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TITLE: Development of a Novel Therapeutic Paradigm Utilizing a Mammary Gland-Targeted, Bin1-Knockout Mouse Model

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Development of a Novel Therapeutic Paradigm Utilizing a Mammary Gland-Targeted, Bin1-Knockout Mouse Model

Bin1 is implicated to be an anti-cancer gene in mammary gland epithelial cells. We have discovered that Bin1 loss can promote tumorigenesis through a cell-extrinsic mechanism that involves escape from host cell-mediated anti-tumor immunity. This correlates with the negative regulatory impact that Bin1 exerts on the important immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO). We have demonstrated how, in combination with certain standard chemotherapeutic agents, inhibitors of IDO can be successfully employed in a non-obvious therapeutic regimen to successfully treat established tumors in MMTV-Neu mice – an autochthonous breast cancer model histologically akin to ductal carcinoma in situ (DCIS). We have shown that these tumors respond to IDO inhibitor treatment in combination with certain chemotherapeutics, but do not know if this is due to inhibiting IDO that is directly expressed in the tumor cells or in accessory antigen presenting cells (APCs). The studies we are conducting directly interrogate the role of Bin1 loss and concomitant IDO upregulation in the development of autochthonous breast tumors. To this end, we have developed antibodies that will allow us to immunohistochemically evaluate Bin1 and IDO expression in tumors. Furthermore, we have developed a conditional Bin1 knockout mouse, which will allow us to target Bin1 deletion specifically to the mammary gland epithelium. In conjunction with the bioavailable inhibitor 1-methyl-tryptophan (1MT), we are using a combination of genetic and chemical genetic approaches to establish the pathophysiological relevance of this pathway to mammary gland tumor biology and begin to elucidate the cellular mechanisms by which tumors escape immune rejection.
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INTRODUCTION

Loss or attenuation of expression of the Bin1 anti-cancer gene in patient biopsies has been associated with malignant breast carcinoma\(^1\) as well as other prevalent cancers. Our recently published results indicate that Bin1 loss can have a striking effect in promoting tumoral immune escape and that this can be more important to tumor formation than the impact of Bin1 loss on intrinsic growth properties\(^2\). We have identified the immunomodulatory gene IDO as a negatively-regulated downstream target of Bin1. IDO encodes indoleamine 2,3-dioxygenase, a tryptophan catabolizing enzyme that has been demonstrated to play a physiologically essential role in protecting the allogeneic fetus during pregnancy by suppressing T cell activation. Our work is the first to connect the IDO gene to a known cancer suppression pathway, and dovetails nicely with the observation of increased IDO-mediated tryptophan catabolism that has been frequently reported in cancer patients. In a well-established transgenic mouse model for breast cancer, the MMTV-Neu mouse, we have demonstrated that IDO inhibitors can exhibit impressive therapeutic utility when used in combination with specific chemotherapeutic agents\(^3\). Identification of this non-obvious combination of immunotherapeutic and chemotherapeutic-based regimen presents a clear path forward for translational development. Our current studies are aimed at addressing the links between Bin1 loss, IDO dysregulation, and host immunity in the autochthonous MMTV-Neu mouse breast cancer model. The overall goals are to evaluate how mammary gland-targeted Bin1 loss and pharmacological inhibition of IDO impact tumor development, as well as examine the roles played by specific immune cell populations in anti-tumor rejection and tolerance. These studies are essential for understanding how IDO inhibitors operate in the in vivo context of mammary gland tumorigenesis and will establish the preclinical conceptual basis for future breast cancer drug development.

BODY

Task 1. Examine Bin1 and IDO expression in autochthonous MMTV-Neu tumors

Bin1 is frequently attenuated in malignant human breast cancers, Bin1 loss has been associated with IDO dysregulation, and IDO inhibition cooperates with chemotherapeutic agents to promote regression of the autochthonous mammary gland tumors that develop in MMTV-Neu mice. In order to understand the role of Bin1 and IDO in the MMTV-Neu model, two critical questions remaining to be addressed are: 1) Bin1 is attenuated MMTV-Neu tumors? and 2) is IDO expression dysregulated directly in the tumor cells? To address these questions we have been performing immunohistochemical analysis using mouse monoclonal antibodies (mAbs) to Bin1 (2F11) and IDO (10.1) that we previously generated. The Bin1 staining results are shown in Figure 1. Fixed tissue sections were prepared from an MMTV-Neu mammary gland tumor (derived from a 6 month-old parous female mouse) and from a normal mammary gland (derived from a pregnant 2 month-old female MMTV-Neu mouse). Adjacent tissue sections were stained with H&E to display histology or with secondary antibody treatment alone to document specificity of the \(\alpha\)-Bin1 staining pattern. In normal mammary gland epithelial cells, Bin1 is
robustly expressed in the nucleus and cytosol, whereas in MMTV-Neu epithelial-derived tumor cells, Bin1 expression is less robust and detectable only in the cytosol of the cells. These data suggest that in MMTV-Neu tumors Bin1 function may be attenuated both through decreased expression and mislocalization during the process of malignant transformation.

We have, as of yet, been unable to successfully perform immunohistochemistry on mouse tissues with the monoclonal antibody to IDO that we currently have available (data not shown). The antibody can be used to successfully detect IDO in human tissues, but the mouse origin of the antibody complicates its use on mouse tissues. We are evaluating additional procedures to circumvent this technical difficulty. We are also in the process of acquiring and evaluating other antibodies for formalin fixed mouse tissue staining including a polyclonal antibody that has been generously provided to us by David Munn from the Medical College of Georgia which has been reported to stain endogenous IDO in mouse tissues3.

Task 2. Directly determine the impact of Bin1 loss on tumor development

To investigate how Bin1 loss affects tumorigenesis driven by ectopic expression of the c-Neu proto-oncogene in the mammary gland, we have crossed the MMTV-Neu transgene onto the Wap-Cre+/– Bin1flx/KO background, in which the Bin1 gene undergoes tissue targeted disruption in the mammary epithelial cells of parous female mice. In addition to the MMTV-Neu+/– WapCre+/– Bin1flx/KO experimental group, a second group of MMTV-Neu+/– WapCre+/– Bin1flx/wt females was also produced to control for effects that might occur independently of Bin1 loss. Beginning at 2 months of age, cohorts of 5 mice each from the experimental and control groups were put through two rounds of pregnancy and nursing and were then monitored for tumor development. Under these conditions, 100% of FVB-strain females carrying homozygous copies of the MMTV-Neu transgene developed mammary gland tumors by 8 months (Fig. 2). In the original grant proposal we acknowledged a concern that changes in the genetic context in which the MMTV-Neu gene was expressed might adversely impact tumor formation, but we could only speculate as there has not been any published literature evaluating these parameters for this tumor model. One of our concerns, that the experimental design involved reducing the copy number of the MMTV-Neu transgene to hemizygosity, turned out not to be of much consequence, as this was shown to only have a minor impact on tumor formation reducing the incidence at 8 months from 100% to 85% on the FVB strain background (Fig. 2). However, during these studies, we have found tumor formation to be dramatically suppressed in the context of the mixed strain background. Following 8 months of monitoring none of the mice with a mixed strain background has developed a palpable mammary gland tumor irrespective of Bin1 status (Fig. 2).

Having anticipated the mixed genetic background might be a potential pitfall, we concurrently began working on the backup strategy. We have now completed 5 or more generations of backcrossing for all of the necessary genetic elements so that the genotypic background of any mouse required for breeding is on the order of 97% FVB. We are currently in the process of breeding these mice to obtain the necessary mix of genetic components to repeat the experiment in the FVB strain background.
We have also initiated an additional backup plan that is yielding positive data. We are investigating alternative mechanisms to MMTV-Neu transgene expression for induction of autochthonous mammary gland tumors in mice. In particular we have studies ongoing to evaluate the impact of mammary gland targeted \textit{Bin1} loss on normal mammary gland development and on the development of tumors induced by chemical carcinogenesis.

To evaluate the near-term impact of \textit{Bin1} loss on the mammary gland, Wap-\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} and Wap-\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} female mice were set up for timed pregnancies by monitoring for vaginal plugs. After parturition, litter sizes were normalized to 5 pups. Nursing was continued for 7 days to achieve full lactation after which the pups were removed to induce mammary gland involution. Mammary gland tissues were isolated for analysis by PCR analysis and histology from euthanized animals at various stages including; virgin (control), 18.5 dpc (date post coitus), 7.5 dpp (date post partum; full lactation), 10.5 dpp (early involution), 17.5 dpp (late involution), and 27.5 dpp (full regression). Virgin Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} mice showed diminished ductal development compared to virgin Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} mice. This difference was more pronounced in pregnant mice at 18.5 dpc at which time Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} mice showed significantly less lobular development than Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} mice (Fig. 3). In contrast, no difference was apparent during nursing or involution in the mammary gland of Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} mice (data not shown). Taken together, these results suggest that, although Bin1 is not essential, it facilitates lobular development prior to and during pregnancy but that compensatory development minimizes this difference following parturition. In long term experiments, over a period of nearly 2 years, no significant impact of mammary gland targeted \textit{Bin1} loss has been observed on the spontaneous development of breast cancer in either virgin or parous females (data not shown).

To evaluate \textit{Bin1} as a negative modifier of tumorigenesis, we are assessing the effect of \textit{Bin1} loss on the mammary gland tumors initiated by treatment with the carcinogen 7, 12-dimethylbenz[\textit{\alpha}]anthracene (DMBA). In these experiments, the synthetic progesterone medroxyprogesterone acetate (MPA) has also been administered to increase tumor frequency, decrease tumor latency and reduce non-tumor related morbidity and mortality. After weaning their first litter, uniparous Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} and Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} mice received subcutaneous implants in the intrascapular area of two 20 mg MPA compressed pellets. Three weeks later, mice received the first of four 1x/week doses of 50 mg/kg DMBA, administered by oral gavage in cottonseed oil, with the three subsequent doses delivered 1, 3 and 4 weeks after the first dose. Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} and Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} groups showed similar tumor latency, multiplicity. However, while Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/wt}} mice developed well-differentiated mammary tumors with robust tubule formation, minimal nuclear pleomorphism, and low mitotic indices, Wap\textit{Cre}^{+/−}\textit{Bin1}^{\text{flox/KO}} mice developed poorly differentiated mammary tumors with limited tubule formation, marked nuclear pleomorphism, and high mitotic indices (Table 1 and Fig. 4).

Task 3. Investigate the chemopreventative activity of IDO inhibitor treatment in relation to \textit{Bin1} status

No activity to report on this Task.
Task 4. Profile tumor-associated immune cell populations and functionally characterize the involvement of specific T cell populations.

No activity to report on this Task.

KEY RESEARCH ACCOMPLISHMENTS

Progress on Tasks outlined in the Statement of Work timeline

Task 1. Examine Bin1 and IDO expression in autochthonous MMTV-Neu tumors (Months 1-12).

- **Completed**
  - Collect malignant and normal mammary gland samples from MMTV-Neu females (Months 1-6)
  - Perform immunohistochemical analysis of Bin1 protein expression (Months 7-9)

- **In Progress**
  - Perform immunohistochemical analysis of IDO protein expression (Months 7-9)
  - Perform Western blot and Northern blot analysis to assess Bin1 and IDO expression levels in MMTV-Neu tumors (Months 10-12)

Task 2. Directly determine the impact of Bin1 loss on tumor development (Months 1-36).

- **Completed**

  **Breeding**
  - Obtain the following founder lines
    a. WapCre(+/–) (purchased from JAX, licensing agreement from Bristol Myers Squibb Co. on file)
    b. Bin1(KO/wt) (generated in house⁵)
    c. MMTV-Neu(+/+) (purchased from JAX, licensing agreement from DuPont Co. on file)
    d. Bin1(flox/flox) (generated in house)
  - Produce the following intermediate lines (Months 1-9)
    a. WapCre(+/+)Bin1KO/wt females (2 generations)
    b. MMTV-Neu(+/+)Bin1(flox/flox) males (2-3 generations)
  - Produce the following experimental and control lines (Months 10-36)
    a. WapCre(+/–)MMTV-Neu(+/–)Bin1(flox/KO) females
    b. WapCre(+/–)MMTV-Neu(+/–)Bin1(flox/wt) females
  - Produce FVB-strain congenic lines carrying the Bin1(KO), Bin1(flox), and Wap-Cre alleles (Months 1-12)
  - Produce experimental and control lines in the FVB-strain background if necessary (Months 13-24)
**Experiment**
- Put experimental and control mice through two rounds of pregnancy and nursing and monitor for tumor development (Months 13-20)

**In Progress**
- Produce experimental and control lines in the FVB-strain background if necessary (Months 13-24)

**Additional relevant research accomplishments**

**Completed**
- Evaluate the short term impact of Bin1 loss on mammary gland histology during lactation and involution.
- Evaluate the long term impact of Bin1 loss on spontaneous mammary gland tumor formation.

**In Progress**
- Evaluate the impact of Bin1 loss on mammary gland tumor formation induced by chemical carcinogenesis.

**REPORTABLE OUTCOMES**

**Manuscripts**


**Abstracts/Presentations**

Abstract presented: “Transcriptional control of IDO by the cancer suppression gene Bin1, a key mechanism for restraining tumor immune escape.” (Minisymposium talk selected from Abstract)

NCI Conference on Translational Immunology Related to Cancer. Bethesda, MD. September 22-23, 2005
Abstract presented: “Pharmacological inhibition of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) cooperatively leverages cytotoxic chemotherapy”
(Poster)

Abstract Presented: “Pharmacological inhibition of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) cooperatively leverages cytotoxic chemotherapy.”
(Plenary session talk selected from Abstract)

AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. Philadelphia, PA. November 14-18, 2005
Abstract Presented: “Development of Brassinin Derivatives as IDO Inhibitors for Combinatorial Cancer Treatment”
(Poster)

CONCLUSION
Consistent with the published data from human breast cancer biopsies1, Bin1 does appear to be attenuated during the process of malignant progression involved in the formation of MMTV-Neu mouse tumors, both through decreased expression as well as through mislocalization. This is encouraging for moving forward with evaluating whether IDO dysregulation occurs in the tumor cells as well. However, we have encountered technical difficulties with the IDO immunohistochemical staining of these tumors and are currently working to resolve this issue.

We successfully performed all of the breeding to combine mammary gland targeted Bin1 disruption with the MMTV-Neu transgene. Unfortunately, the mixed genetic background strongly suppressed the development of tumors driven by the MMTV-Neu transgene. Being cognizant of this possible pitfall, we concurrently began breeding to introduce all of the necessary genetic elements onto the FVB strain background on which the MMTV-Neu transgene efficiently promotes tumor development and are at the stage of performing the crosses necessary to generate the new set of experimental mice.

As an additional contingency, we have induced autochthonous mammary gland tumor formation in the context of conditional Bin1 loss through mechanism that is independent of MMTV-Neu transgene expression. In the context of chemical carcinogenesis initiated with DMBA, Bin1 loss did not alter tumor incidence or latency but consistently resulted in tumors that were identified histopathologically as being more malignant both in terms of their differentiation status as well as their individual and combined clinical scores. This may prove to be a complementary model to the MMTV-Neu model, and may become the primary model if studies in the FVB strain background fail to produce any differences in tumor formation associated with Bin1 loss.

REFERENCES

APPENDICES


Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors

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Indoleamine 2,3-dioxygenase (IDO) is an interferon (IFN)-γ-inducible, extrahepatic enzyme that catalyses the initial and rate-limiting step in the degradation of the essential amino acid tryptophan. Elevated tryptophan catabolism mediated by IDO is associated with a wide variety of human cancers and has historically been thought to be a tumoricidal consequence of IFN-γ exposure. Evidence of a physiological requirement for IDO activity in protecting the allogeneic fetus from rejection by the maternal immune system has stimulated a radical shift in thinking about the role of IDO in cancer. Evidence now suggests that tumours can exploit IDO-mediated peripheral tolerance to promote immune escape. This review summarises key studies that implicate IDO as an important mediator of peripheral immune tolerance as well as the development of a promising new anticancer modality that incorporates the use of IDO inhibitors. The second part focuses on the current state of development of IDO inhibitory compounds as potential pharmaceutical agents.

