numerous IDO inhibitors [10–15]. In contrast, lack of evidence for the implication of TDO in cancer, in combination with the predominant expression of the enzyme in liver, has engendered less interest in targeting TDO for inhibition. Despite the likelihood of TDO involvement in tumour immune escape, only in 2011 did a brain tumour study formally associate TDO with cancer [16], making the enzyme an interesting pharmacological target. This work not only implicates TDO in cancer biology but also provides details regarding the tumour cells' mechanism of action. More specifically, TDO-derived kynurenine acts as an endogenous ligand for the human aryl hydrocarbon receptor (AHR), leading to activation of the receptor and suppression of the antitumour immune response. Interestingly, another study which examined the expression of enzymatically active TDO in various cancers showed that the enzyme is expressed in a significant proportion of human tumours including bladder carcinoma, hepatocarcinoma and melanoma [17]. Clearly such findings have great implications for the potential use of TDO inhibitors as anti-tumour therapeutic agents. The aim of the work reported here was to identify TDO lead inhibitors that can be used as templates for the development of new selective inhibitors of the enzyme. This has revealed seven TDO inhibitors with inhibition potencies between 30 nM and 16 mM, none of which have previously been identified as a TDO inhibitor. In order to examine the specificity of the newly identified compounds for TDO, they were also tested against human IDO and screened against *Pseudomonas fluorescens* kynurenine 3-monoxygenase (KMO). In comparison with TDO, these inhibitors have decreased IDO inhibition potencies (2-8-fold) while in the case of KMO they show negligible inhibition at 10 μM concentration in the kynurenine monoxygenation assay. Study of the flavonoid compound *NSC 36398* (dihydroquercetin, taxifolin) revealed the first selective inhibitor of TDO that has been reported *in vitro*. This compound will be studied further towards the development of a new class of TDO-selective inhibitors.

2. Materials and methods

Most of the chemicals used (L-Trp, L-ascorbate, bovine liver catalase, methylene blue, mitomycin C), including those for buffers

Abbreviations: TDO, human tryptophan 2,3-dioxygenase; IDO, human indoleamine 2,3-dioxygenase; KMO, kynurenine 3-monoxygenase; NCI, National Cancer Institute USA.

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(Sigma–Aldrich), were of the highest analytical grade (⩾97% purity) and were used without further purification. Compounds received from the National Cancer Institute (USA), were used without any further purification.

The expression systems used for production of recombinant human TDO and IDO were as described previously [18,19]. Additionally, the growth medium was supplemented with 5 μM hemin in order to maximize incorporation of heme. Harvested cells were resuspended in 20 mM Tris–HCl buffer pH 8.0, 300 mM in NaCl, 10 mM in imidazole, 1 mM in tris(2-carboxyethyl)phosphine (TCEP) (buffer A). Cell lysis was initiated by incubating the cell suspension for 30 min with hen egg white lysozyme (1 mg per ml of suspension), phenylmethylsulfonyl fluoride (PMSF; 2 mg per ml of suspension) and 1 complete protease inhibition tablet (Sigma). Lysis was completed by ultrasonication of the suspension on ice. Cell debris was then removed *via* centrifugation for 1 h at 28,000 g and 4 °C, and the supernatant collected. For TDO-containing supernatant batches of 100 ml were loaded at a time (to avoid aggregation on the Ni-agarose column) while for IDO-containing supernatant the entire volume (200–250 ml) was loaded onto a Ni-agarose column. For TDO purification the column was washed with 1 column volume (CV) of buffer A, and eluted using the same buffer but with a higher concentration of imidazole (250 mM). For IDO the column-bound protein was washed using 5 CV of buffer A and eluted in the same way. Following elution of TDO or IDO, size exclusion chromatography (Superdex 200 for TDO and Superdex 75 for IDO) was used in order to remove imidazole. In either case the column was pre-incubated in 20 mM Tris–HCl pH 8.0, 1 mM in TCEP for at least 1 CV and the proteins collected were judged to be pure and homogeneous. Enzyme concentrations were determined spectrophotometrically using absorption coefficients for the ferric form of the enzymes: TDO, ε_{408} = 196,000 M^{-1} cm^{-1} IDO, ε_{406} = 172,000 M^{-1} cm^{-1}.

Both TDO and IDO inhibition assays were carried out according to the method of Takikawa et al. with minor modifications [20]. The assays were carried out in 96-well microplates with the components dissolved in 100 mM KP_i buffer, pH 6.5. Each well contained 200 μl of assay mixture. The reaction mixture was composed of 20 μl of 400 mM ascorbic acid, 4 μl of 1 mM methylene blue, 4 μl of 10 mg/ml catalase, 20 μl of L-tryptophan at final assay concentrations from 0–800 μM for TDO and 0–45 μM for IDO, 2 μl of the inhibitor dissolved in DMSO (1% final DMSO concentration in the assay) and 50 μl of TDO or IDO at final concentrations of 20 and 10 nM respectively. The reaction mixture was incubated at room temperature for either 20 min (TDO) or 15 min (IDO) and the reaction was terminated by adding 40 μl of trichloroacetic acid (30 % w/v) into each well. Subsequently the microplate was transferred into an oven and incubated at 50 °C for 30 min. The microplate was then centrifuged for 15 min at 4000 rpm and 125 μl of the supernatant transferred to a new microplate and mixed with an equal volume of 4-dimethylaminobenzaldehyde (DMAB) in acetic acid (2 % w/v). Finally, the absorbance was measured at 490 nm, where the kynurenine-DMAB adduct has an absorbance maximum. For purposes of accuracy and reliability, multiple data sets for each enzyme/inhibitor combination were obtained and compared with either published results (IDO) or cuvette assay results (TDO) and found to be in agreement. Any contribution to absorbance at 490 nm caused by the presence of the NCI compound was eliminated by subtracting the absorbance from a control well that was identical in composition except for the absence of substrate.

