Cancer Immunotherapy: Selected Targets and Small-Molecule Modulators

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Introduction

Cancer immunotherapy has generated a lot of excitement lately in the scientific community along with new hope for many cancer patients due to the recent clinical successes with immune checkpoint inhibitors,[1] chimeric antigen receptor T cell therapy,[2] and adoptive cell therapy with tumor-infiltrating lymphocytes[3] in various tumor types. After more than 100 years of intense research effort,[4] the field is entering an exciting new phase; it has been widely recognized that cancer immunotherapy has the potential to change clinical treatment of cancer patients either as a standalone therapy or in combination[5] with more established targeted therapies, chemotherapeutic agents, and radiotherapy to overcome tumor-induced immunosuppression.[6–8] The currently available immuno-oncology therapeutics in clinical development and on the market are mostly biologics (antibodies,[9] proteins, engineered cells, and oncolytic viruses).[10] However, modulation of the immune system with small molecules offers several advantages that might be complementary and potentially synergistic to large biological molecules. For example, small-molecule drugs offer opportunities to tackle intracellular targets that are inaccessible to protein therapeutics. They can be administered orally and can potentially reach high exposure levels inside the tumor microenvironment. Clinical development studies of small molecules and their application are often relatively straightforward due to well-established formulations and dosing regimes to cope with pharmacokinetic and pharmacodynamic challenges. In general, small-molecule drugs can be prepared with lower costs of goods and overall decreased costs of drug delivery (oral versus infusion). These might be additional factors that make cancer immunotherapy affordable for broader application and useful in a variety of combinations in the future. This review provides a brief introduction into recent trends related to some selected targets for cancer immunotherapy and their small-molecule pharmacological modulators.

Checkpoint Inhibitors

PD-1 and PD-L1

The protein PD-1 (Programmed Death-1, CD279) belongs to the CD28 family and is an inhibitory receptor.[11, 12] It is expressed at the surfaces of various immune cells including activated T cells, B lymphocytes, dendritic cells, monocytes, and macrophages. It is also upregulated in activated T cells of cancer patients.[13, 14] The corresponding ligand PD-L1 (Programmed Death Ligand-1, B7-H1, CD274) is expressed on immune or non-hematopoietic cells.[15]

Activation of T cells triggers the expression of PD-1, whereas PD-1 ligands such as PD-L1 and PD-L2 are upregulated by interferon γ (IFNγ), interleukin-4 (IL-4), and other cytokines produced after T cell activation. PD-L1 is responsible for a co-inhibitory signal in activated T cells and promotes T cell apoptosis, anergy, and functional exhaustion.[16, 17] This gives rise to a feedback loop that weakens immune responses so that immune-mediated damage of tissues is limited without the presence of strong co-stimulatory signals. PD-L1 expression in various tumors is driven by oncogenic signaling pathways (e.g., PI3K-AKT, ALK, and STAT-3 pathways).[18–20] The PD-1–PD-L1 interaction is an important mechanism of cancer immune evasion in adaptive immune resistance. Tumor-associated immune suppression by activation of PD-1–PD-L1 signaling results in T cell exhaustion (deficient cellular immunity). Therefore, blockade of PD-1 or its ligands is an attractive approach to enhance antitumor immunity.

Highly durable clinical responses through the inhibition of immune checkpoint proteins with antibodies or fusion proteins have been achieved recently, inspiring an optimistic outlook for cancer therapy.[21] Despite the fact that first-generation compounds have reached the market, the race for best-in-class PD-1–PD-L1 checkpoint inhibitors and their application in various cancer indications is still ongoing. Unfortunately, despite impressive clinical activity, severe immune-related adverse events (irAEs) due to the disruption of immune self-tolerance have become evident in certain cases. Sustained target inhibition as a result of a long half-life and strong target occupancy for months likely contribute to the irAEs observed in the clinic with antibodies against immune checkpoint proteins.[22]

In a complementary approach to biologics, the use of small-molecule modulators has been described recently. Sharpe et al. at Harvard University prepared and tested small-molecule PD-1 modulators (Figure 1). These compounds are derivatives of sulfamonomethoxine 1 and sulfamethizole 2, which are both
active as antagonists in an IFNγ-release assay in transgenic mouse T cells that express PD-1. The activity of these compounds is in the micromolar range, and therefore may be useful as lead structures for the development of more potent PD-1 inhibitors.\textsuperscript{[23]}

Researchers at Aurigene Discovery Technologies and Laboratoires Pierre Fabre recently announced a collaboration on the development of the peptide AUNP-12 (Aur-012, Aurigene-012, Aurigene NP-12) for the treatment of cancer. To address the limitations of the current antibody PD-1 modulators, Aurigene’s scientists focused on the development of peptides with potent antitumor activity but with a more appropriate pharmacokinetic profile as a strategy to better control irAEs. Sequences of the extracellular domain of PD-1 critical for the PD-L1–PD-L2 binding interaction, overlapping or in close proximity to the known ligand binding regions,\textsuperscript{[24]} were identified and used as starting points for the design and evaluation of 7- to 30-mer peptides derived from human and murine PD-1 sequences. These efforts culminated in the discovery of AUNP-12 (a branched 29-residue peptide sequence). It blocks the PD-1/PD-L1, PD-1/PD-L2 and PD-L1/CD80 pathways. AUNP-12 is highly effective in antagonizing PD-1 signaling, with in vivo exposure upon subcutaneous dosing. It is claimed to inhibit tumor growth and metastasis in preclinical models of cancer and to be well tolerated with no obvious toxicity at any of the tested doses. AUNP-12 (3) is postulated to have the structure shown in Figure 2.\textsuperscript{[25–27]}

Besides AUNP-12, other peptides and peptidomimetics have been discovered by Aurigene researchers with activities in mouse splenocyte proliferation assays, human peripheral blood mononuclear cell (PBMC) proliferation assays, IFNγ production in a CTL assay, and inhibition of tumor growth after subcutaneous injection of mouse melanoma cells into mice. In a series of linear hepta- and octapeptides derived from the PD-1 BC loop including a variety of central linkers they found compound 4 to be the most active (Figure 3). The compound is also active in vivo in a lung metastasis model of B16F10 melanoma in mice (64% reduction in metastasis at 5 mg·kg\textsuperscript{-1}, subcutaneous application, once daily dosing, 14 days treatment).\textsuperscript{[28]}

Similar hepta- to nonapeptides derived from the same PD-1 BC loop have been rigidified by connecting the side chains of lysine and glutamate residues or by cyclizing the N-terminal serine and a C-terminal arginine through amide bonds. These compounds inhibit the PD-1–P-L1 interaction measured by a mouse splenocyte proliferation assay. Compound 5 (Figure 4) is active in vivo in a lung metastasis model of B16F10 melanoma.