Keywords: 1-methyl-tryptophan (1MT), antigen-presenting cell (APC), Bin1, chemotherapy, dendritic cell (DC), Indo, indoleamine 2,3-dioxygenase (IDO), macrophage, tryptophan, tumour


1. Introduction

Tumour interactions with the host immune system are complex and dynamic. Inflammation produces a tumour-promoting environment comprised of cytokines, chemokines and growth factors, activated stroma, and DNA damaging agents [1]. However, tumours are also subject to immune surveillance; in particular, the expression of tumour antigens means that cancer cells must evolve mechanisms to escape or subvert antitumour immunity in order to successfully progress [2,3]. This process of ‘immune editing’, whereby immune-mediated destruction of nascent cancer cells provides selective pressure that shapes the immunogenic phenotype of the growing tumour [4], has been clearly demonstrated in mouse tumour models [5]. Studies of human tumours provides further evidence of a microenvironment of immune privilege that protects cancer cells from immune destruction [6-8]. Now widely recognised as an additional ‘hallmark of cancer’ [9], immune escape is proving to be an important obstacle to the development of immunotherapeutic protocols such as adoptive immunotherapy of in vitro activated T cells, which has been only marginally successful despite evidence that the transferred T cells can localise to tumours [10,11]. A promising target for attacking tumoural immune escape, reviewed here, is the enzyme indoleamine 2,3-dioxygenase (IDO). In particular, recent data show that IDO inhibitors can cooperate with cytotoxic agents to more effectively destroy tumours, in line with the burgeoning notion that combining immunotherapeutic and chemotherapeutic treatment modalities can be remarkably effective.
2. Indoleamine 2,3-dioxygenase, immune regulation and cancer

2.1 Indoleamine 2,3-dioxygenase: background

Elevated tryptophan catabolism, a condition previously associated with microbial infections such as tuberculosis, was observed in patients with bladder cancer in the 1950s [12]. By the 1960s, elevated levels of tryptophan catabolites had been documented in the urine of patients with a variety of malignancies including leukaemia, Hodgkin’s disease, prostate disorders, and breast cancer [13-18]. The hepatic enzyme tryptophan dioxygenase (TDO2; EC 1.13.11.11) was known at the time to carry out the catabolism of dietary tryptophan, having been the first inducible mammalian enzyme to be isolated back in the 1930s [19,20]. TDO2 catalyses the initial and rate-limiting step in the degradation of tryptophan to N-formylkynurenine. However, no increase in TDO2 activity was detected in patients presenting with elevated tryptophan catabolites, implying the activity of a second enzyme.

In 1963 the isolation of a non-hepatic tryptophan catabolising enzyme, D-tryptophan pyrrolase, was first reported [21,22]. Renamed indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.17), this enzyme also converts tryptophan to N-formylkynurenine. However, despite producing the same reaction product as TDO2, IDO is otherwise remarkably dissimilar [23]. IDO is a monomeric 41 kDa enzyme whereas the active TDO2 enzyme is a tetramer totaling 320 kDa in size. The two proteins are antigenically distinct [24] and share no significant amino acid sequence homology (as determined by standard comparative analysis using the NCBI ‘BLAST 2 Sequences’ online program). IDO has less stringent substrate specificity, cleaving a number of indole-containing compounds that are not recognised by TDO2. This is an advantageous consideration in the development of compounds that will selectively inhibit IDO but not TDO2, because the IDO active site is likely to accommodate a wider spectrum of inhibitory compounds than TDO2. IDO is a haem-containing enzyme that utilises superoxide anion for activity, whereas TDO2 does not use superoxide as an oxygen donor. In vitro, IDO enzyme reactions are performed by substituting ascorbic acid for superoxide. The IDO enzyme also requires methylene blue as a cofactor in the reaction to maintain full activity. In vivo, the role of methylene blue is thought to be assumed by either a flavin or tetrahydrobiopterin. The cofactor binding site is distinct from the substrate binding site [25] and may represent an opportunity for the development of distinct classes of noncompetitive IDO inhibitors.

Indo is the official designation for the gene encoding the IDO enzyme. In humans, Indo is a single copy gene comprised of 10 exons spanning ~ 15 kb which maps to 8p12-p11 [26,27]. The mouse gene, also located on chromosome 8, has a similar genomic organisation. There is, however, a good deal of divergence at the primary amino acid sequence level between species, with the human and mouse Indo genes sharing only 62.5% identity. The Indo gene is found early in evolution with a homologous gene present in the yeast Saccharomyces cerevisiae (Genbank no. – Z49578). It appears, however, to have undergone functional divergence during the course of the evolution of archaegastropod mollusks (including Sulculus, Notois, Battilus, Onophasium and Chlorostoma), which express a unique form of myoglobin derived from the primordial Indo gene [28]. This abalone myoglobin provides useful structure/function data regarding the mammalian IDO enzyme. In particular, a mutation of a conserved histidine, which was determined to be the most likely iron-bound proximal histidine for the abalone myoglobin, has been shown to also be critical for mammalian IDO activity ([29] and PS Donover, J DuHadaway, AJ Muller, GC Prendergast, unpublished results). Solving the crystal structure of human IDO (for which diffraction data at 2.3 angstrom resolution has been collected according to a recent web posting (S Oda, H Sugimoto, T Yoshida, Y Shiro, unpublished results) will be immensely valuable for structure–activity relationship-based modelling of inhibitory compound interactions.

The cytokine IFN-γ is a major inducer of IDO, especially in antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) [30-33]. Transcriptional induction of the Indo gene is mediated through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway; in particular, JAK1 and STAT1α [34]. STAT1α appears to act to induce Indo gene expression both directly through binding of IFN-γ activation sites (GAS) within the Indo promoter as well as indirectly through induction of interferon regulatory factor (IRF)-1 which binds the Indo promoter at two IFN-stimulated response element sites (ISRE) [34-38]. NF-κB also contributes to Indo induction [34]. In particular, IFN-γ and TNF (which signals through NF-κB) appear to act synergistically to induce expression of IRF-1 through a novel composite binding element for both STAT1α and NF-κB in the IRF-1 promoter (termed a GAS/NFκB element) that combines a GAS element overlapped by a non-consensus site for NF-κB [39]. A possible alternative to the development of inhibitors that block IDO enzyme activity directly might be to develop compounds that block the induction of Indo gene expression. Given the number of other targets regulated by IFN-γ signalling, however, it is likely that blocking the entire signalling pathway would have pleiotropic consequences unrelated to IDO inhibition.

2.2 IDO promotes peripheral immune tolerance

The topic of the role of IDO in peripheral tolerance has already been extensively covered in a number of recent reviews including [40-42], and so is not treated exhaustively here. IDO activity is ubiquitously present, albeit at differing levels, in mammalian organs [43,44]. Like TDO2, IDO catalyses the initial and rate limiting step in the de novo biosynthesis of the critical coenzyme nicotinamide adenine dinucleotide (NAD). Unlike TDO2, however, IDO is unresponsive to changes in tryptophan or glucocorticoid levels [43] and is, therefore, unlikely to be responsible for metabolic processing of dietary tryptophan uptake. N-formylkynurenine generated by IDO is rapidly converted by a ubiquitously expressed formamidase to kynurenine,
but most mammalian tissues outside of liver lack the subsequent downstream enzymes necessary for de novo NAD biosynthesis. Instead, the kynurenine generated by IDO predominantly enters the bloodstream for urinary elimination [44] although some other cell types, including certain immune and neuronal cells, can further metabolise tryptophan to quinolinate and even NAD [45]. Particularly high basal levels of IDO activity have been observed within the epididymis, the placenta of pregnant females and at other sites of immune privilege [23,44-49]. Bacterial lipopolysaccharide (LPS) exposure elevates IDO activity in a variety of mouse tissues, most notably colon and lung [43,44,50]. The pattern of IDO expression suggested early on a possible role in inflammation [51], and accumulating experimental evidence supported the hypothesis that IDO might protect the host from auxotropic pathogens by depleting the local tryptophan pool and/or the production of toxic catabolites [52,53]. After IDO activity was found to be elevated in various cancer patients, the hypothesis was expanded to include the idea of IDO upregulation being a tumoricidal consequence of IFN-γ exposure through both starvation of the proliferating tumour cells of the essential amino acid tryptophan as well as exposure to cytotoxic tryptophan catabolites [54-58].

In 1998 Munn, Mellor and co-workers published the seminal finding that IDO activity appears to be essential for protecting the allogeneic fetus from the maternal immune system [59]. This study was performed based, in part, on in vitro findings indicating that T cells are exquisitely sensitive to tryptophan depletion by macrophages. Upon encountering an activation signal in a low tryptophan environment, T cells were found to be unable to complete progression through the cell cycle, arresting in mid-G1. If not activated again in the presence of sufficient tryptophan, these T cells subsequently underwent apoptosis [60]. The demonstration that macrophages are imbued with the ability to suppress T-cell proliferation when differentiated in vitro through exposure to macrophage colony-stimulating factor (MCSF) [61,62] had indicated that this might be a useful experimental system to study the mechanistic basis for the establishment of peripheral tolerance. Tryptophan depletion mediated by increased IDO activity was shown to be upregulated in MCSF-differentiated macrophages responding to IFN-γ stimulation [60]. In this in vitro study, the small-molecule IDO inhibitor 1-methyl- DL-tryptophan (1MT) was used to block macrophage IDO activity. 1MT had been identified as the most effective inhibitor among a small series of tryptophan analogues evaluated for IDO inhibitory activity [63]. A detailed review of the literature on small-molecule inhibitors of IDO can be found in Section 3.

1MT became the key tool employed in vivo to demonstrate the essential role of IDO in protecting the developing fetus from maternal immunity [60]. 1MT was delivered by subcutaneous implantation of time-release pellets, which permitted continuous dosing to be achieved. An important caveat with regard to this study, as well as all subsequent studies employing 1MT that have been published, to date, is that none has actually demonstrated that direct inhibition of the intended target, IDO, is responsible for the observed biological consequences. For instance, it has been reported that the transport system L [64,65], which transports L-tryptophan into cells, can also be inhibited by 1MT. This could contribute to the apparent biological activity of 1MT, independent of any direct inhibitory effect on IDO, by potentially limiting access of IDO to substrate and perhaps even directly blocking access of T cells to tryptophan. It has not yet even been demonstrated that IDO is inhibited by 1MT in vivo at the dose levels delivered nor has there been any reported attempt to correlate biological effects of 1MT with IDO pharmacodynamics. Therefore, the possibility that 1MT may be acting through an off-target mechanism of action has yet to be rigorously addressed.

In another ground-breaking finding, IDO has been implicated in the suppression of T-cell activation by cytotoxic T lymphocyte antigen (CTLA)-4 [60]. CTLA-4 is an important mediator of peripheral immune tolerance, and mice that are genetically deficient for CTLA-4 develop fatal autoimmune disease [67,68]. CTLA-4 belongs to the CD28 family of proteins. CD28 is an important co-stimulatory molecule for activation of T cells through engagement of the T-cell receptor (TCR). Both CD28 and CTLA-4, which are expressed on the surface of T cells, bind the B7 ligands, B7-1 and B7-2, expressed on the surface of APCs. The soluble fusion protein CTLA-4-immunoglobulin (CTLA4-Ig), which also binds B7-1 and B7-2, can prevent allograft and xenograft rejection in mouse transplantation models [69-71]. It has generally been accepted that CTLA-4 is directly antagonistic to CD28 in T cells, either through out-competing CD28 for access to B7 ligand, inducing immunosuppressive cytokines, or directly interfering with CD28-mediated and/or TCR-mediated signalling [72]. The first evidence that IDO might be an important mediator of CTLA-4-Ig-induced tolerance was the observation that, in a diabetic mouse model, the ability of CTLA-4-Ig to effectively suppress immune rejection of pancreatic islet allografts was lost if IDO activity was concurrently inhibited by treatment with 1MT [60]. The study by Grohmann et al. further suggested that CTLA-4-Ig-mediated tolerance occurs through a heterodox mechanism of ‘reverse’ signalling through B7 molecules on APCs, which promotes IFN-γ production to induce IDO. Subsequent studies have provided further support for and refinement of this model [73-78], which is consistent with CTLA-4 expression at the maternal–fetal interface during gestation [79].

2.3 IDO in tumoural immune escape

The concept that IDO activity is physiologically important for establishing peripheral tolerance to alloantigens expressed by the fetus has engendered a complete rethinking of the implications of the elevated IDO activity observed in cancer patients. Induction of IDO was generally thought to be a tumoricidal consequence of IFN-γ exposure as the growing tumour cells were starved of an essential amino acid as well as exposed to...
Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors

toxic products of tryptophan degradation [55,80-82]. However, cancer cells are highly adaptive, compensating for a low tryptophan environment, for instance, by upregulating tryptophan tRNA synthetase induction in response to IFN-γ [83]. If IDO can block immune responses to the highly antigenic paternal-ly derived alloantigens expressed by the fetus, it should also be capable of blocking responses to much weaker tumour antigens. Therefore, tumours that can survive the deleterious consequences of IDO upregulation may benefit from its immune suppressive activity. The idea that IFN-γ exposure can have diametrically opposed consequences for tumours is already well-established in the literature. For instance, IFN-γ has been shown to cooperate with lymphocytes to protect against the development of both spontaneous as well as chemically-induced tumours, but the tumours that do grow out in this context are more aggressive when transplanted into a syngeneic, immunocompetent host [5]. This is consistent with positive selection for reduced immunogenicity, a phenomenon that has been termed ‘immune editing’ [4].

Does the relevant upregulation of IDO activity occur in the tumour cells themselves or in the adjacent stroma? Two competing, although not necessarily mutually exclusive explanations have developed regarding this question. Experimental evidence supports the idea that cancer cells with active IDO enzyme can promote immune suppression. A fibrosarcoma cell line ectopically expressing IDO has been shown to directly inhibit T-cell responses [84]. In a separate study, ectopic expression of IDO in a mastocytoma cell line promoted tumour formation in mice that should otherwise have been rendered resistant because of preimmunisation [85]. Coupled with the reports of high IDO expression in many tumour-derived cell lines [20] as well as in a high proportion of primary tumour cells from a wide range of tissues [85], it appears likely that direct expression of IDO in tumours can contribute significantly to immune escape.

This raises the question of how IDO becomes dysregulated in tumour cells. One possible answer to this question has come from the authors’ own studies of the Bin1 cancer suppression gene. Loss or attenuation of normal Bin1 protein expression during malignant progression has been described in a variety of human tumours including breast cancer, prostate cancer, melanoma, neuroblastoma [86-89] and colon cancer (K Xie, L Wang, JD, AJ Muler, GC Prendergast unpublished results). To study the mechanistic basis for the apparent selective pressure against Bin1, the authors used a combination of Mysc and Ras oncopgenes to transform primary epithelial skin cells (keratinocytes) obtained from Bin1-deficient neonates as well as heterozygous control littermates. The growth characteristics of these transformed cells were virtually indistinguishable in vitro; however, when transplanted subcutaneously into syngeneic animals, the Bin1-null cells were aggressively tumorigenic whereas the Bin1-expressing cells were not. Immune escape was implicated as Bin1-expressing cells showed equivalent tumorigenicity when injected into nude mice. Prompted by reports that Bin1 could impact STAT and NF-κB signalling pathways, the authors discovered that Bin1 is involved in the regulatory control of IDO. IFN-γ-mediated induction of IDO is enhanced in Bin1-null keratinocytes and in Bin1-null macrophages as well. As anticipated, treatment with the IDO inhibitor 1MT significantly suppressed the outgrowth of Bin1-null MR-transformed keratinocyte tumours in syngeneic animals but had no significant impact on their outgrowth in athymic nude mice [90]. These data provide further support for the conclusion that direct expression of IDO in tumour cells is capable of promoting immune escape and indicate that loss of Bin1 is one mechanism through which IDO dysregulation may occur.

On the other hand, IDO has been implicated in immune escape by tumours that show no direct evidence of Indo gene expression. It has been argued that this may even be the more relevant means of establishing tolerance [91], although the details on how this might be achieved are still sketchy. Tumours formed by Lewis lung carcinoma cell line, which did not directly express detectable IDO, induced IDO upregulation in the draining lymph nodes and treatment of mice with the IDO inhibitor 1MT delayed the outgrowth of these tumours [92]. The stromal cells most likely to be providing the IDO activity in this scenario are APCs such as DCs or macrophages. Not all APCs appear to induce IDO, however, and a number of cell surface markers that may help characterise particular APC subsets that are key to mediating this immune regulatory mechanism have been reported [73,76,93-95]. In particular, a plasmacytoid class of mouse dendritic cells, which express B cell surface markers and may originate from the B cell lineage, appear to be important expressors of IDO [94,95]. Speculative mechanisms for how tumours induce IDO in proximal APCs are suggested by experimental systems in which evidence for IDO-mediated tolerance has been demonstrated. As described previously, CTLA-4 co-receptor has been implicated in the induction of IDO in APCs through B7 ligation. CTLA-4 is highly expressed on regulatory T cells (Treg) which have been implicated in mediating IDO induction in the DC population [75]. Treg recruitment or generation at the tumour site might thus be a mechanism for cancer cells to indirectly promote local upregulation of IDO activity. Another co-receptor, 4-1BB, has recently been implicated in the promotion of tolerance in the collagen-induced arthritis model in mice [96]. In this case, ligation of 4-1BB with an antibody stimulates the accumulation of CD11b+ CD8+ cells that produce high levels of IFN-γ. This induces IDO in responsive APCs which leads to tolerisation. Interestingly, aberrant expression of 4-1BB ligand has been reported in solid tumours [97,98], suggesting the possibility that tumours that do not directly express IDO might signal through 4-1BB as an alternative mechanism to induce local IDO activity.