3. Results

Plate screening of ~2800 potential inhibitor compounds obtained from the National Cancer Institute indicated that 7 of these compounds (Fig. 1) displayed promise as reversible competitive inhibitors of TDO, as evidenced by inhibition constants either in the nanomolar or low micromolar range (Table 1).

NSC 26326, known as β-lapachone, is a natural occurring quinone that can be isolated from the lapacho tree (*Tabebuia avellanedae*). A series of studies have shown that *NSC 26326* affects the survival rate of cancer cells such as pancreatic, breast, colon, retinoblastoma, leukemia and non-small-cell lung cancer, with LD_{50} and IC_{50} values in the region of 1–4 μM, depending upon cell type, and likely *via* activation of a noncaspase proteolytic pathway [21–26]. Of all the reported inhibitors in this paper, *NSC 26326* is the strongest inhibitor of both IDO and TDO with inhibition constants of 97 ± 14 nM and ~30–70 nM respectively (see Supplementary material for Dixon plots for all compounds). Like *NSC 26326*, *NSC 36398* (dihydroquercetin, taxifolin) is another natural product that belongs to the class of flavonoids. Among the several flavonoids examined *NSC 36398* is the most potent inhibitor of TDO with a K_i of 16.3 ± 3.8 μM (Fig. 2). In contrast with TDO, IDO was not inhibited by *NSC 36398* at concentrations up to 100 μM. The low toxicity of flavonoids in combination with their previously reported anticancer function makes *NSC 36398* an attractive target in cancer therapy [27]. *NSC 267461* or nanaomycin A (in use as an animal antibiotic) is a naphthoquinone based inhibitor, inhibiting IDO and TDO with K_i values of 950 ± 270 and 360 ± 30 nM respectively. *NSC 267461* is active in 59 tumour cell lines, killing several types of cancer cells with IC_{50} values lying somewhere 400 nM and 4 μM [28].

NSC 111041 also inhibits TDO and IDO with inhibition constants of 1.1 ± 0.3 and 4.3 ± 0.9 μM respectively. Examination of this compound revealed activity against colon and breast tumour cell lines [28]. *NSC 255109* (17-aminodemethoxygeldanamycin) is a strong inhibitor for both TDO and IDO, with inhibition constants in the nanomolar and micromolar ranges respectively. For TDO, K_i was found to be 600 ± 70 nM and for IDO it was 1.4 ± 0.5 μM. Tests on tumour cell lines showed that *NSC 255109* is active in 65 different cell lines (with IC_{50} values between ~200 nM and 8 μM in breast cancer cell lines [29]) and the types of cancer cells that this compound is effective on are given in Table 1 [28]. *NSC 261726*, or 3-deazaguanine, is an inhibitor with activity in the lower micromolar range (K_i values of 5.6 ± 0.4 and 21.4 ± 2.4 μM for TDO and IDO respectively). In addition, *NSC 261726* activity in colon and

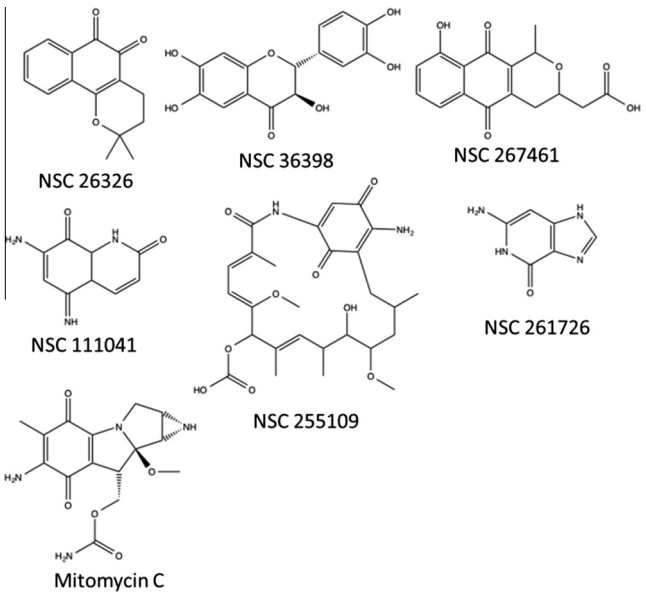

Fig. 1. Structures of the seven TDO and IDO inhibitors as identified by screening of NCI compounds.