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ma in mice (54% reduction in metastasis at 5 mg kg\(^{-1}\), subcutaneous, once daily, 14 days).\[29\]

Further extensions of these studies toward (cyclo)peptide inhibitors of the PD-1–PD-L1 interaction (compounds 6 and 7, Figure 5) were described in another recent patent from Aurigene with both cyclized and open-chain derivatives.\[30\] The pharmacological activity of these compounds was tested in a rescue assay of mouse splenocyte proliferation in the presence of recombinant PD-L1/PD-L2. For cyclopeptide 6 an 82% rescue of splenocyte proliferation (at 100 nM compound concentration) was described. For the linear peptide 7 an 81% rescue of splenocyte proliferation in the same assay was reported. 2-(2-Aminoethoxy)ethanol- and 2-(2-aminoethoxy)hydrazino linkers were also introduced into the cyclopeptidic core structures by Aurigene researchers (Figure 6, compound 8).\[31\]

Taking the peptidomimetic concept one step further, the Aurigene scientists designed oxadiazole and thiadiazole moieties into the core structure of the peptide backbone (Figure 8, compounds 12–15). These compounds were shown to be active in rescuing splenocyte proliferation up to 93% at 100 nM. The compound’s effect is examined by adding the required concentration of compound to anti-CD3/CD28 stimulated splenocytes in the presence of PD-L1.\[34, 35\]

Scientists from Bristol-Myers Squibb also recently published two different approaches toward immunomodulating inhibitors of the PD-1–PD-L1 interaction. In a major effort they claimed highly potent macrocyclic peptidic inhibitors of the PD-1–PD-L1 and CD80(B7-1)–PD-L1 protein–protein interactions.\[36\] Two highly potent macrocyclic examples from this recent patent application are shown in Figure 9 (compounds 16 and 17). The IC\(_{50}\) values for both compounds are summarized in Table 1.

In their second approach, the Bristol-Myers Squibb scientists described a series of substituted biaryl derivatives as inhibitors of the PD-1–PD-L1 interaction. Many of these compounds
showed high activity in a PD-1–PD-L1 homogeneous time-resolved fluorescence (HTRF) binding assay, with IC₅₀ values of the best compounds being in the range of 6–100 nM.⁴⁻⁵ A few selected examples are shown in Figure 10 (compound 18: IC₅₀ = 6–100 nM, compound 19: IC₅₀ = 18 nM, compound 20: IC₅₀ = 22 nM). These compounds clearly demonstrate that it is feasible to find non-peptide-derived small-molecule leads and highly potent inhibitors of protein–protein interactions relevant for immunomodulating checkpoint targets for cancer treatment. It is highly probable that these recent results will trigger further activities toward small-molecule inhibitors for PD-1–PD-L1 interactions but also for other checkpoint targets with pharmacological relevance.

Recently Liu and colleagues (Tsinghua University) reported the discovery of hydrolysis-resistant α-peptide antagonists to target the PD-1–PD-L1 checkpoint by applying mirror-image phage display technology.⁶ The optimized compound ßPPA-1 was reported to bind PD-L1 in vitro with an affinity of 0.51 μM. It was further demonstrated in CT26-tumor-bearing BALB/c mice that ßPPA-1 also effectively disrupts the PD-1–PD-L1 interaction in vivo. Therefore, α-peptide antagonists may provide novel starting points for low-molecular-weight drug candidates for cancer immunotherapy.

Overall inhibition of the interaction between PD-L1 and PD-1 is a viable and promising therapeutic approach for the treatment of cancer which can now also be tackled by small molecules. In particular, the recently published molecular-level details of the human PD-1–PD-L1 interaction based on an X-ray structure of the complex by Dömling, Dubin, Holak, and co-workers should prove very useful for future inhibitor design for this important protein–protein interaction.⁷ The authors elucidated that the ligand binding to human PD-1 is associated with significant plasticity within the receptor. A detailed molecular map of the interaction surface is described, which enables the definition of regions within both interacting protein partners that could be targeted by small molecules.

Kynurenine Pathway Modulators

Indoleamine 2,3-dioxygenase (IDO) has recently emerged as another key target in cancer immunotherapy, as it plays an important role in enabling tumor cells to evade the immune system. Van den Eynde and co-workers reported the expression of indoleamine 2,3-dioxygenase 1 (IDO1) in cancer cells and how this enzyme contributes to the capacity of these tumor cells to evade immune-mediated rejection.⁸ They demonstrated that many human tumor cells express IDO1 and that expression of IDO1 in immunogenic mouse tumor cells prevented tumor rejection by pre-immunized mice.

IDO1 belongs to three heme-dependent dioxygenases that catalyze the first step of the metabolism of L-tryptophan in the kynurenine pathway (Scheme 1). Despite the fact that tryptophan 2,3-dioxygenase (TDO), IDO1, and indoleamine 2,3-dioxygenase (IDO2) catalyze the same reaction, they have distinct substrate specificities and are localized in different subcellular compartments. TDO is primarily expressed in the liver, whereas IDO1 and IDO2 are expressed in immune cells and tumors, respectively. IDO1 is also expressed in non-immune cells, such as epithelial cells and neurons, under conditions of inflammation or stress. The expression of IDO1 is induced by various stimuli, including pro-inflammatory cytokines, growth factors, and deactivation of immune cells. The induction of IDO1 expression is mediated by transcription factors, such as NF-κB and STAT-1, which bind to specific DNA sequences in the IDO1 gene promoter.