Divergent opinions also exist as to the mechanism by which IDO promotes immune suppression, namely whether this is due to the local depletion of tryptophan levels or the local accumulation of toxic tryptophan catabolites. Of course, these two possibilities are not necessarily mutually exclusive.
In vitro data favouring each model has been reported. Shortly after publishing the fetal protection study, Munn, Mellor and co-workers published an in vitro study that demonstrated that T cells are exquisitely sensitive to tryptophan levels during activation [60]. Low tryptophan levels in the media promoted cell cycle arrest and eventually apoptosis if a subsequent activating signal in the presence of sufficient tryptophan was not encountered. These and subsequent experiments indicating that metabolic products were neither necessary nor sufficient for IDO-mediated inhibition in mixed lymphocyte reactions but that the tryptophan depleting effect of IDO was required [99] formed the basis for the tryptophan depletion model. However, evidence reported from other laboratories that tryptophan catabolites are primarily responsible for suppressing T cell activation [100-102], has bolstered the counter argument that tryptophan depletion by IDO is unlikely to account for the observed biology [45]. It is apparent that in vitro systems are too malleable to convincingly resolve this issue and that clear and definitive in vivo experiments will be required.

2.4 Targeting IDO as a therapeutic strategy for cancer treatment

The idea that IDO activity might protect tumours from the host immune system suggests that IDO inhibitors might have utility as anticancer agents. Given that 1MT is known to be biologically active in defeating immunological tolerance of allogeneic concepti, it has clearly been the compound of choice to perform pilot studies. The authors’ own work and that of others has shown that 1MT exhibits some efficacy as a monotherapy. In tumour growth inhibition studies (treatment initiated prior to or concurrent with tumour challenge [103]) involving tumour models that either directly or indirectly utilise IDO for immune escape, 1MT treatment did cause significant tumour growth delays, but failed to block establishment [85,92]. In a more stringent type of study in which treatment was initiated on established tumours, inhibition of tumour growth with 1MT treatment alone was also observed, however, regression of tumours, a critical preclinical criterion, was not achieved [90]. These findings suggest that IDO inhibitor-based, single-agent immunotherapy may have only limited antitumour activity. Pilot experiments performed in our laboratory combining 1MT treatment with injection of IFN-γ or IL-12 did not achieve any stronger effects than 1MT alone (AJ Muller, J DuHadaway, GC Prendergast, unpublished results). It is not particularly surprising that the response of tumours is less dramatic than that of allogeneic concepti, because the tumour antigens that they express are substantially less antigenic than alloantigens and the tumours may be more flexible in employing alternative mechanisms to protect themselves from immune responses as well.

The authors have further explored the use of 1MT in combination with other agents to treat established tumours in the well-accepted MMTV-Neu ‘oncomouse’ model of breast cancer in which overexpression of the HER2/ErbB2/Neu proto-oncogene drives the formation of mammary gland adenocarcinomas that closely resemble human ductal carcinoma in situ [104]. In particular, the authors have investigated the antitumour effects of combining 1MT with paclitaxel and other cytotoxic chemotherapeutic drugs. Such drugs might appear to be a counterintuitive choice because they can kill the immune cells that IDO inhibitors are supposed to activate. However, reports that cytotoxic drug regimens can actually promote immune cell infiltration of tumours and antitumour responses [105-108] largely prompted this line of investigation. Paclitaxel treatment by itself produced only growth inhibition of MMTV-Neu tumours consistent with published evidence that Neu overexpression in breast cancer cells confers paclitaxel resistance [109]. 1MT was delivered to tumour-bearing MMTV-Neu mice by subcutaneous introduction of time-release pellets: the same delivery route that achieves sufficient 1MT exposure for rejection of allogeneic concepti. In contrast to results obtained with 1MT monotherapy, treatment of tumour-bearing MMTV-Neu mice with a combination of 1MT + paclitaxel resulted in tumour regression [90].

Control experiments in which pellets were infused with D,L-tryptophan (analogous to the D,L racemic mixture of the 1MT used) did not replicate the observed cooperative effect of 1MT. Thus, the observed effect could not be trivially ascribed to a nonspecific toxicity caused by high-dose of the tryptophan-like compound. The authors did not rule out the possibility of a pharmacokinetic effect of 1MT on paclitaxel, which might increase its effective dose in the mouse. However, this explanation seems unlikely because no evidence of neuropathy (e.g., hind leg dragging) that would be produced in mice by a higher effective dose of paclitaxel was observed. Immune depletion experiments as well as grafting experiments in nude mice confirmed that T-cell-mediated immunity is essential for the combination therapy to elicit tumour regression.

Similar cooperativity was observed with other chemotherapeutic agents tested, but was not universal. The DNA damaging agents cisplatin, cyclophosphamide and doxorubicin exhibited cooperativity, but the antimetabolites 5-fluorouracil and methotrexate did not. Interestingly, the other mitotic inhibitor tested, vinblastine, did not show cooperativity. Overt toxicity was not evident in any of these trials. Doxorubicin itself produced tumour regression in the MMTV-Neu model at higher doses, but this was associated with severe side effects (slumping and inactivity of treated mice). At a lower dose of doxorubicin, 1MT enhanced regression without increasing evident toxicity. Of two signal transduction inhibitors tested, rapamycin showed no evidence of cooperativity while a farnesyltransferase inhibitor (FTI) did. This latter observation might be explained by accumulating evidence that anticancer effects of FTIs appear not to be mediated through inhibition of Ras signalling, as originally thought, and that a DNA damage mechanism has instead recently been invoked [110]. As expected, the iron chelator tetrathiomolybdate, which has been reported to block angiogenesis, also
showed no cooperativity with 1MT. The implications of these findings are striking as they suggest, perhaps counterintuitively, that modulating immunity with small-molecule inhibitors of IDO in conjunction with conventional cytotoxic chemotherapeutic drug-based treatments might have clinical relevance. This notion that immunotherapy and chemotherapy can be effectively combined to destroy cancer cells is consistent with other preclinical studies that have focused on different immunotherapeutic principles [106,107,111] and the idea that immunotherapy might cooperate with chemotherapy appears to be gaining currency as evidenced by the appearance of review articles addressing this topic [112-114].

3. Indoleamine 2,3-dioxygenase inhibitors: chemistry and pharmacology

3.1 Structural classes of IDO inhibitory molecules

There are only a small collection of reports describing inhibition studies of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17). Not surprisingly, the studies have focused primarily on derivatives of tryptophan (Trp) and structurally related heterocycles like β-carboline, despite the reported [23,115,116] promiscuity of IDO compared with the related tryptophan 2,3-dioxygenase (TDO2, EC 1.13.11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, whereas noncompetitive inhibitors are derivatives of β-carboline.

3.1.1 Competitive inhibitors

Substrate inhibition with high concentrations (> 0.2 mM) of L-Trp was reported [117,118] during early enzymological studies; therefore, it is not surprising that Trp derivatives have been extensively studied. Derivatisation of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid side chain, and modifications of the indole ring.

3.1.1.1 Tryptophan indole ring substitutions

Substitution of the indole ring of Trp (Figure 1) has afforded the most commonly used inhibitor of IDO: 1-methyl-Trp (1) [63]. A racemic mixture was originally used by Munn and co-workers in their seminal study of the fetal survival paradox, but subsequent studies [119] with isolated IDO have revealed a slight preference for the natural L (S) isomer of 1 (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D.L. system). Stereochemical preference for the natural isomer was also reported with the 6-nitro derivative 18 (Table 1). On the other hand, some cellular studies [76,93,94] demonstrate greater activity for the R (D) isomer of 1 (1-MT). Given the more complex nature of cellular studies, IDO inhibitory activity may not be the primary reason for the better activity of the R isomer of 1. Nevertheless, based on these conflicting results, future inhibition studies should carefully consider both stereoisomers of Trp analogues.

Table 1 comprehensively summarises the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (1), 5-bromo (9), 6-fluoro (17), 6-nitro (18, S isomer), 7-fluoro (20), and the two difluorinated derivatives, 4,7-difluoro (8) and 5,7-difluoro (15). Excluding the 1-methyl derivative, the six others are electron withdrawing groups [120-122]. Because the three proposed mechanisms [123,124] for IDO catalysis of the conversion of Trp to N-formyl-kynurenine all begin with nucleophilic attack of the pyrrole ring of Trp, electron withdrawing groups on the indole ring would make this step less favourable and slower. Nevertheless, the activity data in Table 1 indicates that the 5-bromo (9) and the 6-fluoro (17) derivatives still undergo oxidation, therefore some of these compounds still behave as substrates despite their deactivating substitution.

Several compounds, notably the 5-bromo (9) and 2-hydroxy (6) derivatives, have significantly different IDO inhibition values reported by different sources. Some of the variability may be due to the different IDO sources and different assay conditions used in different studies. Peterson and co-workers extracted IDO from human monocyte/macrophage cells induced by IFN-γ [119]. They monitored IDO activity by detecting kynurenine product with a radioimmunoassay or HPLC assay. Southan and co-workers used recombinant human IDO, expressed in and purified from E. coli [125]. They followed IDO activity with a spectrophotometric assay that detected an imine derivative of kynurenine. Several inhibitors reported in later tables were evaluated against IDO isolated from rabbit small intestine using two different detection methods [63,126]. Despite these differences, several compounds show striking consistency, for example, 1 and 13 (Table 1) and 39 (Table 2).

Several electron-releasing substituents in Table 1 are very active as substrates and are oxidised by IDO: 4-methyl (7), 5-methyl (10), 5-methoxy (11), 5-hydroxy (13) and 6-methyl (16). One derivative (5-methyl, 10) is more active than L-Trp. This result is consistent with the mechanistic rational and the outcome described for the electron withdrawing substituents. Electron releasing substituents would be expected to make the indole ring more nucleophilic leading to a faster initial reaction with the oxygen species at the active site.

The 1-methyl derivative 1 defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms [123,124] for IDO involving pyrrole electron donation, actually initiate the nucleophilic attack with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, 1MT prevents the deprotonation from occurring. Similar inhibition is seen with benzofuran (48, Table 3) and benzothiophene (49) analogues of Trp (vide infra). However, there is a limited amount of space in the active site to accommodate N-1 groups as the 1-ethyl (2) and 1-phenyl-sulfonyl (3) derivatives exhibited only weak inhibitory activity.

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Indole ring substitution of Trp derivatives has been extensively explored. Nevertheless, the use of multiple substituents is a strategy that might yield more active inhibitors. Excluding compounds 3, 8 and 15, there are few compounds with multiple substituents that have been synthesised and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site as seen in the weak activity and inhibition with 12. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition.

Table 1. Tryptophan derivatives with indole ring substitution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Indole ring substitution</th>
<th>Stereochemistry at α-position</th>
<th>Inhibition data (%)*</th>
<th>Activity data (%)‡</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-CH₃</td>
<td>S (L)</td>
<td>52.3 (62.9)</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>1</td>
<td>1-CH₃</td>
<td>R,S</td>
<td>26; Kᵢ = 6.6 µM</td>
<td>7</td>
<td>[125]</td>
</tr>
<tr>
<td>1</td>
<td>1-CH₃</td>
<td>R (D)</td>
<td>5.7 (11.6)</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>2</td>
<td>1-CH₂CH₃</td>
<td>S</td>
<td>13.5 (9.9)</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>3</td>
<td>1-SO₂Ph, 6-OCH₃</td>
<td>R</td>
<td>3.2 (28.4)</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>4</td>
<td>2-Cl</td>
<td>S</td>
<td>20</td>
<td>33</td>
<td>[125]</td>
</tr>
<tr>
<td>5</td>
<td>2-Br</td>
<td>S</td>
<td>11</td>
<td>21</td>
<td>[125]</td>
</tr>
<tr>
<td>6</td>
<td>2-OH</td>
<td>S</td>
<td>30</td>
<td>4</td>
<td>[125]</td>
</tr>
<tr>
<td>6</td>
<td>2-OH</td>
<td>R,S</td>
<td>-38.4 (-43.3)</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>7</td>
<td>4-CH₃</td>
<td>R,S</td>
<td>26</td>
<td>33</td>
<td>[125]</td>
</tr>
<tr>
<td>8</td>
<td>4-F, 7-F</td>
<td>S</td>
<td>Kᵢ = 40 µM</td>
<td></td>
<td>[123]</td>
</tr>
<tr>
<td>9</td>
<td>5-Br</td>
<td>R,S</td>
<td>0</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>10</td>
<td>5-CH₃</td>
<td>R,S</td>
<td>56</td>
<td>36</td>
<td>[125]</td>
</tr>
<tr>
<td>11</td>
<td>5-OCH₃</td>
<td>R,S</td>
<td>6</td>
<td>123</td>
<td>[125]</td>
</tr>
<tr>
<td>12</td>
<td>5-OCH₃Ph</td>
<td>R,S</td>
<td>35</td>
<td>70</td>
<td>[125]</td>
</tr>
<tr>
<td>13</td>
<td>5-OH</td>
<td>S</td>
<td>2</td>
<td>1</td>
<td>[125]</td>
</tr>
<tr>
<td>13</td>
<td>5-OH</td>
<td>S</td>
<td>12</td>
<td>59</td>
<td>[125]</td>
</tr>
<tr>
<td>14</td>
<td>5-F</td>
<td>R,S</td>
<td>32</td>
<td>46</td>
<td>[125]</td>
</tr>
<tr>
<td>15</td>
<td>5-F, 7-F</td>
<td>S</td>
<td>Kᵢ = 24 µM</td>
<td></td>
<td>[123]</td>
</tr>
<tr>
<td>16</td>
<td>6-CH₃</td>
<td>R,S</td>
<td>20</td>
<td>72</td>
<td>[125]</td>
</tr>
<tr>
<td>17</td>
<td>6-F</td>
<td>R,S</td>
<td>54</td>
<td>38</td>
<td>[125]</td>
</tr>
<tr>
<td>18</td>
<td>6-NO₂</td>
<td>S</td>
<td>52</td>
<td>2</td>
<td>[125]</td>
</tr>
<tr>
<td>19</td>
<td>7-CH₃</td>
<td>R,S</td>
<td>7</td>
<td>0</td>
<td>[125]</td>
</tr>
<tr>
<td>20</td>
<td>7-F</td>
<td>S</td>
<td>36</td>
<td>18</td>
<td>[125]</td>
</tr>
</tbody>
</table>

*Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an in vitro competitive inhibition assay with 1 mM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

‡Per cent compound oxidized relative to L-tryptophan.

§100 µM inhibitor concentration used in inhibition assay.

¶Kᵢ determined at pH 8.0 in [63].

Figure 1. Structure of the tryptophan amino acid, including the indole ring. Substitution positions are labelled 1 – 7.
Unlike the β-carboline derivatives (vide infra), there has been no indication of slow-binding inhibition from Trp derivatives; the preincubation inhibitory data in Tables 1 – 3 does not substantially differ from the percentage inhibition found in standard competition assays.

### 3.1.1.2 Tryptophan side chain modifications

A range of Trp side chain modifications have been explored as illustrated in Table 2. However, relatively few of these have afforded compounds with promising inhibition. Modest inhibition was realised with the addition of a methyl group to either the α-amine (27) or the α-acid (29). One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative 40 (Figure 2) [90]. Further modification of the thiohydantoin ring might provide even more potent inhibitors.