Table 1
Inhibition constants for TDO and IDO inhibition by seven NCI inhibitors. Information includes K_i values and which tumour cell lines are affected (details of cell lines can be obtained from the National Cancer Institute USA [28]).

NSC	K_i (TDO)	K_i (IDO)	Active against tumour cell lines
26326	30–70 nM	97 ± 14 nM	Pancreatic, breast, colon, retinoblastoma, leukemia and non-small-cell lung cancer
36398	16.3 ± 3.8 μM	>100 μM	Ovarian cancer
267461	360 ± 30 nM	950 ± 270 nM	Non-small cell lung, melanoma, prostate, central nervous system, small cell lung, colon, breast, ovarian, leukemia and renal cancer
111041	1.1 ± 0.3 μM	4.3 ± 0.9 μM	Colon and breast cancer
255109	600 ± 70 nM	1.4 ± 0.5 μM	Non-small cell lung, melanoma, prostate, central nervous system, colon, breast, ovarian, leukemia and renal cancer
261726	5.6 ± 0.4 μM	21.4 ± 2.4 μM	Colon cancer and leukemia
Mitomycin C	2.86 ± 0.03 μM	24.2 ± 1.2 μM	Non-small cell lung, melanoma, prostate, central nervous system, small cell lung, colon, breast, ovarian, leukemia and renal cancer

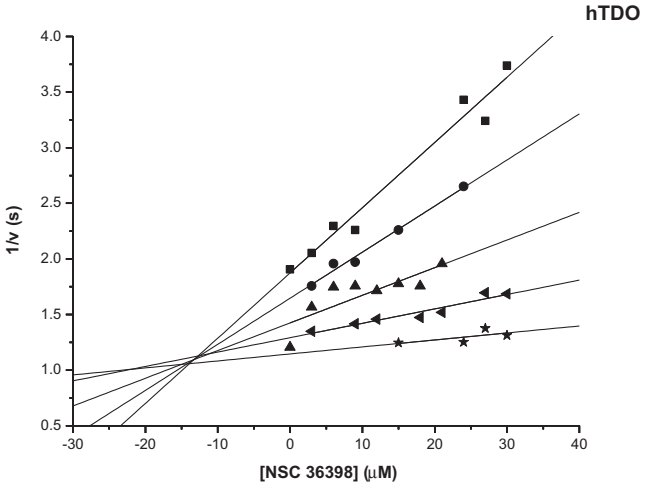

Fig. 2. Dixon plot for determination of K_i value for inhibition of TDO by *NSC 36398*. Substrate concentrations (μM) were ■ = 300, ● = 350, ▲ = 400, ◀ = 500, ★ = 600.

leukemia tumour cell lines has further increased interest in this compound [28]. Despite the structural similarities between *NSC 261726* and guanine, the latter showed no inhibitory activity on either TDO or IDO. Indeed, none of the heterocyclic nucleic acid bases indicated inhibition of IDO or TDO. Finally, among the compounds identified by screening is mitomycin C, an approved oncology drug with action in 74 tumour cell lines [28]. Mitomycin C is a known IDO inhibitor (K_i = 24.2 ± 1.2 μM). Interestingly, our study shows that mitomycin C is about 8 fold more potent inhibitor of TDO with K_i = 2.86 ± 0.03 μM.

4. Discussion

The proposed immunosuppressive involvement of TDO in cancer [16,17] has led us to concentrate our efforts in discovering and optimizing new inhibitors of the enzyme. High-throughput screening of 2800 compounds led to the identification of seven potent TDO inhibitors, increasing at the same time our understanding about their action *in vivo*. The significant number of cancer cell lines that most of these compounds are active in suggests that their *in vivo* action is not completely understood. The action of β-lapachone (*NSC 26326*), for example, was proposed to be highly correlated with the expression of NAD(P)H: quinone oxidoreductase-1 (NQO1) [30], an enzyme that catalyses reduction of quinones to hydroquinones. This work shows that the antitumour potency of the compound is also possibly related with the function of TDO and IDO *in vivo*. *NSC 267461* is another example where its action was proposed to be related DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B) [31]. Herein it is shown that TDO and IDO are also two potent targets of the compound *in vivo*. Similar to *NSC 26326* and *NSC 267461*, the *in vivo* anti-tumour action of the other five compounds has not been related to TDO activity. *In vitro* studies on purified enzyme samples can provide a quantitative assessment of relative affinities of inhibitors for the enzyme of interest (in our case for TDO), which may help to explain the results obtained from mouse model experiments and cell-based assays. The parallel examination of TDO and IDO led to the identification of the first TDO selective inhibitor with inhibitory potency in the low micromolar range. To our knowledge dihydroquercetin (taxifolin, *NSC 36398*) is the first compound identified with *in vitro* selectivity for TDO over IDO. Structure-activity relationship (SAR) studies for optimization of *NSC 36398* inhibition activity will be the next goal of this work, and in order to obtain structural insights into the interaction between *NSC 36398* and TDO, co-crystallization trials will also be carried out.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.037.

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