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ONC201 and imipridones: Anti-cancer compounds with clinical efficacy

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Abstract

ONC201 was originally discovered as TNF-Related Apoptosis Inducing Ligand (TRAIL)-inducing compound TIC10. ONC201 appears to act as a selective antagonist of the G protein coupled receptor (GPCR) dopamine receptor D2 (DRD2), and as an allosteric agonist of mitochondrial protease caseinolytic protease P (ClpP). Downstream of target engagement, ONC201 activates the ATF4/CHOP-mediated integrated stress response leading to TRAIL/Death Receptor 5 (DR5) activation, inhibits oxidative phosphorylation via c-myc, and inactivates Akt/ERK signaling in tumor cells. This typically results in DR5/TRAIL-mediated apoptosis of tumor cells; however, DR5/TRAIL-independent apoptosis, cell cycle arrest, or antiproliferative effects also occur. The effects of ONC201 extend beyond bulk tumor cells to include cancer stem cells, cancer associated fibroblasts and immune cells within the tumor microenvironment that can contribute to its efficacy. ONC201 is orally administered, crosses the intact blood brain barrier, and is under evaluation in clinical trials in patients with advanced solid tumors and hematological malignancies. ONC201 has single agent clinical activity in tumor types that are enriched for DRD2 and/or ClpP expression including specific subtypes of high-grade glioma, endometrial cancer, prostate cancer, mantle cell lymphoma, and adrenal tumors. Synergy with radiation, chemotherapy, targeted therapy and immune-checkpoint agents has been identified in preclinical models and is being evaluated in clinical trials. Structure-activity relationships based on the core pharmacophore of ONC201, termed the imipridone scaffold, revealed novel potent compounds that are being developed. Imipridones represent a novel approach to therapeutically target previously undruggable GPCRs, ClpP, and innate immune pathways in oncology.

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Abbreviations: 5-FU, 5-fluorouracil, A2A, Adenosine 2A receptor, ALCL, anaplastic large cell lymphoma, ALL, acute lymphoblastic leukemia, AML, acute myeloid leukemia, AMPK, AMP kinase, ATRT, atypical teratoid rhabdoid tumor, AUC, area under the curve, BRD, bromodomain, cAMP, cyclic AMP, cCK18, caspase-cleaved cytokeratin 18, CK18, cytokeratin 18, CLL, chronic lymphocytic leukemia, ClpP, caseinolytic protease P, ClpX, caseinolytic mitochondrial matrix peptidase chaperone subunit X, CML, chronic myelogenous leukemia, CRC, colorectal cancer, CSC, cancer stem cell, CTCL, cutaneous T-cell lymphoma, DIPG, diffuse intrinsic pontine glioma, DLBCL, diffuse large B-cell lymphoma, DNA-PKcs, DNA-activated protein kinase catalytic subunit, DR5, death receptor 5, DRD1, dopamine receptor D1, DRD2, dopamine receptor D2, DRD3, dopamine receptor D3, DRD4, dopamine receptor D4, DRD5, dopamine receptor D5, DSRCT, desmoplastic small round cell tumor, EC, endometrial cancer, EGFR, epidermal growth factor receptor, FLAIR, fluid-attenuated inversion recovery, GBM, glioblastoma multiforme, GDSC, Genomics of Drug Sensitivity in Cancer, GIRK, G protein-coupled inwardly rectifying potassium channel, GnRH, gonadotropin-releasing hormone receptor, GPCR, G protein coupled receptor, HCC, hepatocellular carcinoma, IHC, immunohistochemistry, HGG, high-grade glioma, ISR, integrated stress response, MCL, mantle cell lymphoma, MM, multiple myeloma, MTD, maximum tolerated dose, NHL, non-Hodgkin's lymphoma, NK, natural killer, NOAEL, no-observed-adverse-event-level, NSCLC, non-small cell lung cancer, OS, overall survival, OXPHOS, oxidative phosphorylation, PC-PG, pheochromocytoma-paraganglioma, PD, pharmacodynamic, PDX, patient-derived xenograft, PFS, progression-free survival, PK, pharmacokinetic, PLC, phospholipase C, RANO, Response Assessment in Neuro-Oncology, RECIST, Response Evaluation Criteria in Solid Tumors, rhTRAIL, recombinant human TRAIL, RP2D, recommended phase II dose, SAR, structure–activity relationship, SCLC, small-cell lung cancer, TIC10, TRAIL-inducing compound 10, TMZ, temozolomide, TNBC, triple-negative breast cancer, TRAIL, TNF-associated apoptosis-inducing ligand, TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling, WHO, World Health Organization