Induction of IDO1 expression leads to the production of kynurenine, which is further metabolized by the kynurenine pathway to produce several metabolites, including kynurenic acid, quinolinic acid, and 3-hydroxyanthranilic acid. These metabolites are known to have immunosuppressive effects, such as inhibiting T-cell proliferation, downregulating the expression of co-stimulatory molecules on antigen-presenting cells, and inducing the production of regulatory T cells.

IDO1 is an attractive target for cancer immunotherapy, as its inhibition can improve the function of immune cells and enhance the efficacy of immunotherapy. Several small-molecule inhibitors of IDO1 have been identified, and some of these inhibitors have shown promising results in preclinical studies. However, the development of IDO1 inhibitors for clinical use is challenging, as IDO1 is also expressed in non-cancerous tissues, such as the intestine and brain, where it plays a physiological role in maintaining homeostasis.

A recent study by Liu and colleagues at Tsinghua University reported the discovery of hydrolysis-resistant α-peptide antagonists to target the PD-1–PD-L1 checkpoint by applying mirror-image phage display technology. The optimized compound ßPPA-1 was reported to bind PD-L1 in vitro with an affinity of 0.51 μM. It was further demonstrated in CT26-tumor-bearing BALB/c mice that ßPPA-1 also effectively disrupts the PD-1–PD-L1 interaction in vivo. Therefore, α-peptide antagonists may provide novel starting points for low-molecular-weight drug candidates for cancer immunotherapy.
genase 2 (IDO2) all catalyze the same biochemical transformation, they share only limited structural similarity.

The kynurenine pathway plays a major role in immune response regulation.\(^ {42-44}\) IDO1, which is the most broadly expressed enzyme for the first step in the pathway, has been evaluated as a potential target for cancer therapy and other immunosuppressive disease states. According to a number of studies, the activity of IDO1 seems to be directly connected to the immune system evasion of tumor cells; therefore, the inhibition of IDO1 may give access to new cancer therapies.

**Indoleamine-2,3-dioxygenase (IDO)**

Inhibitors of indoleamine 2,3-dioxygenase 1 (IDO1) are of high interest as small-molecule cancer immunotherapeutics\(^ {45}\) and potential combination partners for checkpoint inhibitors. A tool compound that has been used in a wide variety of pharmacological and mechanistic studies is the competitive inhibitor of IDO1, 1-methyltryptophan \(^ {24}\) (1-MT, Figure 11). The absence of T cell accumulation within the tumor could be partially reversed by treating mice with 24.

Further structural derivatives of 24 have also been used as IDO1 inhibitor tool compounds. Methylthiohydantoin-$\delta$,L-tryptophan 25 (MTH-trp, necrostatin 1, Figure 11) has been tested in combination with paclitaxel. Compound 25 has an IDO $K_i$ of 11.4 $\mu$M. Unfortunately, in addition to IDO1, 25 also inhibits receptor-interacting serine/threonine protein kinase 1 (RIPK1), and therefore the results of the in vitro and in vivo experiments with 25 must be interpreted with caution.\(^ {46,47}\) Several excellent reviews on IDO1 inhibitors were recently published,\(^ {42-44,45}\) and thus the focus of this section is on important tool compounds and inhibitors that have recently reached preclinical and clinical development phases.

In pioneering biological studies to elucidate the role of IDO1, derivatives of methyltryptophan (24 and 25) and phenylimidazole (PIM, 26, Figure 11) have been used as tool compounds.\(^ {48}\) PIM was used as a starting point for further structure–activity relationship (SAR) studies. With an IDO IC$\text{S}_{50}$ value of $\sim$48 $\mu$M, PIM is a rather weak tool compound. It was found that 2-hydroxyphenyl substitution led to improved potency, and these results triggered further syntheses of substituents at this position. Researchers at Newlink Genetics described a series of ureas (e.g., 27) and amides (e.g., 28) in a patent application with clear structural relation to 2-hydroxyphenylimidazole (Figure 12).\(^ {49}\) Some of these compounds demonstrated IC$\text{S}_{50} < 1 $ $\mu$M in a human IDO1 enzyme assay. In another patent application by Newlink Genetics, a tricyclic scaffold based on the PIM structure (examples 29 and 30, Figure 12) was described.\(^ {50}\) Compounds with a secondary hydroxy group display human IDO1 IC$\text{S}_{50}$ values of $< 1 $ $\mu$M. According to the SAR

![Scheme 1. Metabolism of L-tryptophan in the kynurenine pathway.](image1)

![Figure 11. Tool compounds for IDO1 inhibition.](image2)

![Figure 12. Phenylimidazole derivatives as IDO1 inhibitors (Newlink Genetics).](image3)
data published in another patent application by Newlink Genetics, it seems that the hydroxy moiety in 29 is necessary for potency in the IDO1 assay in this structural class.\(^{[58]}\)

IDO1 potency is decreased by oxidation of the hydroxy group to the ketone (30, IC\(_{50}\): 1–10 \(\mu\)M). Interestingly, the potency against TDO activity (IC\(_{50}\) < 1 \(\mu\)M) remains unchanged by this chemical transformation. Newlink Genetics selected their clinical candidate NLG919 from this tricyclic structural series. NLG919 is a potent IDO1 inhibitor (\(K_i = 7\) nM) and is currently in phase I clinical trials for adult patients with recurrent advanced solid tumors.\(^{[59]}\)

In another study, Röhrig et al. optimized the unsubstituted 4-phenyl-1,2,3-triazole 31 (Figure 13) which is a weak IDO1 inhibitor with an IC\(_{50}\) value of ~83 \(\mu\)M. By systematic and structure-guided introduction of substituents at the phenyl ring, they arrived at the highly potent (IDO1 IC\(_{50}\): 0.08 \(\mu\)M) chlorophenol derivative 32 (Figure 13) with complete selectivity for TDO.\(^{[60]}\)

![Figure 13. 4-Phenyl-1,2,3-triazole derivatives as IDO1 inhibitors.](image)