### 3.1.1.3 Indole ring modifications

Modifications of the indole ring have afforded a few novel competitive inhibitors (Table 3). Most notable amongst this group are the benzofuran (48) and benzothiophene (49) derivatives described earlier. These two compounds, like

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**Table 2. Tryptophan side chain modifications.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R = *</th>
<th>Stereochemistry at α-position</th>
<th>Inhibition data (%)‡</th>
<th>Activity data (%)§</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>-CH₂CH₂NH₂</td>
<td></td>
<td>28</td>
<td>32</td>
<td>[125]</td>
</tr>
<tr>
<td>22</td>
<td>-CH₂CH₃NH₂; (5-OCH₃)</td>
<td></td>
<td>-43.9¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>23</td>
<td>-CH₂CH₃NH₂; (2-CO₂H)</td>
<td></td>
<td>16.3 (17.9)¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>24</td>
<td>-CH₂CH₃NH₂; (2-CO₂H, 5-OCH₃)</td>
<td>10.8 (3.4)¶</td>
<td></td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>25</td>
<td>-CH₂CH₂CO₂H</td>
<td></td>
<td>0</td>
<td>8</td>
<td>[125]</td>
</tr>
<tr>
<td>26</td>
<td>-CH₂C(CH₃)₃(NH₂)CO₂H</td>
<td>(R,S)</td>
<td>1</td>
<td>35</td>
<td>[125]</td>
</tr>
<tr>
<td>27</td>
<td>-CH₂CH(NHCH₃)CO₂H</td>
<td>S</td>
<td>33</td>
<td>21</td>
<td>[125]</td>
</tr>
<tr>
<td>28</td>
<td>-CH₂CH(NHCOCH₃)CO₂H</td>
<td>S</td>
<td>7</td>
<td>3</td>
<td>[125]</td>
</tr>
<tr>
<td>29</td>
<td>-CH₂CH(NH₃)CO₂CH₃</td>
<td>S</td>
<td>30</td>
<td>15</td>
<td>[125]</td>
</tr>
<tr>
<td>30</td>
<td>-CH₂CH(NH₂)CO₂CH₂CH₃</td>
<td>S</td>
<td>7</td>
<td>14</td>
<td>[125]</td>
</tr>
<tr>
<td>31</td>
<td>-CH₂CH(OH)CO₂H</td>
<td>(R,S)</td>
<td>9.7 (1.4)¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>32</td>
<td>-CH₂N(CH₃)₂</td>
<td></td>
<td>-6.6¶</td>
<td></td>
<td>[119]</td>
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<td>-CH₂CN</td>
<td></td>
<td>3.5¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>34</td>
<td>-(O)NH₂; (5-OH)</td>
<td></td>
<td>0¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>35</td>
<td>-CHO</td>
<td></td>
<td>4.4¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>36</td>
<td>-CH = CHCO₂H</td>
<td></td>
<td>2.5 (3.2)¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>37</td>
<td>-CH = CHCO₂CH(CH₃)₂</td>
<td></td>
<td>15.2 (11.6)¶</td>
<td></td>
<td>[119]</td>
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<tr>
<td>38</td>
<td>-(E)-CH = CH-(3-pyridinyl); (6-F)</td>
<td>0</td>
<td></td>
<td></td>
<td>[127]</td>
</tr>
<tr>
<td>39</td>
<td>-CH(CH₃)CH(NH₃)CO₂H</td>
<td>α-S,β-S; α-R, β-R</td>
<td>0.0 (-2.7)¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>39</td>
<td>-CH(CH₃)CH(NH₃)CO₂H</td>
<td>α-S,β-R; α-R, β-S</td>
<td>9.8 (3.6)¶</td>
<td></td>
<td>[119]</td>
</tr>
<tr>
<td>39</td>
<td>-CH(CH₃)CH(NH₃)CO₂H</td>
<td>R,S</td>
<td>7</td>
<td>32</td>
<td>[125]</td>
</tr>
<tr>
<td>40</td>
<td>-CH₂·5'-(3'·methyl-2'·thioxo-4'·imidazolinone)</td>
<td>(R,S)</td>
<td>Kᵢ = 11.4 µM</td>
<td>[90]</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>-CH₂CH(NH₃)CO-(S)-Trp</td>
<td>S</td>
<td>Kᵢ = 147 µM</td>
<td></td>
<td>[119]</td>
</tr>
</tbody>
</table>

* Additional indole substituents are added in brackets.

† Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an in vitro competitive inhibition assay with 1 mM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

§ Per cent compound oxidised relative to L-tryptophan.

¶ 100 µM inhibitor concentration used in inhibition assay.

---

![Tryptophan](image1.png) ![Compound 40](image2.png)

Figure 2. Tryptophan side chain and modification in compound 40.
1-methyl-Trp (1), lack an N-1 proton and, therefore, cannot be deprotonated; the initial step in the proposed catalytic mechanism of IDO indole oxidation \[123,124\]. Attempts at identifying feedback inhibition from subsequent intermediates in the kynurenine pathway failed with the kynurenine analog 46 and the 3-hydroxykynurenine analog 47. Surprisingly, based on the success of electron withdrawing groups on the benzene portion of the indole (Table 1), a π-deficient analog of indole, 7-azaindole (43), also failed to demonstrate inhibitory activity. Similarly, modifications of either the pyrrole portion of the indole ring, i.e., reduction (44) or incorporation of another nitrogen (42), also failed to afford inhibition. The majority of the data from Table 3 indicates that the indole ring is almost essential for the creation of a competitive inhibitor.

### 3.1.1.4 Miscellaneous structures

A small selection (Table 4) of structures unrelated to Trp has been tested for competitive inhibition. Similar to the modified indole ring structures in Table 3, the majority of the structures have not shown any inhibitory activity. Feedback inhibition was not detected with kynurenic acid (54) or quinolinic acid (57), nor was inhibition seen with the structurally related analogs 53, 55 and 56. Two interesting exceptions were discovered with 52 and 58. 3-Amino-2-naphthoic acid (52) is an analog of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound 52 is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogs. A second unique inhibitor was pyrrolidine dithiocarbamate (58) \[129\]. This antioxidant demonstrated notable inhibitory activity of IDO generated from IFN-γ treatment of human monocyte-derived macrophages. It is possible that the sulfur of the dithiocarbamate is binding to the haem iron in the active site of IDO. This binding mode would be consistent with the well-known affinity of sulfur for iron in biological systems (e.g., ferrodoxin).

#### 3.1.2 Noncompetitive inhibitors

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 \[126\]. Initially, they were reported to exhibit uncompetitive inhibition but β-carboline (59), also known as norharman, was subsequently reclassified as a noncompetitive inhibitor \[130\]. β-Carboline derivatives (Table 5, Figure 3) continue to be the most common type of noncompetitive inhibitor, but there are three novel structures (Table 6, Figure 4) that have also been reported \[131\].

### Table 3. Indole ring modifications of tryptophan.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Side chain (CH$_2$-)</th>
<th>Stereochemistry at α-position</th>
<th>Inhibition data (%)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>3-(1H-Indazolyl)-</td>
<td>R,S</td>
<td>0.0</td>
<td>[119]</td>
</tr>
<tr>
<td>43</td>
<td>3-(7-Azaindolyl)-</td>
<td>R,S</td>
<td>-1.6</td>
<td>[119]</td>
</tr>
<tr>
<td>44</td>
<td>3-Indolinyl</td>
<td>S</td>
<td>0.4 (3.0)</td>
<td>[119]</td>
</tr>
<tr>
<td>44</td>
<td>3-Indolinyl</td>
<td>R</td>
<td>-2.4 (-1.2)</td>
<td>[119]</td>
</tr>
<tr>
<td>45</td>
<td>3-Quinolinyl</td>
<td>S</td>
<td>0</td>
<td>[119]</td>
</tr>
<tr>
<td>45</td>
<td>3-Quinolinyl</td>
<td>R</td>
<td>0</td>
<td>[119]</td>
</tr>
<tr>
<td>46</td>
<td>(2-Amino-phenyl)methyl</td>
<td>S</td>
<td>-0.3</td>
<td>[119]</td>
</tr>
<tr>
<td>47</td>
<td>(2-Amino-3-hydroxy-phenyl)methyl</td>
<td>R,S</td>
<td>-0.4</td>
<td>[119]</td>
</tr>
<tr>
<td>48</td>
<td>3-Benzofuranyl</td>
<td>R,S</td>
<td>43±5.5</td>
<td>[125]</td>
</tr>
<tr>
<td>48</td>
<td>3-Benzofuranyl</td>
<td>R,S</td>
<td>$K_i = 25$ µM</td>
<td>[63]</td>
</tr>
<tr>
<td>49</td>
<td>3-Benzothiophenyl</td>
<td>R,S</td>
<td>16±5.8</td>
<td>[125]</td>
</tr>
<tr>
<td>49</td>
<td>3-Benzothiophenyl</td>
<td>R,S</td>
<td>$K_i = 70$ µM</td>
<td>[63]</td>
</tr>
<tr>
<td>50</td>
<td>1-(1,4-Cyclohexadienyl)</td>
<td>S</td>
<td>$K_i = 230$ µM</td>
<td>[128]</td>
</tr>
</tbody>
</table>

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an in vitro competitive inhibition assay with 100 µM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.
* 1 mM inhibitor concentration used in inhibition assay.
* 22% of 44 was oxidised by indoleamine 2,3-dioxygenase.
* 19% of 45 was oxidised by indoleamine 2,3-dioxygenase.
been explored. The most potent IDO inhibitors have larger alkyl substituents in the C-3-position (e.g., 74 and 76). There appears to be a hydrophobic pocket in the active site capable of accommodating these alkyl groups. Fluorine and the isothiocyanate group were present in several potent C-6 substituted β-carboline derivatives (e.g., 68, 76 and 77).

As noncompetitive inhibitors, β-carboline derivatives do not compete for the same active site location as Trp or other indoleamine substrates. Nevertheless, there is experimental evidence that indicates that β-carboline 59 binds directly to the haem iron at the active site as a nitrogen ligand and competes with oxygen for binding at the active site iron [130]. Some has determined that the β-carboline occupies another binding site close to the L-Trp binding region and he hypothesises that this space may be available for a natural cofactor or a regulator of the enzyme [25]. Interestingly, several of the β-carboline inhibitors (i.e., 61, 64 and 66) demonstrated considerably greater potency on preincubation with IDO. This is suggestive of slow-binding inhibition and may indicate these inhibitors need time to settle into the second binding pocket near the haem iron. One important liability of β-carboline derivatives is the reported neuroactivity of these structures as benzodiazepine receptor ligands [132-135]. In fact, many previous IDO inhibitor studies were focused on developing treatments for neurological disorders (e.g., excitotoxic brain lesions) where penetration of the central nervous system may have been necessary. However, an IDO inhibitor that was able to penetrate the CNS could cause problematic side effects in cancer therapy.

### 3.1.3 Summation

Although a selection of compounds have been investigated for IDO inhibition, submicromolar inhibition has not yet been achieved. A few unique structures have been discovered to have IDO inhibitory activity, nonetheless the majority of the most active structures contain the indole system or resemble L-Trp. Clearly, one important goal in the development of IDO inhibition as a cancer therapy will be to discover more potent inhibitors, and it seems that a diversification of IDO inhibitor structures may be necessary to achieve this goal.

### 3.2 Indoleamine 2,3-dioxygenase inhibitors in vivo

#### 3.2.1 Administration of IDO inhibitors

Until recently, 1MT has been the only compound evaluated as an IDO inhibitor in vivo. The means of delivery used in the majority of studies has been the same as that used in the original allogeneic conceptus rejection study [59]. This involves encapsulating the 1MT compound in a polymer matrix. The pellets (as prepared by Innovative Research, Inc.) are claimed to provide a steady-state release rate of 10 mg/day, although no data directly demonstrating this has been provided. During the course of tumour treatment studies, the authors have evaluated the serum levels of 1MT achieved with subcutaneous pellet implants. Two 140 mg pellets per mouse resulted in a steady-state level of 1MT in the serum of 100 μM within 24 h after implantation. At the published compound release rate these pellets were expected to maintain a constant level of serum 1MT for a period of 2 weeks. The authors found, however, that this steady-state level of 1MT was maintained for only 5 – 7 days postimplantation after which time serum 1MT rapidly dropped to negligible levels. An alternative procedure for delivering 1MT in drinking water has been reported in one study [85]. However, this method of compound delivery provides only limited control over the frequency and amount of each administered dose and can reportedly result in dehydration of the animals.

The authors’ own recent work has determined that efficacious administration of 1MT can be achieved by oral gavage
bolus dosing of a bead-milled suspension prepared in methocel/tween (0.5% methylcellulose/1% tween 80). Single-dose intravenous and oral pharmacokinetic profiles of 1MT administered at 40 mg/kg in mice have been published [90].

Based on the favourable oral bioavailability and relatively slow clearance of 1MT, we have developed an oral dosing schedule that, in combination with paclitaxel administration, produces regression of MMTV-Neu tumours (AJ Muller, J DuHadaway, E Sutanto-Ward and GC Prendergast, manuscript in preparation). The ability to effectively deliver 1MT in a controlled manner through oral administration will greatly facilitate further acquisition of preclinical data regarding dosing and scheduling parameters for the development of an optimized combinatorial treatment protocol that will provide a necessary contextual framework within which to evaluate new, more effective, IDO inhibitors as they are identified.

Table 5. β-Carboline ring substitution compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>β-Carboline ring substitution</th>
<th>Inhibition data (%)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>None</td>
<td>50.3 (57.0); $K_i = 178 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>60</td>
<td>3-OCH$_2$CH$_3$</td>
<td>5.5 (21.2)</td>
<td>[131]</td>
</tr>
<tr>
<td>61</td>
<td>3-OCH$_2$CH$_2$CH$_3$</td>
<td>16.7 (76.7); $K_i = 98.0 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>62</td>
<td>3- OCH$_2$CH$_2$OH</td>
<td>6.7 (11.0)</td>
<td>[131]</td>
</tr>
<tr>
<td>63</td>
<td>3-CO$_2$t-Bu</td>
<td>7.0 (7.2)</td>
<td>[131]</td>
</tr>
<tr>
<td>64</td>
<td>3-C(O)CH$_2$CH$_2$CH$_3$</td>
<td>-4.1 (44.9)</td>
<td>[131]</td>
</tr>
<tr>
<td>65</td>
<td>3-NH$_2$</td>
<td>0.9 (-19.4)</td>
<td>[131]</td>
</tr>
<tr>
<td>66</td>
<td>3-N = C = S</td>
<td>26.7 (86.1)</td>
<td>[131]</td>
</tr>
<tr>
<td>67</td>
<td>3-OH</td>
<td>30.1 (-5.3)</td>
<td>[131]</td>
</tr>
<tr>
<td>68</td>
<td>3-CO$_2$CH$_3$, 6-F</td>
<td>40.4 (49.2); $K_i = 7.4 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>69</td>
<td>3-CO$_2$CH$_3$, 6-Br</td>
<td>-4.9 (13.4)</td>
<td>[131]</td>
</tr>
<tr>
<td>70</td>
<td>3-CO$_2$H</td>
<td>$K_i = 40.6 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>71</td>
<td>3-CO$_2$CH$_3$</td>
<td>$K_i = 259 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>72</td>
<td>3-CO$_2$CH$_2$CH$_3$OH</td>
<td>$K_i = 98.0 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>73</td>
<td>3-CO$_2$C(CH$_3$)$_3$</td>
<td>$K_i = 89.7 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>74</td>
<td>3-CH$_2$CH$_2$CH$_2$CH$_3$</td>
<td>$K_i = 3.3 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>75</td>
<td>3-NO$_2$</td>
<td>$K_i = 37.5 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>76</td>
<td>3-CO$_2$CH$_2$CH$_3$, 6-F</td>
<td>$K_i = 21.0 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>77</td>
<td>3-CO$_2$CH$_3$, 6-N = C = S</td>
<td>$K_i = 8.5 \mu M$</td>
<td>[131]</td>
</tr>
<tr>
<td>78</td>
<td>1-CH$_3$, 7-OCH$_3$</td>
<td>10*</td>
<td>[126]</td>
</tr>
<tr>
<td>79</td>
<td>1-CH$_3$, 2-O, 7-OCH$_3$</td>
<td>46*</td>
<td>[126]</td>
</tr>
<tr>
<td>80</td>
<td>1-CH$_3$, 7-OH</td>
<td>-11*</td>
<td>[126]</td>
</tr>
<tr>
<td>81</td>
<td>1-CH$_3$</td>
<td>-13*</td>
<td>[126]</td>
</tr>
<tr>
<td>82</td>
<td>1- CO$_2$CH$_3$, 7- OCH$_3$</td>
<td>25*</td>
<td>[126]</td>
</tr>
<tr>
<td>83</td>
<td>1-CH$_3$, 7-OCH$_3$, 3,4-dihydro</td>
<td>4*</td>
<td>[126]</td>
</tr>
<tr>
<td>84</td>
<td>1-CH$_3$, 7-OH, 3,4-dihydro</td>
<td>21*</td>
<td>[126]</td>
</tr>
<tr>
<td>85</td>
<td>1,2,3,4-tetrahydro</td>
<td>0*</td>
<td>[126]</td>
</tr>
<tr>
<td>86</td>
<td>1-OH, 7-OCH$_3$, 3,4-dihydro</td>
<td>-13*</td>
<td>[126]</td>
</tr>
</tbody>
</table>

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an in vitro competitive inhibition assay with 100 μM of inhibitor. Per cent in parenthesis indicates inhibition data with 2 h preincubation of inhibitor with indoleamine 2,3-dioxygenase.