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GPCRs in oncology

GPCRs represent the largest class of cell surface receptors (>800 members) in humans [1]. These receptors regulate a variety of physiological processes by controlling diverse cell signaling pathways through a wide array of signal transduction mechanisms [1]. GPCRs regulate signaling by changing their conformation following binding to a range of extracellular ligands, which alters coupling to G proteins and other intracellular proteins. Consequently, 30–50% of marketed drugs target GPCRs that span a multitude of disease areas such as high blood pressure, diabetes, motion sickness, acid reflux, depression, migraine, asthma and allergy [2].

GPCRs regulate signaling pathways responsible for proliferation, apoptosis, metastasis, self-renewal, angiogenesis and immune regulation [3]. GPCRs and G-proteins are less frequently mutated in cancers compared

to many other oncology drug targets; however, their expression is often dysregulated in a broad range of cancers depending on their functions as tumor suppressors or oncogenes [4]. Despite being rational drug targets, GPCRs are underexploited in oncology. Drug discovery efforts for GPCR ligands have traditionally been challenging due to multiple factors. These include the paucity of structural information associated with the difficulty of crystallizing these transmembrane receptors, the high degree of homology within GPCR ligand binding sites that underpins pleiotropy among competitive ligands, overlapping biological functions as well as signaling pathways for GPCRs, and common inability to generate functional and specific antibodies due to their minimal extracellular domains. As a result, a large number (~80%) of GPCRs are currently not targeted by any approved therapies. Despite being traditionally ignored in oncology due to their low frequency of mutations, GPCRs are increasingly recognized as oncology targets as evidenced by the clinical translation of immune checkpoint targeting of adenosine 2A receptor (A2A) and gonadotropin-releasing hormone receptor (GnRH) [1,5,6].

ClpP in oncology

Caseinolytic protease P (ClpP) is a serine protease located in the mitochondrial matrix that regulates several mitochondrial functions [7–10]. ClpP forms a large tetradecameric ATP-dependent protease complex involving two homo-heptamer rings, which not only interface with each other, but also each associate a ring of ClpX proteins that function as a regulatory cap [7]. This complex regulates oxidative phosphorylation (OXPHOS) by controlling the degradation of the respiratory chain complex subunits and can trigger the mitochondrial unfolded protein response [7–10]. The ClpP protease complex is conserved in bacteria and is also a target of interest for antibiotics [8]. ClpP is structurally similar to the cytoplasmic/nuclear proteasome complex in human cells, a well-established therapeutic target used in the treatment of certain hematologic malignancies [7,11].

In acute myeloid leukemia (AML), mitochondrial ClpP is overexpressed in 45% of primary samples, is equally expressed in stem cell and bulk tumor cell populations, and its overexpression is evident across the spectrum of cytogenetic and molecular mutations that are common in this disease [9]. Chemical or genetic modulation of this protease leads to impaired OXPHOS and selectively kills AML cells *in vitro* and *in vivo* [9]. Similar treatment of normal cells does not induce cell death despite degradation of ClpP client proteins, suggesting that the therapeutic window of this approach may be due in part to an apparently decreased dependency on OXPHOS. Therapeutic targeting of ClpP represents a novel approach for oncology that has not been previously explored in the clinic [7,9,10].