Further structurally diverse lead series for novel IDO1 inhibitors have been identified from high-throughput screens by various companies. Incyte scientists discovered hydroxyamidine 33 (Figure 14) with an IDO1 IC\(_{50}\) value of 1 \(\mu\)M.\(^{[61]}\) This lead series was optimized further and resulted in compound 34. The 4-fluoro substituent at the aromatic ring improved in vitro human clearance. Tumor growth inhibition was demonstrated with 34 in C57BL/6 mice bearing granulocyte macrophage colony-stimulating factor (GMCSF)-secreting B16 tumors. The best effect of 50% tumor growth inhibition was observed at a dose of 75 mg kg\(^{-1}\) subcutaneously, twice daily. Compound 34, which has been used extensively as a tool compound, was further optimized by Incyte researchers into the clinical candidate INCBO24360 (epacadostat, 35).\(^{[62,63]}\) Compound 35 has an IDO1 IC\(_{50}\) value of 72 nM and has been tested in preclinical studies,\(^{[64]}\) it has been evaluated in various phase I/II clinical trials for metastatic melanoma\(^{[65]}\) and as a monotherapy for ovarian cancer.\(^{[66]}\) Furthermore, Incyte and Bristol-Myers Squibb have evaluated the combination of 35 with the PD-1 immune checkpoint inhibitor nivolumab in various tumor types including melanoma, non-small-cell lung (NSCLC), ovarian, and colorectal cancer (CRC), as the combination of these two drugs could lead to synergistic antitumor immune responses.\(^{[67]}\)

From a high-throughput screen of their compound library, Amgen scientists recently discovered another lead series with a thiazolotriazole scaffold.\(^{[68]}\) AMG-1 (36, Figure 15) has an IC\(_{50}\) value of 3 \(\mu\)M in an IDO1 assay and high selectivity against IDO2 (IC\(_{50}\) > 250 \(\mu\)M) and TDO (IC\(_{50}\) > 62.5 \(\mu\)M).

![Figure 15. Thiazolotriazole scaffold as IDO1 inhibitor (Amgen).](image)

In a structure-driven approach, Tojo et al. optimized an imidazothiazole lead series further and designed novel and potent IDO1 inhibitors.\(^{[69]}\) The starting point for their optimization efforts was the imidazothiazole 37 (Figure 16). Expansion of the imidazothiazole methyl group by introduction of a variety of linkers and aryl groups resulted in optimized compounds such as 38 (IC\(_{50}\) = 1.9 \(\mu\)M). A major improvement in this series was the introduction of a urea side chain, which resulted in the most potent compound 39 with an IC\(_{50}\) value of 77 nM (Figure 16).

Röhrig et al. reported detailed analysis and follow-up studies of a high-throughput screen for IDO1 inhibitors. They screened a library of 1200 US Food and Drug Administration (FDA)-approved drugs and 14,000 compounds from the Maybridge Hit-
They also described several challenges related to such screens: for example, oxidative proteins are often challenging screening targets, as compounds capable of redox cycling can indirectly inhibit target activity by interfering with the reduced state of the enzyme and result in false positives. Iron chelators could interact with the heme cofactor, which is necessary for enzyme activity. By applying several assays for post-screen hit confirmation, miconazole 40 (IDO1 IC\(_{50}\) = 6.7 μM, Figure 17) and several other imidazole-containing anti-fungal agents were discovered as IDO1 inhibitor hits.

Scientists at Bristol-Myers Squibb recently reported derivatives of a 2-aminophenylurea scaffold\(^\text{[71–74]}\) as highly potent IDO1 inhibitors with cellular IC\(_{50}\) values in the low nanomolar to picomolar range (Figure 18, examples 41–43). IC\(_{50}\) values had been determined in an IDO kynurenine assay with human ID01/HEK293 cells.

**TDO inhibitors**

IDO1 has thus far been the most extensively studied target for cancer immunotherapy of the three enzymes that catalyze the first degradation step of l-tryptophan in the kynurenine pathway. TDO has also attracted interest in the past few years, as Platten and co-workers found that TDO is highly expressed in human glioma cells and that it is the key enzyme for tryptophan degradation in these cells.\(^\text{[75]}\) Furthermore, Van den Eynde and co-workers demonstrated that TDO is expressed in a wide variety of human tumors.\(^\text{[76]}\)

Several TDO inhibitors have been described and used as tool compounds to elucidate pharmacological effects in vitro and in vivo. The fluorinated indole derivatives 680C91 (44)\(^\text{[77]}\) and LM10 (45)\(^\text{[78]}\) are both selective against IDO1, with IC\(_{50}\) values in an hTDO assay in the sub-micromolar range (Figure 19). Van den Eynde and colleagues also demonstrated that mice treated with 45 can reject TDO-expressing tumors. These authors stated that the inhibition of both IDO1 and TDO could further improve the efficacy of this cancer immunotherapy. In an evaluation of 104 human tumor cell lines, they found 20 that expressed only TDO and 17 that expressed only IDO1; 16 cell lines expressed both IDO1 and TDO. Therefore, novel inhibitors with a mixed TDO/IDO1 inhibitor profile would have a chance to be superior and could, in principle, be efficacious on 51% of the tumor cells versus 32% for IDO1 inhibitors and 35% for TDO inhibitors.

Hung, Wu (National Health Research Institutes Taiwan) and co-workers reported the development of a structure-based virtual screening strategy to identify novel TDO inhibitors.\(^\text{[79]}\) An initial hit with IC\(_{50}\) = 711 nM was selected by virtual screening and was subjected to structural modifications and molecular docking studies. This resulted in the identification of a potent TDO-selective inhibitor 46 (Figure 20, TDO IC\(_{50}\) = 30 nM, IDO1 IC\(_{50}\) = 640 nM), which the authors claim could be used as a tool compound and for further investigations as a potential cancer therapeutic.

**IDO2 inhibitors**

IDO2 is another tryptophan-degrading enzyme and is the least-studied member of the kynurenine pathway. IDO2 is a tryptophan dioxygenase, but has much lower catalytic activity in this regard than IDO1. The cellular role of IDO2 is not completely clear. Therefore a selective IDO2 inhibitor could be very useful to clarify the specific physiological roles of IDO1.