† 2 mM inhibitor concentration used in inhibition assay with rabbit intestine indoleamine 2,3-dioxygenase.

§ 1 mM inhibitor concentration used in inhibition assay with rabbit intestine indoleamine 2,3-dioxygenase.

Figure 3. Structure of the β-carboline ring. Substitution positions are labelled 1 – 8.
Table 6. Other compounds demonstrating noncompetitive inhibition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Inhibition data (%)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>4-Phenylimidazole</td>
<td>$K_i = 4.4 , \mu M$</td>
<td>[130]</td>
</tr>
<tr>
<td>88</td>
<td>Camalexin</td>
<td>21.3</td>
<td>[131]</td>
</tr>
<tr>
<td>89</td>
<td>Brassilexin</td>
<td>$K_i = 5.4 , \mu M$</td>
<td>[131]</td>
</tr>
</tbody>
</table>

* Unless otherwise stated, inhibition data are reported as 100 minus percentage of tryptophan metabolised in an in vitro competitive inhibition assay with 100 \( \mu M \) of inhibitor.

As noted in Secton 3.1.1.2, the thiohydantoin side chain modification of tryptophan produced a compound (MTH-Trp, 40) with threefold higher potency against purified human IDO enzyme than 1MT. The differential was even more pronounced in a cell-based assay, in which MTH-Trp was demonstrated to be $\sim 20$-times more potent than 1MT (EC$_{50} = 12.5 \, \mu M$ for MTH-Trp versus $> 200 \, \mu M$ for 1MT [90]). MTH-Trp delivered in time release pellet format in combination with paclitaxel was more effective than a comparable combination with paclitaxel was more effective than a comparable amount of 1MT at producing regression of tumours in the MMTV-Neu model [90]. This demonstration that a structurally distinct IDO inhibitor exhibits a biological effect comparable to that of 1MT bolsters the argument that the proposed mechanism of action is correct. MTH-Trp efficacy by oral gavage delivery has not been evaluated because pharmacokinetic analysis has revealed that MTH-Trp, unlike 1MT, is rapidly cleared from the bloodstream [90]. Thus, it appears unlikely that the efficacy achieved through oral gavage bolus dosing of 1MT in combination with paclitaxel can be replicated with MTH-Trp.

3.2.2 Serum kynurenine as a biomarker for IDO activity

Studies have reported the functional consequences of IDO inhibitor treatment without assessing IDO inhibition in vivo or correlating IDO inhibition with the observed biological consequences. This raises the legitimate concern of ‘off target’ effects (e.g., through interaction with other enzymes that bind tryptophan). The degradation of tryptophan by IDO produces \( \gamma \)-formylkynurenine. This, in turn, is rapidly converted to kynurenine due to high levels of kynurenine formamidase activity, the specific activity of which far exceeds that of IDO. However, other than liver and kidney, most tissues examined exhibit no significant activity associated with other key enzymes on the kynurenine pathway. Rather than being further metabolised in these tissues, kynurenine instead appears to pool in both tissue and blood spaces and systemic induction of IDO in mice by LPS correlated, in large part, with changes in plasma kynurenine levels [44]. Levels of kynurenine as well as tryptophan in the serum can be simultaneously measured by HPLC-based analysis [136-138]. The authors are developing this procedure for use as a pharmacodynamic assay to evaluate inhibition of IDO in vivo by compounds administered to mice. Intraperitoneal administration of bacterial LPS induces IDO activity in a variety of tissues resulting in the production of kynurenine and its release into the bloodstream. Peak kynurenine levels are reached one day after LPS administration [44,50]. The serum kynurenine pool is rapidly turned over with a half-life of $< 10$ minutes [44,139], so pre-existing kynurenine should not unduly mask the impact of IDO inhibitor treatment on kynurenine production. The serum level of compound being tested for IDO inhibitory activity can also be determined by high-performance liquid chromatography analysis of the same sample, permitting concurrent collection of pharmacokinetic data from a single experiment.

In human patients, determination of the serum kynurenine to tryptophan ratio has been performed in several studies to estimate IDO activity independent of baseline tryptophan levels, which can be influenced to some extent by dietary tryptophan uptake [140,141]. Measuring tryptophan and kynurenine levels in plasma or serum is much less invasive than enzyme activity tests performed on tissue samples. Thus, the serum kynurenine to tryptophan ratio can serve as a useful in vivo biomarker for evaluating IDO inhibitors in the clinic. The serum kynurenine to tryptophan ratio also correlates closely with neopterin concentrations for a variety of diseases [142]. IFN-\( \gamma \) stimulates guanosine triphosphate cyclohydrolase I, the key enzyme for pteridine production, and human monocytes/macrophages release large amounts of neopterin in response to IFN-\( \gamma \) stimulation [143]. Therefore, neopterin might serve as a valuable companion marker that responds to conditions that induce IDO activity, but is insensitive to direct IDO inhibition.

4. Conclusion

From the standpoint of therapeutic development, IDO as a drug target offers a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry,
IDO is highly tractable for inhibitor development compared with most other therapeutic targets in cancer. Second, the only other enzyme known to catalyse the same reaction, TDO2, has a much more restricted substrate specificity simplifying the problem of possible ‘off target’ effects. Third, the medicinal chemistry of indoleamines and indoleamine mimetics is well-developed. Fourth, lead inhibitors exist in 1MT and MTH-Trp, both of which are bioactive and orally bioavailable. These inhibitors may offer tools for clinical validation of the novel combination principle reviewed here. Fifth, an Indo gene ‘knockout’ mouse has been reported to be viable and healthy [73], indicating that inhibitors will be unlikely to produce unmanageable mechanism-based toxicities, although promotion of inflammatory conditions remains a valid concern. Sixth, the combination of tryptophan and kynurenine, (the major substrate and downstream product of the IDO reaction, respectively) provides a useful biomarker for the pharmacodynamic evaluation IDO inhibitors. This analysis can be performed on blood samples, which eliminates the need to obtain and analyse tumour biopsy specimens that can be difficult, expensive, technically challenging and troublesome to obtain from patients on trial. Lastly, small-molecule immunomodulatory agents are likely to offer substantial logistical and cost advantages relative to both biologics and cell-based immunotherapies. IDO has clearly become established as an attractive and tractable target for the development of better small molecule inhibitors for possible use as immunomodulatory adjuvants to standard chemotherapy and with perhaps the potential for even broader applicability for use against diseases characterised by immune suppression.

5. Expert opinion

The successful treatment of advanced, metastatic cancers remains an elusive goal. Anticancer drug development still focuses predominantly on producing compounds that elicit direct cytotoxicity against tumour cells. Cytotoxic chemotherapeutic agents are currently among the most effective agents in the clinic but these compounds do not have favourable safety profiles. Recently, attention has been shifting towards finding drugs that target specific signal transduction pathways, but, at least so far, the therapeutic impact of such agents has been limited. A popular truism in the cancer field today is that specific combinations of targeted agents will have to be tailored to individual tumours based on their genetic makeup. Such balkanization of cancer therapy clearly runs counter to the contemporary managed care culture. This reality, in the authors’ opinion, means that the partitioning of cancer patients among a plethora of small niche markets is likely to be a prohibitively expensive proposition.

Strategies aimed at activating antitumour immunity have the potential to be useful against a wide variety of tumours and might be particularly effective for treating disseminated metastases – the overarching problem facing cancer patients. On this basis, many novel anticancer therapies are currently being developed, with the purpose of stimulating immune responses through the use of cytokines, recombinant antibodies directed at tumour cells, antitumour vaccines, or cell-based, tumour-targeted immune therapies [144]. All of these therapies rely on large, complex biologically-based agents or whole cells. Small molecules have clear advantages over biologics in terms of production, delivery and cost. However, few small-molecule agents for stimulating antitumour immunity have been described. In this regard, IDO represents a particularly appealing target. As a classical biochemical enzyme (a rarity amongst cancer targets), IDO is particularly well-suited for pharmacological intervention, there being a wealth of medicinal chemistry knowledge and experience in the successful development of such drugs. A further advantage stems from the determination that IDO inhibitors appear to work most effectively as immunomodulatory adjuncts to conventional chemotherapeutics, as this suggests that use of IDO inhibitors might be successfully added to standard treatment protocols, which should ease their adoption into the clinic.

Like IDO, a number of other factors, upregulated at tumour sites, have been implicated in the establishment of tumoural immune escape including; transforming growth factor B, IL-10, prostaglandin E₂, Fas, tumour necrosis factor-related apoptosis inducing ligand (TRAIL) and receptor-binding cancer antigen expressed on SiSo cells (RCAS)-1 to name a few. Why then focus on IDO? In large measure, it has been a case of the biology leading the way. Many of the same mechanisms implicated in tumoural immune escape are also posited to protect the developing fetus. However, the IMT experiment [59] (which the authors have reproduced [90]) is so dramatic, that it strongly implicates IDO as being a particularly powerful mechanism for immune protection. Studying the mechanistic basis of how Bin1 loss contributes to tumorigenicity has further pointed towards the importance of IDO-mediated immune tolerisation [90]. Re-establishing control over IDO that is lost in Bin1-null cells might even be a therapeutic alternative to direct inhibition of IDO activity, though how this might actually be achieved is as yet undetermined. Understanding how Bin1 exerts control over IDO is currently an area of active research.

Autochthonous MMTV-Neu tumours were found not to be as dependent on IDO activity for survival as the developing fetus. This was not altogether surprising given the weaker nature of the tumour antigens involved and the greater plasticity of cancer cells in responding to immunological pressure. The MMTV-Neu mouse mammary gland tumour model was specifically chosen for study because it is not artificially dependent on IDO for its outgrowth but rather mimics, as closely as possible, breast cancer as it develops in humans. Conceptually it makes sense that the problem of immune escape might have to be attacked on multiple fronts to generate an effective antitumour response. What was perhaps counterintuitive, however, was the finding that standard cytotoxic chemotherapeutic agents show striking cooperativity. Therapies that combine
immunotherapy with cytotoxic chemotherapy have not been widely explored, perhaps because of the assumption that such combinations will work at cross purposes (because cytotoxic chemotherapy kills immune cells that immunotherapy targets for stimulation). This attitude has been challenged recently by a growing number of reports demonstrating striking complementarity between immunotherapeutic and chemotherapeutic strategies \[106,108,111]\). The authors’ findings likewise argue that IDO inhibitor-based immunotherapy can strongly promote antitumour efficacy in combination with chemotherapy. IDO inhibition was found to potentiate the antitumour efficacy of several DNA damaging agents as well as paclitaxel without elevating side effects of these agents. Although the mechanism underlying the observed pattern of cooperation remains to be defined, it is notable that IDO inhibition cooperated with all of the DNA-damaging agents, but none of the antimetabolic agents tested. One interpretation of this pattern of cooperation is that IDO may facilitate tumour survival in response to certain kinds of genotoxic stress, perhaps by attenuating the immune response elicited by cells that display certain types of DNA damage, sustain certain checkpoint responses, or undergo certain kinds of cell death (e.g., apoptosis versus nonapoptotic cell death). A second interpretation is that the chemotherapies that cooperate with IDO inhibition are those that stimulate antitumour immunity in a complementary manner (i.e., combination therapy is really composed of two immunotherapies). Although there is extensive evidence that the efficacy of cytotoxic drugs is based in their ability to directly kill cancer cells, there is also evidence that many of these drugs can also stimulate antitumour immunity. One appealing aspect of this idea is that it addresses what some might view as a paradox of the authors’ findings, namely, how a cytotoxic agent that kills immune cells might possibly cooperate with an immune stimulatory agent. In future work, it will be important to rigorously test these two alternatives, for example, by determining whether or not combinatorial efficacy is retained against tumour cells that are resistant to chemotherapy-induced cell death, and whether or not the cytotoxic components of different effective combinations have similar effects on how the immune system responds to tumours despite diversity in cytotoxic mechanisms.

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- First full report on the isolation of the IDO enzyme.


52. FEEFFERKORN ER: Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells.
Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors


**Demonstration that a wide variety of tumor types overexpress IDO and that IDO activity expressed intrinsically in cancer cells can suppress antitumour immunity.**


**Demonstration that loss of the anti-cancer gene Bin1 promotes tumour outgrowth through IDO deregulation and that IDO inhibitors can cooperatively enhance chemotherapy.**


**Demonstration that IDO activity expressed extrinsically of cancer cells can suppress antitumour immunity.**


**Demonstration that 4-1BB signaling can suppress inflammation through expansion of a specific IFN-γ producing T-cell population that induces IDO in DCs and macrophages.**


**Demonstration that certain T cells are sensitive to the toxic effects of tryptophan catabolites produced by cells overexpressing IDO.**


**Early report of immunological derepression resulting from cyclophosphamide treatment.**


**Demonstration of cooperativity between vaccine-based immunotherapy and taxane-based chemotherapy in the treatment of autochthonous MMTV-Neu mouse tumours.**


109. YU D, LIU B, TAN M et al.: Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to...
Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors


Mechanistic examination of immunotherapy/chemotherapy cooperativity.


Slightly different iron mechanism for IDO reaction proposed.


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Marrying Immunotherapy with Chemotherapy: Why Say IDO?

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Abstract

Activation of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in cancer cells facilitates immune escape. A recent study now shows how small-molecule inhibitors of IDO can be used to leverage the efficacy of traditional chemotherapeutic drugs that are used to treat cancer in the clinic. By promoting antitumor immune responses in combination with cytotoxic chemotherapy, IDO inhibitors may offer a drug-based strategy to more effectively attack systemic cancer. (Cancer Res 2005; 65(18): 8065-8)

Background

During the breakdown in cellular physiology that accompanies malignant tumor development, cancer cells develop certain emblematic characteristics that include inherent cellular properties (cell intrinsic) as well as properties defined through interaction with the host environment (cell extrinsic). Fundamental cell-intrinsic characteristics of cancer cells include immortalization, growth signal self-sufficiency, insensitivity to growth inhibitory signals, and apoptosis resistance, whereas fundamental cell-extrinsic characteristics include the capacity for angiogenesis, invasion, metastasis, and immune escape. Establishment of the importance of immune escape to malignant progression has been relatively recent (1). Indeed, studies of the cell-extrinsic traits of cancer have, in general, tended to lag behind studies of the cell-intrinsic traits, because the former can not be easily evaluated in simple tissue culture systems. Moreover, these processes are generally associated more with epigenetic changes and modifier effects than with mutation of the classically defined oncogene and tumor suppressor pathways that have, until recently, been the major focus of research in molecular cancer biology.

The interactions between developing tumors and the immune system are complex and dynamic. On the one hand, inflammation provides a host of proteumorigenic factors and suppression of immune responses can actually promote tumor regression in some model systems (2). On the other hand, cancer cells are also subject to immune surveillance with pressure on tumors to evade or subvert the immune response that tumor antigens should elicit (3). The development of immunotherapeutic strategies has focused predominantly on stimulating or supplementing immune effector cells. It is becoming increasingly apparent, however, that immune tolerance may be dominant in cancer patients and that it will be essential to breach established immune suppressive mechanisms for immunotherapy to be effective (1).

One strategy of immune escape that is used by cancer cells (Fig. 1) has been adapted from a mechanism that normally exists to prevent maternal immune response to paternal fetal antigens that are present during gestation (4). An inescapable consequence of sexual reproduction among histoincompatible individuals is that some means to circumvent maternal immunity must be hardwired into the system to protect the allogeneic fetus. The catabolic enzyme indoleamine-2,3 dioxygenase (IDO; EC 1.13.11.42) has been implicated in providing immune protection to the developing conceptus. IDO catalyzes the initial step in the degradation of tryptophan in the pathway leading to biosynthesis of NAD⁺. Activation of IDO in placental trophoblast cells has been proposed to lead to the establishment of immune tolerance through either localized depletion of tryptophan or accumulation of toxic catabolites. This process is immune suppressive because T cells undergoing antigen-dependent activation are exquisitely sensitive to local tryptophan catabolism, which can cause them to arrest in G1, become anergic, or die (5–7). In a key experiment, treatment of pregnant female mice with 1-methyl-tryptophan, a small-molecule inhibitor of IDO, has been shown to promote T cell–mediated destruction of allogeneic but not syngeneic concepti (4). IDO has also been more generally implicated in CTL-associated protein-4 (CTLA-4)–induced immune tolerance mediated through reverse B7 signaling in vivo (8).