ONC201 and the imipridone scaffold

ONC201 was originally discovered as a first-in-class TNF-Related Apoptosis Inducing Ligand (TRAIL)-inducing compound (TIC10), later discovered to activate the integrated stress response through ATF4/CHOP, ultimately causing cell death through upregulation of TRAIL receptor DR5. ONC201 appears to be a selective competitive and non-competitive antagonist of dopamine receptor D2 (DRD2), a GPCR that is overexpressed in a broad range of malignancies, and an allosteric agonist of ClpP. With regard to dopamine receptors, DRD2 antagonists that are more potent than ONC201 are not more efficacious anti-tumor agents. Dopamine receptor D3 (DRD3) is also targeted at equipotent concentrations, however this receptor is rarely expressed in oncology despite its functional redundancy with DRD2. Downstream of DRD2 and ClpP engagement, ONC201 activates the ATF4/CHOP-mediated integrated stress response (ISR) pathway and inactivates Akt/ERK signaling, which ultimately results in DR5/TRAIL-mediated apoptosis of tumor cells. Additionally, ClpP engagement inhibits OXPHOS, leading to apoptosis. Imipridones are a class of anti-cancer compounds that share a unique tri-heterocyclic core chemical structure but differ in their peripheral moieties. Additional imipridones were chemically synthesized based on the structure of ONC201 that has shown broad-spectrum efficacy across solid tumors and hematologic malignancies in preclinical models, as well as exceptional safety, pharmacokinetic (PK), pharmacodynamic (PD) and efficacy profiles in Phase I/II trials.

ONC201 mechanism of action

DRD2 antagonism

While initially ONC201 was discovered based on its downstream activation of the TRAIL pathway, efforts focused on identification of its molecular targets. Subsequent efforts led to identification of a subclass of dopamine receptors as molecular targets for ONC201 antagonism. Neurotransmitters, such as dopamine and serotonin, have been reported to play a role in tumor initiation and growth for some forms of cancer [12]. In line with the oncogenic role of the dopamine pathway, epidemiological studies have reported that Parkinson's disease patients have a lower cancer incidence, and that the use of antipsychotic DRD2 antagonists is associated with lower levels of cancer in schizophrenia patients [13,14]. Overexpression of DRD2 is apparent in a number of malignancies (Fig. 1), its expression can increase with disease stage, and its antagonism results in anti-cancer effects in several tumor types [15–18].

Cancer cells can source dopamine by autocrine, as well as paracrine, mechanisms to drive tumor growth [18]. Consistent with an autocrine mechanism, plasma dopamine levels have also been reported to be elevated in some cancer patients [21]. Consistent with a paracrine mechanism, brain tumors located in midline regions where high levels of dopamine is produced physiologically are highly susceptible to DRD2 antagonism [16,22].

The dopamine receptor family has five members, which are grouped into D2-like receptors (DRD2, DRD3, and DRD4), which couple to Gαi and inhibit adenylate cyclase activity, and D1-like receptors (DRD1 and DRD5) that couple to Gαs and activate adenylate cyclase, opposing D2-like receptor signaling [23]. Adenylate cyclase is a key enzyme involved in cyclic AMP (cAMP) synthesis that affects intracellular signaling pathways [23]. Additionally, D2-like receptor signaling through Gβγ can activate PLC signaling, thereby increasing cytoplasmic calcium and other downstream signaling events, and regulate G protein-coupled inwardly rectifying potassium channels (GIRKs) and L- and N-type calcium channels [23]. Lastly, intracellular signaling can occur downstream of D2-like receptor signaling in a G protein-independent manner via β-arrestin, which perform functions related to receptor desensitization and internalization [24].