Figure 17. Miconazole as an IDO1 inhibitor (SIB Swiss Institute of Bioinformatics).

Figure 18. 2-Aminophenylurea scaffold as IDO1 inhibitors (Bristol-Myers Squibb).

Figure 19. Selective TDO inhibitors (Wellcome Research Laboratories; University of Namur).

Figure 20. TDO inhibitor derived from virtual screening (National Health Research Institutes Taiwan).
and IDO2. In initial discovery efforts to identify selective IDO2 inhibitors, Ball et al. tested a library of FDA-approved drugs in a mouse IDO2 assay. The authors reported that the proton pump inhibitor tenatoprazole 47 (Figure 21) exhibited an IC_{50} value of 1.8 μM for IDO2 with no inhibition of IDO1 or TDO2 activity detected at a concentration of 100 μM.[80]

**Figure 21. Tenatoprazole as an IDO2 inhibitor (University of Sydney).**

### Adenosine Pathway

Adenosine triphosphate (ATP), adenosine, and tumor-associated antigens are released into the tumor microenvironment during tumor cell degradation under conditions of hypoxia, increased cellular stress, and chemotherapy.[81, 82] ATP is then further transformed into adenosine by the ectonucleotidases CD39 and CD73, which are upregulated in various cell types within the tumor microenvironment, including regulatory T cells (Tregs), stromal cells, and tumor cells. Adenosine in the tumor microenvironment has significant effects on various phases of immune function. Blocking of adenosine A_2A receptors on effector T cells, Tregs, NK cells, dendritic cells, myeloid-derived suppressor cells, and tumor-associated macrophages can therefore act against the immunosuppressive role of adenosine in the tumor microenvironment and strengthen the immune response. Combining pharmacological blockade of adenosine A_2A receptors with other approaches to cancer therapy might have additive or synergistic effects. It has been demonstrated that blocking the adenosine A_2A receptor can enhance the effect of tumor vaccines during T cell activation. Furthermore, adenosine A_2A receptor inhibition in combination with immune checkpoint inhibitors, such as PD-1 or PD-L1 blockade, drives T cell function during the effector phase of the immune response.[83]

**CD39 and CD73**

CD39 (ectonucleoside triphosphate diphosphohydrolase 1, NTPDase 1), is a cell-surface-bound phosphatase expressed on lymphocytes and tumor cells and is responsible for the conversion of extracellular ATP into adenosine. In vitro and in vivo experiments involving knockout models and surrogate inhibitors of CD39 provide evidence in support of the anticancer activity of CD39 inhibition. Furthermore, the development of CD39-blocking monoclonal antibodies (mAbs) as potential anticancer drugs has been reported.[84]

CD73 (also designated as ecto-5′-nucleotidase or ecto5′Nase) is a cell-surface-bound phosphatase found on lymphocytes and tumor cells and is responsible for the dephosphorylation of extracellular AMP into adenosine. In the tumor microenvironment CD73 plays a critical role in promoting tumor growth due to the release of adenosine and subsequent immunosuppression of tumor-infiltrating immune cells. This catabolic enzyme thereby negatively regulates both the activation and effector phase of CD8+ T cells and natural killer cells and thus contributes to tumor immune system evasion.[85]

Recently the development of potent and selective inhibitors of CD73 starting from αβ-methylene-ADP (AOPCP, adenosine-5′-O-(phosphonomethyl)phosphonic acid) was described by Müller and colleagues.[86] One of the most potent inhibitors was found to be compound 48 (Figure 22) with K_I values in a human CD73 assay of 2 nM and in a rat CD73 assay of 9 nM.

Researchers from Vitae Pharmaceuticals have claimed purine derivatives as CD73 inhibitors for the treatment of cancer in a recent patent application.[87] Compounds such as 49 (Figure 22) reached IC_{50} values of < 100 nM both in biochemical and cellular (U138 neuroglioma) assays.

**A_2A receptor antagonists**

The adenosine A_2A receptor is a member of the G-protein-coupled receptor (GPCR) superfamily. It is an important player in many physiological processes in the central nervous system and in peripheral tissues. A_2A adenosine receptor antagonists are of interest as potential therapeutics for Parkinson’s disease due to interactions between the A_2A receptor and the dopamine D_2 receptor. Adenosine A_2A receptor antagonists have also been studied recently as cancer immunotherapeutics given the specific role of this receptor in the tumor microenvironment.[88, 89] Yuan and Jones recently summarized important aspects of the discovery, development, chemical syntheses, and pharmacological evaluations of next-generation adenosine A_2A receptor antagonists.[90]

SCH-58261 (50, Figure 23) is a potent and selective adenosine receptor A_2A antagonist with more than 50-fold selectivity for A_2A over other adenosine receptors.[91] It has been used as a tool compound by Beavis, Darcy, and colleagues (Peter MacCallum Cancer Centre, Melbourne, Australia) to demonstrate that blockade of the adenosine A_2A receptor increases the efficacy of anti-PD-1 treatment through enhanced antitumor T cell...
Dual blockade of PD-1 and adenosine A<sub>1</sub> receptors significantly enhanced the expression of IFN<sub>γ</sub> and granzyme B by tumor-infiltrating CD8<sup>+</sup> T cells, and as a consequence increased inhibition of tumor growth of CD73-positive tumors and survival of mice. These studies indicate that CD73 expression is a potential biomarker for the efficacy of anti-PD-1 mAb therapy in cancer patients, and that the efficacy of anti-PD-1 mAb can be enhanced by adenosine receptor A<sub>1</sub> antagonists.

In further experiments, they used as an additional tool compound SYN115 (51, tozadenant, hA<sub>2A</sub> K<sub>i</sub> = 5.0 nM, hA<sub>18</sub> K<sub>i</sub> = 700 nM), a drug that has already undergone phase IIb testing for Parkinson’s disease. The authors reported that SYN115 has no single-agent activity in established AT-3ova<sup>TM</sup> CD73-positive tumors, but significantly enhances the antitumor efficacy of anti-PD-1 antibody to a similar extent as SCH-58261 (50).