Immune Escape in Cancer: Modulation of Indoleamine-2,3 Dioxygenase Expression by Bin1

A connection between elevated urinary tryptophan catabolites and bladder cancer was first reported in the 1950s (9). Since then, elevated levels of IDO-generated catabolites have been associated with a number of malignancies. This phenomenon was initially thought to be a tumoricidal consequence of IFN-γ, which stimulates expression of IDO in cells (10). However, a radical rethinking of the significance of IDO in cancer has been engendered by its implication in the prevention of allogenic conceptus rejection and by the evidence that IDO is overexpressed in most tumors and/or tumor-draining lymph nodes (11–13). How does IDO become deregulated in cancer cells? One possible answer has emerged from studies of a gene called Bin1, a cancer suppressive gene that seems to limit cancer to a large extent by limiting immune escape.

Bin1 was initially identified in a two-hybrid screen for c-Myc-interacting proteins (14). Along with the Bin3 gene, Bin1 is one of two related genes that are conserved through evolution to yeast and that define a family of adapter proteins characterized by a unique fold termed the BAR domain (14, 15). Frequent loss or attenuation of Bin1 occurs in advanced breast cancer, prostate cancer, melanoma, astrocytoma, neuroblastoma, and colon cancer (16–19). At least 10 different Bin1 splice isoforms exist in mammalian cells of which two are ubiquitously expressed, whereas the remainder are restricted to specific terminally differentiated tissues including neurons and skeletal muscle cells. The different splice isoforms exhibit different patterns of subcellular localization and cancer suppressive activity, arguing that they have different functions. A precedent for BAR adapter proteins with dual trafficking and transcriptional functions
has been established through studies of APPL, a Rab5-binding endosomal protein that translocates to the nucleus upon epidermal growth factor stimulation to associate with the NuRD/MeCP1 nucleosome remodeling and transcriptional repression complex (20). Likewise, the ubiquitously expressed Bin1 splice isoforms, which encode its anticancer properties, have been implicated in both endosomal trafficking and transcriptional repression (21, 22).

The possibility that Bin1 adapter proteins may affect pathways leading to the nucleus has garnered additional support based on possible involvement in the trafficking of signal transducer and activator of transcription (STAT) and nuclear factor-κB (NF-κB) transcription factors (23, 24).

Studies aimed at understanding how Bin1 restricts tumor outgrowth identified immune tolerance established through IDO deregulation as a likely mechanistic explanation (25). Deleting the Bin1 gene from cells resulted in superinduction of IDO gene expression by IFN-γ, directly suppressing activation of T cells in the local tumor environment. Blocking IDO activity systemically with small molecule inhibitors (e.g., 1-methyl-tryptophan) reverses T-cell suppression that occurs as a result of tryptophan catabolism in both settings.

This dichotomy reflected a difference in immune response to the cells, as Bin1-expressing cells produced rapidly growing tumors when introduced into either athymic nude mice or syngeneic mice depleted of CD4+/CD8+ T cells. Treatment of mice with the small-molecule IDO inhibitor 1-methyl-tryptophan suppressed the outgrowth of Bin1-null MR KEC tumors in syngeneic mice, but had no effect on tumor growth in mice lacking T cells (either nude mice or immunodepleted syngeneic animals). Taken together, these findings indicated that the deregulation of IDO, which accompanies Bin1 loss in these cells, promotes tumorigenicity by enabling immune escape. The frequent Bin1 attenuation and IDO overexpression observed in human cancers warrants further evaluation of the relationship between these two events.

**Cooperation of Indoleamine-2,3 Dioxygenase Inhibitors with Chemotherapy**

The Bin1-IDO studies prompted us to evaluate IDO inhibitors as potential anticancer agents. This effort revealed that immune modulation via IDO inhibition can significantly increase the efficacy of a variety of traditional chemotherapeutic drugs. In several preclinical models of cancer, single-agent therapy with an IDO inhibitor is only marginally efficacious, at best slowing tumor growth...
(11, 12, 25). In contrast, regression of established tumors can be achieved by combining an IDO inhibitor with a cytotoxic chemotherapeutic drug (25). In the MMTV-neu transgenic mouse model of breast cancer (harboring the c-neu proto-oncogene controlled by the mouse mammary tumor virus promoter), which closely resembles human ductal carcinoma in situ, established tumors refractory to single-agent therapy underwent regression when enrolled on the combination regimen. This response could not be explained by drug-drug interactions that might raise effective exposure to the cytotoxic agent, and it was dependent on T-cell immunity because depletion of CD4+ T cells abolished the efficacy of the combination therapy. These results offer an initial step in validating IDO as a drug development target in the context of a cytotoxic combination treatment modality.

As a possible drug development target, IDO has a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry, IDO is highly tractable for developing small-molecule inhibitors compared with most other therapeutic targets in cancer. Second, the only other enzyme that catalyzes the same reaction, TDO2, has a more restricted expression and substrate specificity, mitigating “off-target” issues posed by novel agents. Third, bioactive and orally bioavailable “lead” inhibitors exist that serve as useful tools for preclinical validation studies. Fourth, an Indo gene “knockout” mouse has been reported to be viable and healthy (26), indicating that IDO inhibitors will be unlikely to produce unmanageable mechanism-based toxicities (although promotion of inflammatory conditions would remain a valid concern). Fifth, pharmacodynamic evaluation of IDO inhibitors can be done easily by examining the blood serum levels of tryptophan and kynurenine, the chief substrate and downstream product of the IDO reaction, respectively. Lastly, small-molecule inhibitors of IDO likely offer substantial logistical and cost advantages relative to biological or cell-based therapies that aim at modulating immunity. IDO inhibitors may be useful not only in cancer but also in other pathologic settings, where it is desirable to relieve immune suppression and/or break immune tolerance (e.g., chronic viral infections).

**Future Perspective**

One general question raised by the work on combining IDO inhibitors with cytotoxic agents is how an immunotherapy can effectively enhance the efficacy of chemotherapy. As detailed elsewhere (27), there are at least six critical factors for inducing an antitumor immune response that might be augmented by cytotoxic chemotherapy including antigen threshold, antigen presentation, T-cell response, T-cell traffic, target destruction, and generation of memory. Consensus is lacking as to whether chemotherapy affects immune responsiveness through direct disruption of tolerogenic mechanisms or indirectly through tumor cell killing. In some experimental settings, tumor cell killing by cytotoxic agents has been shown to be critical for cooperation with no evidence of direct effects on cross-presentation by antigen-presenting cells (APC) or on endogenous immune responsiveness (27). The finding that tumor cells killed by alkylating agents such as cyclophosphamide are more effective at activating APCs, when compared with tumor cells killed by antimetabolites or freeze thaw (28), suggests some specificity to this mechanism of immune stimulation. IFN-γ can reportedly sensitize resistant tumor cell lines to apoptosis induction by cytotoxic agents independent of their p53 status (29). In this way, immunotherapy might cooperate with chemotherapy to augment tumor cell killing and indirectly generate additional proinflammatory signals. On the other hand, there is a long history of cyclophosphamide treatment preferentially neutralizing the suppressor arm of the immune system to enhance antitumor responses (30), and such a mechanism of action has been suggested for other cytotoxic agents as well (31). Recently, there has been a growing realization that it is precisely these tolerizing mechanisms that must be overcome for an immunotherapeutic strategy to be successful (1). In this context, both an IDO inhibitor and a cytotoxic agent might be acting as complimentary immunotherapies. Studies have indeed shown that when enhancement of antitumor T-cell responses by immunotherapy with CTLA-4 antibodies (CTLA-4 blockade) was combined with subtherapeutic doses of chemotherapy that shifted the cytokine profile to that of a Th1 response, this potentiated the treatment of established tumors in a mouse model and correlated with enhanced Th1 responsiveness in the treated mice (31). In this context, it is interesting to note that IDO has been proposed to be a downstream effector for the induction of CTLA-4-mediated immune tolerance (8).

IFN-γ may provide a key to understanding how the complex interplay between tumor and stroma is affected by IDO activity and inhibition. A number of reports argue that IFN-γ suppresses tumor outgrowth. Likewise, IDO activity can have antitumor consequences and its up-regulation by IFN-γ may significantly contribute to the negative effect of IFN-γ on tumors (10). These observations seem to run counter to the idea that IDO contributes positively to tumorigenesis, but this interpretation ignores the inherently complex and evolving nature of the interaction between developing tumors and the host immune system. IFN-γ has been directly implicated in the process called immune editing, whereby the immunogenic environment of the host provides positive selection for reduced tumoral immunogenicity (3). Specifically, IFN-γ signaling contributes to an immune-based host environment that suppresses tumor incidence but which can also drive formation of tumors that are more highly aggressive within an immune context (33). At early stages of tumor development, IDO up-regulation by IFN-γ may be detrimental. However, if tumor cells can adapt to the tryptophan poor environment, then keeping IDO under IFN-γ control could give tumor cells the flexibility of turning IDO off and thereby mitigating its negative consequences in the absence of elevated IFN-γ levels that would signal an active Th1 response.

Alternatively, because IDO acts as the rate-limiting enzyme in NAD+ biosynthesis, one can also envision scenarios in which constitutive expression of IDO in cancer cells is intrinsically beneficial (e.g., under hypoxic conditions that tend to confer drug resistance). Notably, poly(ADP-ribose) polymerase (PARP)–mediated NAD+ consumption drives “programmed necrosis” independent of the major apoptotic effectors p53, Bax, Bak, and caspases in cancer cells that have become dependent on glycolysis to maintain ATP levels (34). If tumor cells turn on the NAD+ biosynthesis pathway, may they be able to override sensitization to PARP. In targeting the rate-limiting step for NAD+ biosynthesis, IDO inhibitors would be expected to cooperate with chemotherapeutic drugs by reestablishing the sensitivity of tumor cells to PARP activation by these drugs. Unlike apoptosis, this necrotic form of cell death is highly proinflammatory potentially incorporating an immune component into the therapeutic response as well. By raising these issues, studies of IDO inhibitor cooperativity with chemotherapy should not only provide insights into the mechanistic basis for this new therapeutic approach but may also afford a deeper understanding of the complex contextual relationship between cancer cells and the multifaceted immune/stromal environment.
Acknowledgments

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A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition and therapeutic applications

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Abstract

Indoleamine 2,3-dioxygenase (IDO) has recently been implicated in tumor immune escape. In particular, IDO undermines a vigorous antitumor immune response by promoting peripheral tolerance, thereby shaping the host environment to be more hospitable to tumor survival and growth. Consequently, the development of potent IDO inhibitors that compromise this tolerogenic mechanism is an important therapeutic goal. To assist in the development of more potent IDO inhibitors, the current review presents the proposed catalytic mechanisms of IDO and comprehensively reviews reported IDO inhibitors. Finally, the successful preclinical application of IDO inhibition in a new anticancer modality is described.

Introduction

The treatment of advanced (metastatic) cancers is a major clinical challenge. Current regimens involving chemotherapy and other systemic modalities all too often provide only a limited benefit to the approximately 50% of cancer patients in the U.S. and other developed countries who present with advanced disease at diagnosis. Similarly, current regimens ultimately fail patients who relapse with disseminated disease after treatment of their primary tumors. It has long been recognized that tumors display immunogenic tumor antigens yet escape immune destruction, somehow evading or subverting and perhaps even reprogramming the immune system for their own benefit. This phenomenon of “immune escape” is central to tumor cell survival, but its basis is poorly understood (1). An appropriately activated immune system can eradicate cancer, even when it is aggressive and disseminated, but spontaneous occurrences of this are rare. This has prompted the development of numerous peptide- and cell-based anticancer therapies aimed at boosting the immune response (e.g., the administration of cytokines, tumor-associated antigen peptide/vector vaccines, dendritic cell [DC] vaccines and adoptive transfer of tumor antigen-specific effector T-cells expanded ex vivo from cancer patients [2-8]). These therapies, which are conceptually based on stimulating components of the immune system that produce an effective response, may not, however, be sufficient to overcome tumor immune escape if pathological immune tolerance is dominant in cancer patients, as has been recently proposed (9).

The enzyme indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.42), which appears to play a key role in protecting allogeneic conceptus from the maternal immune system, has been implicated in the establishment of pathological immune tolerance by tumors. The physiological role of IDO, which catabolizes the essential amino acid tryptophan, has been defined in large part through the
use of the bioavailable IDO-inhibitory compound 1-methyltryptophan (1-MT). This review details current thinking regarding the catalytic mechanism of tryptophan degradation by IDO in conjunction with a comprehensive summary of the current literature on small-molecule IDO inhibitors, and concludes with an overview of how IDO-inhibitory compounds might be incorporated into a novel treatment strategy that has the potential to broadly impact standard cancer therapies.

IDO mechanism

IDO is the first and rate-determining step of the kynurenine pathway of L-tryptophan (L-Trp) metabolism. It catalyzes the addition of oxygen across the C-2/C-3 bond of the indole ring in Trp and generates N-formylkynurenine (Scheme 1). In vitro, methylene blue and ascorbic acid are a necessary reductant to maintain maximum catalytic activity, but in vivo a flavin or tetrahydrobiopterin cofactor is believed to serve this role (10, 11).

The rational design and development of IDO inhibitors requires an understanding of the enzyme’s mechanism. Although the exact mechanism of IDO remains unknown, important mechanistic research with IDO and non-enzyme-catalyzed oxidation reactions have led to some understanding of the mechanism and several mechanistic proposals. Much of this work was previously described in an earlier review article (11). The current review will summarize the details in the previous review and provide an update on some recent research.

All the proposed mechanisms begin with the binding of O₂ and Trp at the active site of IDO, although the exact order of binding is uncertain. The active form of IDO has the heme iron in the ferrous (Fe²⁺) oxidation state and, although the enzyme is prone to auto-oxidation, the primary catalytic cycle does not involve redox changes. The ferric (Fe³⁺) form of IDO is inactive and requires reduction to the ferrous form before catalytic activity is returned. The ferric form is also particularly susceptible to substrate inhibition by Trp (12).

After O₂ and Trp bind in IDO’s active site, all the proposed mechanisms proceed through a 3-indolenylperoxy-Fe²⁺ (1, Scheme 2). There are three different proposals for the process to reach 1 (Scheme 2) and there are two different proposed mechanisms for the decomposition of 1 to N-formylkynurenine (Scheme 3). Intermediate 1 is central to all the proposed mechanisms because the related 3-hydroperoxyindolenine structure (not shown) has been shown to be a competent intermediate in the nonenzymatic oxidation of Trp to N-formylkynurenine (13, 14).

Three mechanisms for the formation of intermediate 1

1. Ionic mechanism

   The ionic mechanism (15) has the heme iron serving as a Lewis acid that activates the molecular oxygen (Scheme 2, Path A). Once activated, the electrophilic oxygen undergoes nucleophilic attack by the electron-rich pyrrole portion of the indole to form 1. Based on substrate and inhibitor studies (16), the N-1 proton of the indole ring of Trp is essential for the oxidation to occur. In the ionic mechanism, it has been proposed that a base in the active site deprotonates the indole to generate a more nucleophilic ring.

2. Pericyclic mechanism

   One variant of the ionic mechanism has the distal oxygen of O₂ operating as the basic site (Scheme 2, Path B) (17). Consequently, the reaction is really a pericyclic process and, more precisely, a group transfer reaction similar to an ene reaction (18). Identification of an important basic amino acid in the active site might allow for discrimination between the pericyclic and ionic mechanism. Interestingly, a recent report (19) identified two important amino acids in IDO, His346 and Asp274, through site-directed mutagenesis studies. The authors suggested that the His346 might be the proximal heme iron ligand. The role of the Asp274 is unknown, but the authors suggest it might serve as the distal heme ligand or be important for conformational stability. It is also possible that one of these amino acids is the general base for the deprotonation of the N-1 indole proton.