In another study, Morello and co-workers (University of Salerno, Italy) showed that the adenosine A<sub>2A</sub> receptor antagonist ZM241365, in combination with anti-CTLA4 mAb, inhibits tumor growth and enhances antitumor immune responses in a B16F10 mouse melanoma model.<sup>[93]</sup>

Darcy and co-workers also reported that the blockade of A<sub>2A</sub> receptors potently suppresses the metastasis of CD73-positive tumors.<sup>[94]</sup> Treatment of mice with the selective adenosine A<sub>2A</sub> receptor antagonist SCH-58261 (50, Figure 23) or the adenosine A<sub>18</sub> receptor antagonist PSB1115 (52, hA<sub>18</sub> K<sub>i</sub> = 53 nM, Figure 24) decreases tumor metastasis. Therefore, it seems that both adenosine A<sub>2A</sub> and A<sub>18</sub> receptors play important roles in enhancing CD73-positive tumor cell metastasis.

A structurally related compound is PSB603 (53, Figure 24), which is a potent human A<sub>18</sub> receptor antagonist<sup>[95]</sup> with a K<sub>i</sub> value of 3.6 nM. Kojima and co-workers (Tokyo University of Science) studied the effect of PSB603 on tumor growth in B16 melanoma-bearing C57BL/6 mice.<sup>[96]</sup> Treatment with PSB603 significantly suppressed the increase in tumor volume. Furthermore, an increase in the Treg population in PSB603-treated mice was suppressed, whereas the populations of CD4 and CD8 T cells were higher, and splenic lymphocyte-mediated cytotoxicity toward B16 melanoma was significantly increased. The authors could demonstrate that PSB603 has a beneficial effect in a pulmonary metastasis model in mice intravenously injected with B16 melanoma cells. Metastasis was suppressed in the PSB603-treated mice. Overall, they could demonstrate that the A<sub>18</sub> receptor antagonist PSB603 enhances antitumor immunity by inhibiting differentiation to Tregs, resulting in a delay of tumor growth and a suppression of metastasis.

The application of adenosine A<sub>1</sub> receptor antagonists in cancer immunotherapies is still in its relatively early phase, but is a rapidly growing and promising field. A broader understanding of the specific processes and the systems biology of the tumor microenvironment should be possible with broader application of selective chemical probes and as a consequence their further development into novel treatment options for cancer immunotherapy.

**STING Activators**

Cyclic dinucleotides (CDNs) are ubiquitous small-molecule second messengers produced by bacteria and immune cells. These compounds have been described to activate innate immunity by binding directly to the endoplasmic reticulum (ER)-resident receptor STING (stimulator of interferon genes), thereby activating a signaling pathway that induces the expression of a variety of interferons, cytokines, and T cell recruitment factors.<sup>[97]</sup> The STING signaling pathway plays a major role as a Toll-like receptor (TLR)-independent mediator of host innate defense in response to cytosolic nucleic acids, either through direct binding of CDNs secreted by bacteria, or through binding of a CDN produced by a host cell receptor in response to binding of cytosolic double-stranded DNA.

The structures of some STING-activating ligands are shown in Figure 25. Cyclic-di-GMP 54 is synthesized by bacteria, and cyclic-GMP-AMP (cGAMP) 55 is produced by cellular cyclic GMP-AMP synthase (cGAS), a host cell nucleotidyl transferase that directly binds double-stranded DNA, and in response synthesizes this second messenger. cGAMP activates the STING pathway and induces IFNβ expression.<sup>[90]</sup>

A much less complex small molecule structurally unrelated to CDNs is 5,6-dimethylxanthenone-4-acetic acid (DMXAA, 56, Figure 25). Interestingly, this molecule is capable of stimulating STING signaling in mice, and has been shown to induce an innate immune-mediated antitumor response in mice.<sup>[100]</sup> Unfortunately 56 binds to the human STING receptor without activation, and it failed in combination with chemotherapy in a phase III clinical trial in patients with NSCLC.<sup>[101]</sup>
Toll-Like Receptors

Toll-like receptors (TLRs) play a key role in recognizing pathogen-associated molecular patterns, but are also involved in the recognition of damage-associated molecular patterns. Therefore TLRs control a broad range of biological processes including inflammatory and immune responses during carcinogenesis. The TLR superfamily contains more than ten different members (TLR 1–13 in humans). TLRs are type 1 transmembrane proteins and are localized in the cytoplasm, with the exception of TLR3, TLR7, TLR8, and TLR9, which are localized in the endosomal compartment. The strong expression of various TLRs by antigen-presenting cells and their ability to induce antitumor mediators such as type I interferon triggered efforts to use TLR agonists in tumor therapy in order to stimulate the immune response toward antitumor responses.

Most antitumor clinical trials of TLR agonists have tested the agonists either as vaccine adjuvants or as a monotherapy. The clear focus of these trials was on agonists of endosomal TLRs (TLR3, 7, 8, and 9). Small-molecule heterocyclic structures such as the imidazoquinolines are recognized as agonists by TLR7 and TLR8, whereas for TLR3 and TLR9, only oligonucleotide agonists have been discovered thus far. Antitumor activity of TLR7 and TLR8 agonists is achieved via activation of dendritic cells and natural killer cells to directly eliminate tumor cells and by the suppression of Tregs.

A variety of synthetic and biosynthetic agonists for TLR activation have been described for use both as vaccine adjuvants and for cancer immunotherapy. TLR agonists range from small molecules (e.g., imiquimod, resiquimod, and S-27609) to large and complex bio-macromolecules such as lipopolysaccharides, nucleic acids, and lipopeptides. Imiquimod (Aldara, Graceway Pharmaceuticals) belongs to the imidazoquinoline family and is a small-molecule agonist of the imidazoquinoline family and is a small-molecule agonist.
of TLR7 and TLR8. It stimulates the secretion of various cytokines and chemokines and activates macrophages. Imiquimod has been approved as a topical treatment for actinic keratosis, external genital warts, and basal-cell carcinomas.\(^\text{[119]}\)

Further small-molecule agonists of TLR7 (852A, 62)\(^\text{[120]}\) and TLR8 (VTX-2337, motolimod, 63)\(^\text{[121]}\) have been reported as suitable for systemic administration and have been tested as single agents in both solid and hematological malignancies (Figure 28).

David and colleagues (University of Kansas and University of Tokyo) recently reported a structure-based design approach toward human TLR8-specific agonists with enhanced potency.\(^\text{[122]}\) Crystal structures of the ectodomain of hTLR8 co-crystallized with two regioisomers of a dual TLR7/8-agonistic N1-substituted imidazoquinoline 64 showed small differences in their interactions in the binding site of hTLR8 (Figure 29). Using this structural insight, the authors decorated the quinoline core with alkylamino groups at various positions. These experiments resulted in the discovery of a novel pure TLR8 agonist 65 as a chemical tool compound that was found to be ~20-fold more potent (9 nm) than the parent quinoline compound and demonstrated strong adjuvant activity in a rabbit model of immunization. Oligonucleotide TLR9 agonists such as IMO-2055, CPG7909, and MGN1703 induce type I IFN secretion in dendritic cells and promote the response of cytotoxic dendritic cells, natural killer cells, and T cells, thereby enabling tumor-specific immune responses and the reversal of immune suppression.\(^\text{[123]}\) Overall agonists of TLRs 3, 4, 7, 8, and 9 have been described as important potential immunotherapeutics and were included several years ago in the US National Cancer Institute’s list of therapeutics with the highest potential to treat cancer.\(^\text{[124]}\) Therefore, the future development of small-molecule tool compounds for other TLRs beyond TLR7/8 might be of significant interest and especially rewarding to elucidate the full potential of this target class in cancer immunotherapy.

One major concern for the use of any TLR agonist is that systemic TLR activation could trigger severe side effects with toxic shock caused by cytokine syndrome or cytokine storms, and this has contributed to their limited clinical use so far. Recent research efforts have therefore focused on decreasing and eliminating this systemic toxicity.

The TLR7 agonist resiquimod 60 was tested in combination with radiation therapy, and long-lasting tumor remission in a lymphoma mouse model was observed.\(^\text{[125]}\) No signs of cardiotoxicity were reported, and a tumor-specific memory immune response was generated by the treatment, ensuring that the mice achieved long-lasting protection against tumor reemergence.

Another strategy to overcome systemic toxicity is a tumor-specific targeted delivery of TLR7 agonists with a monoclonal antibody. Edwards and co-workers (University of Reading) recently demonstrated that a successful conjugation of a small-molecule TLR7 agonist to an antitumor monoclonal antibody (the anti-hCD20 rituximab) is feasible without compromising antigen specificity.\(^\text{[126]}\) The TLR7 agonist UC-1V150 was conjugated to rituximab using two conjugation methods. Both conjugation methods produced rituximab–UC-1V150 conjugates 66 (Figure 30) with UC-1V150/rituximab ratios ranging from 1:1 to 3:1. The rituximab–UC-1V150 conjugates showed the expected pro-inflammatory activity in vitro (EC\(_{50}\) = 28–53 nm), with enhanced activity relative to unconjugated UC-1V150 (EC\(_{50}\) = 547 nm). Antigen binding and specificity of the rituximab–UC-1V150 conjugates was unchanged. After incubation with human blood leukocytes, all rituximab–UC-1V150 conjugates bound strongly only to CD20-expressing B cells. No unspecific binding to CD20-negative cells was observed.

**TGFβ and ALK5**

Transforming growth factor β (TGFβ) has been described as one of the most potent immunosuppressive cytokines in the tumor microenvironment.\(^\text{[127]}\) It binds to the TGFβ receptor type 2 and signals via anaplastic lymphoma kinase 5 (ALK5)-mediated phosphorylation of SMAD2/SMAD3.\(^\text{[128]}\) Despite the already longer-lasting interest in this pathway for cancer therapy,\(^\text{[129]}\) development of small-molecule drug candidates and progress toward clinical development\(^\text{[130]}\) has been rather slow, which might be related to the fact that TGFβ can both positively and negatively regulate the immune response. Another critical issue has been to achieve selectivity for inhibitors both within the ALK family (six members) and against other kinases.
that do not belong to this class. There were concerns about the myocardial toxicity of early ALK5 inhibitors which seemed to be due to ALK5 inhibition.\textsuperscript{131} In the meantime the next generation of ALK5 inhibitors has recently reached clinical development. It was reported that the ALK5 inhibitor LY-2157299 (67, galunisertib, Figure 31)\textsuperscript{132} has an improved preclinical safety profile in a number of solid tumors, and clinical trials against pancreatic and other cancer types have been initiated.\textsuperscript{132}

Another potent ALK5 inhibitor EW-7197 (68, Figure 31) demonstrated successfully, in a murine melanoma model, that the inhibition of tumor growth can be achieved by activation of cytotoxic T lymphocytes.\textsuperscript{134} EW-7197 shows an improved safety profile similar to that of LY-2157299 and has also progressed into clinical studies as an immune activator.\textsuperscript{135}

**Novel Trends and Strategies for Small Molecules in Cancer Immunotherapy**

Given the recent successes both in clinical cancer immunotherapy approaches with biologic treatment modalities and in preclinical studies with small molecules in various established targets, it is not surprising that significant efforts are currently underway to rapidly broaden the target space for small-molecule approaches in cancer immunotherapy. The great opportunities for small molecules in immuno-oncology, including an overview on immuno-oncology targets that may be amendable to small-molecule medicines, have been summarized in an excellent review by Hoos and colleagues (GlaxoSmithKline).\textsuperscript{136} The authors focus on pathways and mechanisms that could be best or exclusively targeted by small molecules, such as modulation of the immune response, trafficking to the tumor microenvironment, and cellular infiltration. Novel small-molecule approaches for these mechanisms might have the potential to extend the scope of overall immuno-oncology treatment options and further strengthen the combination potential with tumor-targeting agents and biologics in cancer immunotherapy.