3. Radical mechanism

   The third mechanism (20) (Scheme 2, Path C) involves a one-electron process to reach intermediate 1 and, similar to the ionic mechanism, involves deprotonation of the N-1 indole proton by a base in the active site. However, in the radical mechanism, the indole anion 2 undergoes a one-electron oxidation to generate the intermediate 3. The two radical structures at the active site can combine to generate 1.
Two mechanisms for the transformation of 1 to N-formylkynurenine

1. Criegee-type rearrangement

After formation of the key 3-indolylperoxy-Fe\(^{2+}\) intermediate 1, two possible pathways are proposed. The first and more likely is a concerted Criegee-type rearrangement to afford the labile cyclic hemiacetal intermediate 4 (Scheme 3, Path D) (15, 21). Simple decomposition of 4 leads to formylkynurenine.

2. Dioxetane retrocycloaddition

Alternatively, 1 may have the distal oxygen add to the C-2 position of the indole to form the dioxetane intermediate 5 (Scheme 3, Path E) (15, 21). A retrocycloaddition of 5 yields N-formylkynurenine. Since the formation of the strained intermediate 5 would be thermodynamically unfavorable, this pathway is considered less likely. Furthermore, the highly exothermic decomposition of 5 should lead to light emission, but chemiluminescence has never been detected in the enzyme reaction. Nonenzymatic mechanistic studies also undermine the dioxetane intermediate pathway (22, 23).

The addition of other nucleophiles to the C-2 position of the indole in 1 has also been proposed. Notably, the \(\alpha\)-amino group of Trp might add to generate a tricyclic intermediate, 3a-hydroperoxypyrrolo[2,3-b]indole derivative (6, Fig. 1), which subsequently undergoes conversion to N-formylkynurenine with expulsion of the \(\alpha\)-amino group. Evidence for the existence of 6 has been found in the nonenzymatic oxidation of Trp (13), but not in the process catalyzed by IDO. Amino acid side-chain residues at the active site have also been proposed to transiently add to the C-2 position of the indole, although no experimental evidence exists to support this idea (23).

IDO inhibitors: chemistry and pharmacology

Structural classes of IDO-inhibitory molecules

There exists only a small collection of reports describing inhibition studies of IDO. Not surprisingly, the studies have focused primarily on derivatives of Trp and structurally related heterocycles like \(\beta\)-carboline, despite...
the reported (24-26) promiscuity of IDO compared to the related tryptophan 2,3-dioxygenase (TDO2, EC 1.13.11.11). Both competitive and noncompetitive inhibitors of IDO have been identified. To date, competitive inhibitors are primarily derivatives of Trp, while noncompetitive inhibitors are derivatives of β-carboline; both contain an indole core.

**Competitive inhibitors**

Substrate inhibition with high concentrations (> 0.2 mM) of L-Trp was reported (12, 27) during early enzymological studies, and therefore it is not surprising that Trp derivatives have been extensively studied. Derivatization of the Trp structure has occurred in three areas: substitution of the indole ring, modification of the amino acid side-chain and modifications of the indole ring.

1. Tryptophan indole ring substitutions

Substitution of the indole ring of Trp has afforded the most commonly used inhibitor of IDO: 1-MT (7, Table I) (16). A racemic mixture was originally used by Munn and coworkers in their seminal study of the fetal survival paradox (28), but subsequent studies (29) with isolated IDO have revealed a slight preference for the natural (S)-(L) isomer of 7 (the more precise Cahn-Ingold-Prelog system of configurational assignment will subsequently be used in preference to the historic D,L system). Furthermore, the (S)-isomer of the natural substrate Trp has 10-50 times smaller \( K_m \) values than (R)-Trp (30). Sterechemical preference for the natural isomer was also reported with the 6-nitro derivative 24 (Table I). On the other hand, some cellular studies (31-33) demonstrate greater activity for the (R)-(D) isomer of 7 (1-MT). Given the more complex nature of cellular studies, IDO-inhibitory activity may not be the primary reason for the better activity of the (R)-isomer of 7. Nevertheless, based on these conflicting results future inhibition studies should carefully consider both stereoisomers of Trp analogues.

Table I comprehensively summarizes the range of substituents that have been tested on the indole ring of Trp. The seven most potent compounds based on the reported inhibition data are the five monosubstituted derivatives, 1-methyl (7), 5-bromo (15), 6-fluoro (23), 6-nitro (24), ([S]-isomer), 7-fluoro (26), and the two difluorinated derivatives 4,7-difluoro (14) and 5,7-difluoro (21). Excluding the 1-methyl derivative, the other six are electron-withdrawing groups (34-36). Since the proposed mechanisms for IDO-catalyzed conversion of Trp to N-formylkynurenine (see above) all begin with electron donation from the pyrrole ring of Trp, electron-withdrawing groups on the indole ring would make this step less favorable and slower. Nevertheless, the activity data in Table I indicates that the 5-bromo (15) and the 6-fluoro (23) derivatives undergo oxidation; therefore some of these compounds still behave as substrates despite their deactivating substitution.

Several compounds, notably the 5-bromo (15) and 2-hydroxy (12) derivatives, have significantly different IDO inhibition values reported by different sources. Some of the variability may be due to the different IDO sources and assay conditions used in the different studies. Peterson and coworkers extracted IDO from human monocyte/macrophage cells induced by interferon gamma (29). They monitored IDO activity by detecting kynure-
substituents would be expected to make the indole ring more nucleophilic, leading to a faster initial reaction with the oxygen species at the active site.

The 1-methyl derivative \( \text{7} \) defies the trend seen with substituents on the benzene portion of the indole ring. The proposed mechanisms (see above) for IDO involving pyrrole electron donation actually initiate the reaction with deprotonation of the N-1 hydrogen of Trp. Without a hydrogen, \( \text{7} \) prevents the deprotonation from occurring.

Similar inhibition is seen with benzofuran (\( \text{54} \)) and benzothiophene (\( \text{55} \)) analogues of Trp (Table III; see below). However, there is a limited amount of space in the active site to accommodate N-1 groups, as the 1-ethyl (\( \text{8} \)) and 1-phenylsulfonyl (\( \text{9} \)) derivatives exhibited only weak inhibitory activity.

Indole ring substitution of Trp derivatives has been extensively explored; nevertheless, the use of multiple

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**Table I: Trp derivatives with indole ring substitution.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Indole ring substitution</th>
<th>Stereochemistry at α position</th>
<th>Inhibition data (%) ( \text{a} )</th>
<th>Activity data (%) ( \text{b} )</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 1-CH(_3)</td>
<td>S (L)</td>
<td>52.3 (62.9)( \text{c} ); ( K_\text{f} = 34 \mu\text{M} )</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 1-CH(_3)</td>
<td>R,S</td>
<td>26; ( K_\text{f} = 6.6 \mu\text{M} )</td>
<td>7</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>7 1-CH(_3)</td>
<td>R (L)</td>
<td>5.7 (11.6)( \text{c} )</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 1-CH(_2)CH(_3)</td>
<td>S</td>
<td>13.5 (9.9)( \text{c} )</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 1-SO(_2)Ph, 6-OCH(_3)</td>
<td>R</td>
<td>3.2 (28.4)( \text{c} )</td>
<td>29</td>
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<tr>
<td>10 2-Cl</td>
<td>S</td>
<td>20</td>
<td>33</td>
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<tr>
<td>11 2-Br</td>
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<td>11</td>
<td>21</td>
<td>37</td>
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</tr>
<tr>
<td>12 2-OH</td>
<td>S</td>
<td>30</td>
<td>4</td>
<td>37</td>
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<tr>
<td>12 2-OH</td>
<td>R,S</td>
<td>-38.4 (-43.3)( \text{c} )</td>
<td>29</td>
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<tr>
<td>13 4-CH(_3)</td>
<td>R,S</td>
<td>26</td>
<td>33</td>
<td>37</td>
<td></td>
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<tr>
<td>14 4-F, 7-F</td>
<td>S</td>
<td>( K_\text{f} = 40 \mu\text{M} )</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>15 5-Br</td>
<td>R,S</td>
<td>0( \text{c} )</td>
<td>29</td>
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</tr>
<tr>
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<td>R,S</td>
<td>56</td>
<td>36</td>
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<tr>
<td>16 5-CH(_3)</td>
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<td>123</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>17 5-OCH(_3)</td>
<td>R,S</td>
<td>35</td>
<td>70</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>18 5-OCH(_3)Ph</td>
<td>R,S</td>
<td>2</td>
<td>1</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>19 5-OH</td>
<td>S</td>
<td>12</td>
<td>59</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>19 5-OH</td>
<td>S</td>
<td>14( \text{c} )</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 5-F</td>
<td>R,S</td>
<td>32</td>
<td>46</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>21 5-F, 7-F</td>
<td>S</td>
<td>( K_\text{f} = 24 \mu\text{M} )</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22 6-CH(_3)</td>
<td>R,S</td>
<td>20</td>
<td>72</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>23 6-F</td>
<td>R,S</td>
<td>54</td>
<td>38</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>24 6-NO(_2)</td>
<td>S</td>
<td>52</td>
<td>2</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>24 6-NO(_2)</td>
<td>R</td>
<td>7</td>
<td>0</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>25 7-CH(_3)</td>
<td>R,S</td>
<td>38</td>
<td>18</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>26 7-F</td>
<td>S</td>
<td>( K_\text{f} = 37 \mu\text{M} )</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( \text{a} \)Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an in vitro competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

\( \text{b} \)Percent compound oxidized relative to \( \text{L-tryptophan} \).

\( \text{c} \)100 \( \mu\text{M} \) inhibitor concentration used in inhibition assay.

\( \text{d} \)\( K_\text{f} \) determined at pH 8.0 in reference (16).
of these have afforded compounds with promising inhibition. Modest inhibition was realized with the addition of a methyl group to either the α-amine (33) or the α-acid (35). One notable derivative with interesting activity and a novel structure is the thiohydantoin derivative (46) (39). Further modification of the thiohydantoin ring might provide even more potent inhibitors.

2. Tryptophan side-chain modifications

A range of Trp side-chain modifications have been explored, as illustrated in Table II. However, relatively few substituents is a strategy that might yield more active inhibitors. Excluding compounds 9, 14 and 21, few compounds with multiple substituents have been synthesized and tested. The synthetic challenge posed by polysubstituted indoles is probably one reason that these examples are limited. Another limitation would appear to be the space available in the indole binding region of the active site, as seen in the weak activity and inhibition with 18. Despite these limitations, it is clear that a range of substituents has been accommodated and therefore combinations of these might afford synergistic inhibition. Unlike the β-carboline derivatives (see below), there has been no indication of slow-binding inhibition from Trp derivatives; the preincubation inhibitory data in Tables I-III do not substantially differ from the percent inhibition found in standard competition assays.

### Table II: Trp side-chain modifications.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R(^a)</th>
<th>Stereochemistry at α position</th>
<th>Inhibition data (%)(^b)</th>
<th>Activity data (%)(^c)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>-CH(_2)CH(_2)NH(_2)</td>
<td>28</td>
<td>32</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>28</td>
<td>-CH(_2)CH(_2)NH(_2); (5-OCH(_3))</td>
<td>-43.9(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>29</td>
<td>-CH(_2)CH(_2)NH(_2); [2-CO(_2)H]</td>
<td>16.3 (17.9)(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>30</td>
<td>-CH(_2)CH(_2)NH(_2); [2-CO(_2)H, 5-OCH(_3)]</td>
<td>10.8 (3.4)(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>31</td>
<td>-CH(_2)CH(_2)CO(_2)H</td>
<td>0</td>
<td>8</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>-CH(_2)C(CH(_3))(NH(_2))CO(_2)H</td>
<td>R,S</td>
<td>1</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td>33</td>
<td>-CH(_2)CH(NHCH(_3))CO(_2)H</td>
<td>S</td>
<td>33</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td>34</td>
<td>-CH(_2)CH(NHCOCH(_3))CO(_2)H</td>
<td>S</td>
<td>7</td>
<td>3</td>
<td>37</td>
</tr>
<tr>
<td>35</td>
<td>-CH(_2)CH(NH2)CO(_2)CH(_2)H</td>
<td>S</td>
<td>30</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td>36</td>
<td>-CH(_2)CH(NH)CO(_2)CH(_2)CH(_2)H</td>
<td>S</td>
<td>7</td>
<td>14</td>
<td>37</td>
</tr>
<tr>
<td>37</td>
<td>-CH(_2)CH(OH)CO(_2)H</td>
<td>R,S</td>
<td>9.7 (1.4)(^d)</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>38</td>
<td>-CH(_2)N(CH(_3))(_2)</td>
<td>-6.6(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>39</td>
<td>-CH,CN</td>
<td>3.5 (^e)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>40</td>
<td>-CONH(_2); (5-OH)</td>
<td>0(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>41</td>
<td>-CHO</td>
<td>4.4(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>42</td>
<td>-CH=CHCO(_2)H</td>
<td>2.5 (3.2)(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>43</td>
<td>-CH=CHCO(_2)CH(CH(_3))(_2)</td>
<td>15.2 (11.6)(^d)</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>44</td>
<td>-(E)-CH=CH-(3-pyridinyl); (6-F)</td>
<td>0</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>-CH(_2)CH(NH)CO(_2)H</td>
<td>α-S,β-β-S,α-R,β-R</td>
<td>0.0 (2.7)(^d)</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>45</td>
<td>-CH(_2)CH(NH)CO(_2)H</td>
<td>α-S,β-β-R,α-R,β-S</td>
<td>9.8 (3.6)(^d)</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>45</td>
<td>-CH(_2)CH(NH)CO(_2)H</td>
<td>R,S</td>
<td>7</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>46</td>
<td>-CH(_2)S-(3-methyl-2-thioxo-4′-imidazolinone)</td>
<td>R,S</td>
<td>K(_i) = 11.4 µM</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>47</td>
<td>-CH(_2)CH(NH)CO-(S)-Trp</td>
<td>S</td>
<td>K(_i) = 147 µM</td>
<td></td>
<td>29</td>
</tr>
</tbody>
</table>

\(^a\) Additional indole substituents are added in brackets.

\(^b\) Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an in vitro competitive inhibition assay with 1 mM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

\(^c\) Percent compound oxidized relative to l-tryptophan.

\(^d\) 100 µM inhibitor concentration used in inhibition assay.

![Fig. 2. Compound 46.](image)
kynurenic acid (60) or quinolinic acid (63), nor was inhibition seen with the structurally related analogues 59, 61 and 62. Two interesting exceptions were discovered with 58 and 64. 3-Amino-2-naphthoic acid (58) is an analogue of anthranilic acid, an intermediate in the aromatic pathway of Trp metabolism. Although assay differences preclude direct comparisons of the potency of IDO inhibitors, compound 58 is one of the most potent inhibitors yet reported in the literature. It is clearly one of the most interesting lead compounds, notwithstanding the synthetic challenge of constructing 3-amino-2-naphthoic acid analogues. A second unique inhibitor was pyrrolidine dithiocarbamate (64) (42). This antioxidant demonstrated notable inhibitory activity against IDO generated from interferon gamma treatment of human monocyte-derived macrophages. It is possible that the sulfur of the dithiocarbamate is binding to the heme iron in the active site of IDO. This binding mode would be consistent with sulfur’s well-known affinity for iron in biological systems, e.g., ferrodoxin.

**Noncompetitive inhibitors**

The first class of structures exhibiting IDO inhibition was a series of β-carboline structures reported in 1984 (38). Initially, they were reported to exhibit uncompetitive inhibition, but β-carboline (65), also known as norharman,
β-Carboline derivatives

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-3. The benzene ring has been subsequently reclassified as a noncompetitive inhibitor (43). β-Carboline derivatives (Table V) continue to be the most common type of noncompetitive inhibitor, but three novel structures (Table VI) have also been reported (44).