One of these novel mechanisms for cancer immunotherapy has been described in a recent patent application by researchers from Genentech and Constellation Pharmaceuticals, in which the use of CBP/EP300 bromodomain inhibitors for cancer immunotherapy was claimed.\textsuperscript{137} According to the authors, CBP/EP300 bromodomains play important roles in Tregs and CD8\textsuperscript{+} T cells. CBP/EP300 bromodomains control the differentiation of Tregs and the expression of critical genes that control important biological functions in these cells. Furthermore, inhibition of CBP/EP300 bromodomains results in an impairment of the suppressive function of Tregs. In CD8\textsuperscript{+} cells CBP/EP300 bromodomains control some genes that are important in the control of exhaustion. Therefore, inhibition of CBP/EP300 bromodomains results in both suppression of Treg function and reversal of CD8\textsuperscript{+} T cell exhaustion. Both effects might play a beneficial role in cancer immunotherapy approaches for the treatment of human cancers. The tool compounds (IC\textsubscript{50} potency range between 0.27 \textmu M and > 20 \textmu M) used in this study were taken from the subset shown in Figure 32 (examples 69–76).

The recent successful clinical applications of antibodies and biologics for cancer therapy have also triggered efforts to prepare small molecules with the targeting and effector functions of antibodies to combine the best of both worlds. Spiegel and co-workers (Yale University and Bristol-Myers Squibb) designed and prepared a novel class of molecules of intermediate size (~7000 Da), which possess both the targeting and effector functions of antibodies.\textsuperscript{138} These compounds, termed synthetic antibody mimics targeting prostate cancer (SyAM-Ps), can bind both to prostate-specific membrane antigen and Fc \gamma receptor I, which is a cell-surface receptor found on immune cells responsible for initiating pro-inflammatory responses. Fc \gamma receptor I activates immune cells to initiate phagocytosis. SyAM-Ps (e.g., 77, SyAM-P3, Figure 33) induce the formation of three-component complexes between effector and target cells. By this simultaneous binding and activation of Fc receptors, they trigger highly selective cancer cell phagocytosis. Unlike many conventional small molecules, SyAM constructs require neither cell permeability nor the ability to interfere with protein–ligand interactions in order to recruit immune cells and function effectively as cytotoxic agents. These synthetic antibodies are expected to exhibit high selectivity for a range of cancer-relevant targets in contrast to many traditional chemotherapeutics or toxin conjugates, which often exhibit “off-target” toxicity due to either nonspecific uptake of lethal toxins or low levels of target antigen expression. The convergent synthesis of these synthetic antibodies should enable rapid modification and adjustment against a wide variety of disease-associated cells, viruses, or proteins. These novel molecules could provide interesting opportunities for the development of next-genera- tion customizable immunotherapeutics.

Figure 30. TLR7 agonist UC-1V150 conjugated to rituximab (University of Reading).

Figure 31. ALKS inhibitors.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure30.png}
\caption{TLR7 agonist UC-1V150 conjugated to rituximab (University of Reading).}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure31.png}
\caption{ALK5 inhibitors.}
\end{figure}
Cell-based therapies have emerged as a novel treatment option for cancer and autoimmune diseases. T cells engineered with synthetic receptors (chimeric antigen receptors (CARs)) have demonstrated efficacy in eliminating several forms of B cell cancers resistant to chemotherapy. These CAR T cells can recognize antigens on the surface of tumor cells and as a consequence start to eliminate them. Creating therapeutic CAR T cells requires extracting cells from patients, genetically modifying them and then re-infusing the engineered cells. This approach has started to deliver some initial excellent results from clinical studies for rare cancers with particularly bad prognoses, extending patients' lives and delivering complete remissions. Unfortunately, CAR T cells can also trigger severe toxicity, including life-threatening inflammatory side effects due to excessive immune response. This potential risk which stems from a lack of precise control over the activity of the therapeutic cells once they are infused into patients is a key challenge to the routine administration of such cell-based therapies. It
would therefore be highly desirable to control the location, duration, and intensity of the therapeutic activities of engineered cells by an exogenously added specific regulator. Onuffer, Lim, and colleague (University of California, San Francisco) have developed “ON-switch” CARs that enable small-molecule control over T cell therapeutic functions while still retaining antigen specificity. In these specific receptors the antigen binding and intracellular signaling components assemble only in the presence of a heterodimerizing small molecule. As a prototype, the authors used a set of structurally well-defined heterodimerizing components. The FK506 binding protein (FKBP) domain and the T2089L mutant of FKBP-rapamycin binding domain (FRB*) heterodimerize in the presence of a rapamycin analogue AP21967 (78, Figure 34). Depending on the added amount of small molecule, the ON-switch CAR T cells exhibited titratable therapeutic activity, ranging from undetectable to as strong as that of conventional CAR T cells. This work illustrates that even in complex cellular systems like engineered CAR T cells, small molecules can play an important role as chemical tools and might result in overall safer and better controlled therapeutics for antitumor therapy.

Summary and Outlook

In the last few years exciting progress in the development of immunotherapy for the treatment of cancer patients has been achieved. Small molecules have just recently begun to grow into another important treatment modality in this field, in addition to antibodies, engineered cells, and vaccines. This opens new opportunities for future combination therapies, and initial clinical studies have already been started. Due to the great clinical success that has been observed with antibodies for checkpoint inhibition and with gene-modified T cells, combinations of small-molecule drug candidates with these treatment options is a particularly attractive opportunity to extend their scope and efficacy. No less exciting are the possibilities for drug discovery toward novel intracellular targets of the innate immune system which are accessible to small-molecule modulators and will further extend the options to reverse immune tolerance in the treatment of cancer patients. Finally, small-molecule modulators even for checkpoint targets in immunoncology might be of high interest if the advantages of small molecules can be combined with the high potency and selectivity of biological entities against protein–protein interaction targets. Overall small-molecule drug discovery in cancer immunotherapy has rapidly evolved into an important research area for medicinal chemists and will probably provide many more exciting opportunities in the future.

Keywords: antitumor agents · cancer immunotherapy · immune system modulators · small molecules

References