Table IV: Other compounds tested for competitive inhibition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Inhibition data (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>1-amino-2-naphthoic acid</td>
<td>-2.0 (11.2)</td>
<td>29</td>
</tr>
<tr>
<td>58</td>
<td>3-amino-2-naphthoic acid</td>
<td>74.2 (75.2)</td>
<td>29</td>
</tr>
<tr>
<td>59</td>
<td>3-quinolinicarboxylic acid</td>
<td>-2.6</td>
<td>29</td>
</tr>
<tr>
<td>60</td>
<td>4-hydroxy-2-quinolinicarboxylic acid</td>
<td>1.1</td>
<td>29</td>
</tr>
<tr>
<td>61</td>
<td>4,8-dihydroxy-2-quinolinicarboxylic acid</td>
<td>2.9</td>
<td>29</td>
</tr>
<tr>
<td>62</td>
<td>2-picolinic acid</td>
<td>1.5</td>
<td>29</td>
</tr>
<tr>
<td>63</td>
<td>quinolinic acid</td>
<td>6.8</td>
<td>29</td>
</tr>
<tr>
<td>64</td>
<td>pyrrolidine dithiocarbamate</td>
<td>44b; IC&lt;sub&gt;50&lt;/sub&gt; = 6.5-12.5 µM</td>
<td>42</td>
</tr>
</tbody>
</table>

*Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an in vitro competitive inhibition assay with 100 µM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

Table V: β-Carboline ring substitution compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>β-Carboline ring substitution</th>
<th>Inhibition data (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>none</td>
<td>50.3 (57.0); K&lt;sub&gt;i&lt;/sub&gt; = 178 µM</td>
<td>44</td>
</tr>
<tr>
<td>66</td>
<td>3-OCH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>5.5 (21.2)</td>
<td>44</td>
</tr>
<tr>
<td>67</td>
<td>3-OCH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>16.7 (76.7) ; K&lt;sub&gt;i&lt;/sub&gt; = 98.0 µM</td>
<td>44</td>
</tr>
<tr>
<td>68</td>
<td>3-OCH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>6.7 (11.0)</td>
<td>44</td>
</tr>
<tr>
<td>69</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;Bu</td>
<td>7.0 (7.2); K&lt;sub&gt;i&lt;/sub&gt; = 89.7 µM</td>
<td>44</td>
</tr>
<tr>
<td>70</td>
<td>3-COCH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-4.1 (44.9)</td>
<td>44</td>
</tr>
<tr>
<td>71</td>
<td>3-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.9 (-19.4)</td>
<td>44</td>
</tr>
<tr>
<td>72</td>
<td>3-N=N=S</td>
<td>26.7 (86.1)</td>
<td>44</td>
</tr>
<tr>
<td>73</td>
<td>3-OH</td>
<td>30.1 (-5.3)</td>
<td>44</td>
</tr>
<tr>
<td>74</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt; 6-F</td>
<td>40.4 (49.2); K&lt;sub&gt;i&lt;/sub&gt; = 7.4 µM</td>
<td>44</td>
</tr>
<tr>
<td>75</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt; 6-Br</td>
<td>-4.9 (13.4)</td>
<td>44</td>
</tr>
<tr>
<td>76</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 40.6 µM</td>
<td>44</td>
</tr>
<tr>
<td>77</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 259 µM</td>
<td>44</td>
</tr>
<tr>
<td>78</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 98.0 µM</td>
<td>44</td>
</tr>
<tr>
<td>79</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;br&gt;CH</td>
<td>= 3.3 µM</td>
<td>44</td>
</tr>
<tr>
<td>80</td>
<td>3-NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 37.5 µM</td>
<td>44</td>
</tr>
<tr>
<td>81</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt; 6-F</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 21.0 µM</td>
<td>44</td>
</tr>
<tr>
<td>82</td>
<td>3-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt; 6-N=N=S</td>
<td>K&lt;sub&gt;i&lt;/sub&gt; = 8.5 µM</td>
<td>44</td>
</tr>
<tr>
<td>83</td>
<td>1-CH&lt;sub&gt;3&lt;/sub&gt;, 7-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>84</td>
<td>1-CH&lt;sub&gt;3&lt;/sub&gt;, 2-O, 7-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>85</td>
<td>1-CH&lt;sub&gt;3&lt;/sub&gt;, 7-OH</td>
<td>-11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>86</td>
<td>1-CH=CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>87</td>
<td>1-CO&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;, 7-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>88</td>
<td>1-CH&lt;sub&gt;3&lt;/sub&gt;, 7-OCH&lt;sub&gt;3&lt;/sub&gt;, 3,4-dihydro</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>89</td>
<td>1-CH&lt;sub&gt;3&lt;/sub&gt;, 7-OH, 3,4-dihydro</td>
<td>21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>90</td>
<td>1,2,3,4-tetrahydro</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>91</td>
<td>1-OH, 7-OCH&lt;sub&gt;3&lt;/sub&gt;, 3,4-dihydro</td>
<td>-13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38</td>
</tr>
</tbody>
</table>

<sup>a</sup>Unless otherwise stated, inhibition data are reported as 100 minus percent of tryptophan metabolized in an in vitro competitive inhibition assay with 100 µM of inhibitor. Figures in parentheses indicate inhibition data with 2-h preincubation of inhibitor with IDO.

<sup>b</sup>2 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

<sup>c</sup>1 mM inhibitor concentration used in inhibition assay with rabbit intestine IDO.

Modifications to the β-carboline structure have occurred in both the pyridine and the benzene rings. The pyridine ring has been reduced and substituted at C-1 and C-
IDO is an extrahepatic enzyme that catalyzes the initial and rate-limiting step in the degradation of tryptophan along the kynurenine pathway that leads to the biosynthesis of NAD⁺ (nicotinamide adenine dinucleotide) (26, 49). IDO does not, however, handle dietary catabolism of tryptophan, which is instead the role of the structurally unrelated liver enzyme tryptophan dioxygenase (TDO2). Moreover, NAD⁺ levels in mammalian cells are predominantly maintained by salvage pathways. Thus, for many years the biological role of IDO remained unclear. Recently, however, it has been demonstrated that IDO modulates immune function by suppressing cytotoxic T-cell activation (reviewed in 50). The physiological relevance of IDO-mediated immunosuppression was confirmed in a seminal study which demonstrated that administration of the bioactive IDO inhibitor 1-MT (16) can elicit MHC-restricted, T-cell-mediated rejection of allogeneic mouse concepti (28, 51).

**Genetic control of IDO by the tumor suppressor gene Bin1**

Elevated tryptophan catabolism in cancer patients, first reported in the 1950s (52), was generally ascribed to be a tumoricidal effect of interferon gamma elevation operating through IDO to starve the rapidly growing tumor cells of the essential amino acid tryptophan (53). However, the elucidation of IDO’s tolerogenic role has recently prompted the opposing hypothesis that elevated IDO can promote tumor cell immune escape by suppressing the activation of cytotoxic T-cells that could recognize and destroy them.

A key finding in support of this hypothesis has been the discovery of a regulatory link between IDO elevation and in situ expression of IDO inhibitors that potentially can block IDO activity.
in tumor cells and an established cancer suppression signaling pathway controlled by the adaptor protein Bin1 (39). Bin1 was originally identified through its ability to interact with and inhibit the oncogenic activity of the c-Myc oncoprotein (54, 55). Subsequent studies have indicated complex splice regulation of Bin1 protein isoforms in cells, which are linked to diverse cellular processes, and systemic disruption in homozygous Bin1 knockout mice results in perinatal lethality associated with severe cardiomyopathy (56). Existing studies in human prostate and breast cancers support the candidacy of Bin1 as a tumor suppressor or negative modifier gene. Loss or attenuation of Bin1 expression occurs in >50% of primary human breast tumors and in all breast tumor cell lines examined to date (57). The 2q14-21 region, where Bin1 is located, is frequently deleted in breast cancers (58, 59), particularly in tumors that contain BRCA1 mutations or have metastatic capacity (58-61). In prostate cancers, Bin1 shows frequent loss of heterozygosity (LOH) and loss of expression, especially in advanced cases with metastatic capacity (62, 63). Studies utilizing Bin1 knockout mouse-derived cell lines corroborate the hypothesis that Bin1 has an antiprogession role in cancer. In particular, Bin1 loss provides a dramatic cell-extrinsic benefit to in vivo tumor growth that is explained by IDO dysregulation (39).

Preclinical studies combining IDO inhibitors with breast cancer chemotherapy

Based on these pivotal studies linking Bin1 loss to IDO upregulation and immune escape by tumors, critical proof-of-principle studies have been performed. These studies have led to the discovery of a novel therapeutic strategy whereby IDO inhibitors in combination with standard chemotherapeutic agents cooperatively produce dramatic regression of established tumors in preclinical studies (39). One prediction of the hypothesis framed in the previous section is that an IDO inhibitor might break immune tolerance and promote tumor regression. We employed the well-accepted mouse model of breast cancer, the MMTV-Neu “oncomouse” that develops mammary gland tumors closely resembling human ductal carcinoma in situ (DCIS), to investigate this idea. The possible antitumor effects of the well-established IDO inhibitor 1-MT were evaluated either alone or in combination with other agents. 1-MT treatment alone slowed tumor growth but did not reverse it, consistent with other recently published observations (64, 65). This finding suggests that single-agent IDO inhibitor-based immunotherapy has limited antitumor efficacy when applied to established tumors. In contrast, treatment of tumor-bearing MMTV-Neu mice with a combination of 1-MT + paclitaxel produced an average decrease of approximately 30% in tumor volume at the 2-week endpoint, while paclitaxel treatment by itself produced only tumor growth inhibition. Histological and immunohistochemical examination revealed evidence of increased cell death in tumors from mice treated with 1-MT + paclitaxel (39). Consistent with host immunity being critical for the therapeutic regression of tumors, immunodepletion of either CD4+ T-cells (39) or CD8+ T-cells (unpublished) abrogated the ability of 1-MT to cooperate with paclitaxel. Similar cooperativity was observed with some but not all chemotherapeutic agents tested (39).

In summary, IDO inhibition produces dramatic antitumor efficacy in the autochthonous MMTV-Neu tumor model when combined with certain cytotoxic chemotherapeutic agents. This finding is striking as it supports what has generally been viewed as a counterintuitive notion, that combining immunotherapy with chemotherapy can be used to effectively promote tumor regression. Immunotherapy and chemotherapy have generally been thought to work at cross purposes; however, the case for complementarity has been gaining credence as of late, based on experiments employing increasingly sophisticated models and tools to monitor the progress of antitumor immune responses (66).

While a useful tool for proof-of-principle studies, 1-MT is not the ideal compound for development as a clinical agent. 1-MT is a weak inhibitor, especially in cell-based assays (where the EC50 is >200 µM), and has poor solubility characteristics. To address these issues, we have conducted enzyme- and cell-based screens of commercially available compounds and have identified a series of thiohydantoin derivatives of tryptophan that are pharmacologically superior to 1-MT. The most potent of these is the compound 3’-methylthiohydantointryptophan (termed MTH-trp), with an EC50 in the cell-based assay of 12 µM (39), being approximately 20-fold more potent than 1-MT.

A trial of MTH-trp in the MMTV-Neu autochthonous tumor model revealed that it has biological activity similar to or better than that of 1-MT. Over the 2-week course of treatment, MTH-trp alone promoted tumor growth delay but in combination with paclitaxel promoted tumor regression. MTH-trp combination therapy achieved an overall reduction in mean tumor volume over the 2-week treatment period of 58% (including one complete regression) as compared to 30% with 1-MT. As with 1-MT combination therapy, tumor regression was found to be associated with increased tumor cell death (39).

Therapeutic potential of IDO inhibitors based on market analysis

Cancer is the second leading cause of death in the U.S., with over 500,000 people dying each year. Of the approximately 1.4 million new cases of cancer diagnosed in 2004, 563,700 will have died in 2004 (41% death rate). Of these deaths, 28% will have been from lung cancer, 10% from colon cancer, 7% from breast cancer and 5% from prostate cancer. These cancers, often referred to as “The Big Four”, represent the major targets to positively impact the cancer survival rate. Breast cancer is the most frequently diagnosed cancer in women. In 2002, approximately 200,000 new cases were diagnosed in the U.S.
Table VII: U.S. market for cancer therapies.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>1995</th>
<th>2000</th>
<th>2005*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic drugs</td>
<td>$1,397</td>
<td>$1,863</td>
<td>$1,975</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>$265</td>
<td>$393</td>
<td>$355</td>
</tr>
<tr>
<td>Antitubulin agents</td>
<td>$580</td>
<td>$783</td>
<td>$846</td>
</tr>
<tr>
<td>Alkylation agents</td>
<td>$220</td>
<td>$281</td>
<td>$295</td>
</tr>
<tr>
<td>Others</td>
<td>$305</td>
<td>$378</td>
<td>$412</td>
</tr>
</tbody>
</table>

and over 1 million new cases worldwide. Treatment for breast cancer is correlated to the disease stage and patient hormone receptor status, with specific therapy decisions based on an individual patient’s likelihood of response. Typical interventions for breast cancer range from surgical resection, for the treatment of localized stage 1 tumors, to combination treatment strategies that employ surgery, radiotherapy and multidrug therapy for patients with advanced breast cancer. Current treatment of metastatic (stage IV) disease, in particular, must balance prolonging the patient’s life with the impact of treatment on the patient’s quality of life. In breast cancer, fewer than 15% of patients who develop metastatic disease survive for 5 years after diagnosis, irrespective of treatment.

The extraordinary size of the cancer market becomes apparent in Table VII, which shows sales (in millions) of chemical anticancer drugs in the U.S., with 2005 projected (as denoted by the asterisk).

The market for cancer chemotherapies in 2001 reached $4 billion in the U.S. and $10.8 billion worldwide, and is growing at an annual rate of 10%. Of this market, breast cancer therapies represented approximately $250 million annual sales in the U.S. The antitubulin segment, of which Taxol® (paclitaxel) is a member, is projected to be the fastest growing segment of the cancer therapies market. Taxol® sales reached $1.2 billion in 1998 and $1.7 billion in 2001. The expanded use of Taxol® (and the newer taxanes), as represented by the over 20% per year increase in sales since its introduction, reflects both its utility and the very limited alternatives available for the effective treatment of solid tumors. IDO inhibitors, which are likely to work most effectively as immunomodulatory adjuncts to conventional chemotherapeutics, represent particularly attractive candidates for clinical development in this context. As such, IDO inhibitors have tremendous potential to cooperatively leverage taxane-based breast cancer therapy, as well as other chemical therapies for a variety of cancer indications.

References

Indoleamine 2,3-dioxygenase as a new cancer immunosuppression target


Figure 1. Immunohistochemical analysis reveals an abnormal Bin1 staining pattern in MMTV-Neu mouse mammary gland tumor cells. Panels on the right are from a representative autochthonous MMTV-Neu mouse tumor, panels on the left are from an adjacent normal mammary gland. Formalin-fixed sections were stained with; hematoxalin + eosine (top panels), α-Bin1 antibody 2F11 (middle panels), or secondary antibody alone (bottom panels).
Figure 2. Impact of Neu transgene copy number and strain background on mammary gland tumor development. Following two rounds of pregnancy and nursing initiated at 2-3 months of age, mice were monitored for the appearance of mammary gland tumors. N/N = homozygosity and N/wt = hemizygosity for the MMTV-Neu transgene. The mixed background includes C57BL/6, 129SvIm/J, and FVB strain alleles.

Figure 3. Aberrant mammary gland development associated with Bin1 loss. H&E stained sections of representative formalin-fixed mammary gland tissues from nonparous (left panels) and 18.5 dpc late-term pregnancy (right panels) female mice. Bin+ and Bin− indicate WapCre<sup>+/−</sup> Bin1<sup>flox/wt</sup> and WapCre<sup>−/−</sup> Bin1<sup>flox/KO</sup> genotypes respectively.
Table 1. Summary of DMBA/MPA-induced breast tumor data in Wap-Cre$^{+/−}$ uniparous females

<table>
<thead>
<tr>
<th>Bin1 Genotype</th>
<th>n</th>
<th>Mice w/ tumors</th>
<th>Tumors /mouse</th>
<th>Onset mos</th>
<th>Latency days$^a$</th>
<th>Grade$^b$</th>
<th>Differentiation status</th>
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<tr>
<td>flox/wt</td>
<td>8</td>
<td>8 (100%)</td>
<td>2.3±1.3</td>
<td>11.5±1.8</td>
<td>128±47</td>
<td>1.6 1.5 2.3 5.4</td>
<td>Well</td>
</tr>
<tr>
<td>flox/KO</td>
<td>14</td>
<td>14 (100%)</td>
<td>2.2±1.4</td>
<td>11.9±2.6</td>
<td>115±36</td>
<td>2.3 2.5 2.9 7.8</td>
<td>Poorly</td>
</tr>
</tbody>
</table>

$^a$ Latency for tumor development from the end of 4th DMBA treatment.

$^b$ Mammary tumors are graded according to the Nottingham modification of the Bloom-Richardson system. The grading is based on 3 parameters:
- T(tubule formation): point 1-3
- N(nuclear pleomorphism): nuclear variation in size and shape. point 1-3
- M(mitotic count): mitoses. point 1-3

Figure 4. Bin1 loss is associated with poorly differentiated tumor histopathology. H&E stained sections of DMBA-induced mammary gland tumors. Bin+ and Bin1− indicate WapCre$^{+/−}$ Bin1$^{flox/wt}$ and WapCre$^{+/−}$ Bin1$^{flox/KO}$ genotypes respectively.