A machine learning-based drug-target identification algorithm predicted that ONC201 may antagonize DRD2 and DRD3 [25]. β-Arrestin recruitment, Ca^{+2} flux, and cAMP report assays supported the prediction that ONC201 may selectively antagonize DRD2/3 in human cells [25]. By contrast to antipsychotics that competitively antagonize DRD2, ONC201 does not antagonize other dopamine receptors or other GPCRs and is a more potent anti-tumor agent as compared to stronger DRD2 antagonists. Moreover, ONC201 exhibits a wide therapeutic index as compared to antipsychotics in tumor versus normal cell viability assays [26]. Schild analyses and radioligand competition assays revealed competitive and non-competitive DRD2 antagonism by ONC201 at concentrations that coincide with its anti-cancer activity. Molecular docking and mutagenesis experiments revealed ONC201 may antagonize DRD2 through a unique binding pocket that involves orthosteric and allosteric

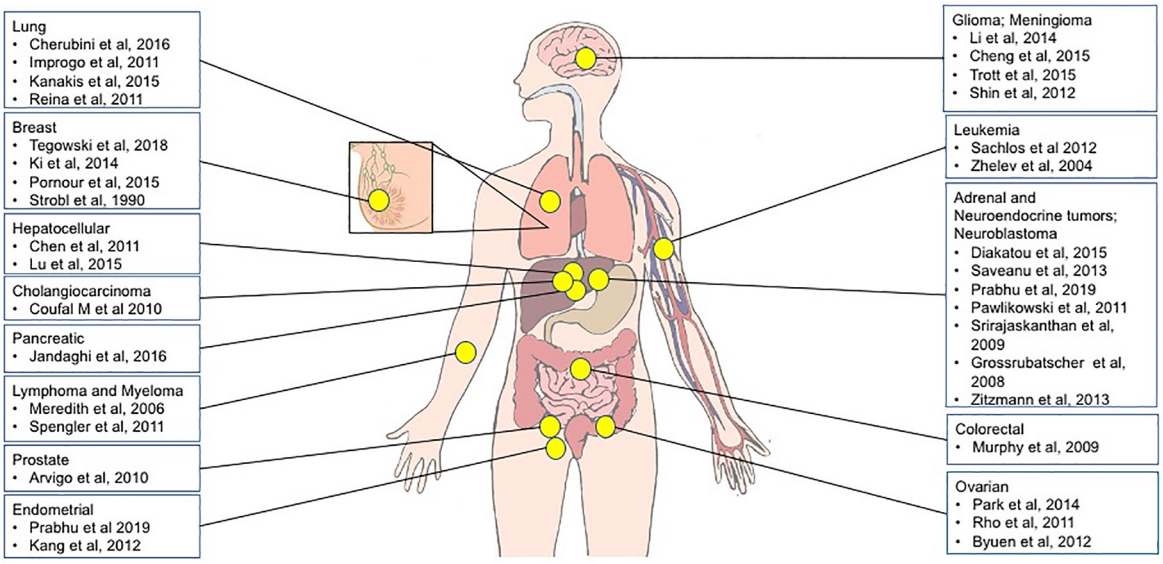

Fig. 1. DRD2 Overexpression in Human Cancer. The indicated publications for each tumor type were identified through a PubMed search as of July 2020, as well as recent reviews of DRD2 in oncology [19,20].

residues, some of which are not conserved in other GPCRs and are not required for other DRD2 antagonists [27]. There is no crystal structure of ONC201 bound to DRD2. DRD2 knockdown appeared to phenocopy downstream signaling by ONC201 involving ISR activation and inactivation of Ras signaling in glioblastoma cells, while CRISPR-mediated knockout of either DRD2 or DRD3 did not impact ONC201 sensitivity in breast cancer cells. ONC201-mediated cancer cell death was modulated by transient changes in DRD2 expression in isogenic studies, suggesting that DRD2 expression may influence ONC201 anti-cancer efficacy [26,28]. Thus, ONC201 anti-cancer efficacy and safety is driven, at least in part, in some tumor types, by antagonism of DRD2 that may be relevant in dopamine pathway-dysregulated tumors. The connections between dopamine receptor antagonism and downstream anti-tumor effects of ONC201 remain under investigation, as do the connections, if any, between dopamine receptors and the second direct molecular target of ONC201, ClpP, described below. Whether and how these molecular targets are linked mechanistically to the integrated stress response leading to the apoptotic and growth arrest pathways remains under investigation.

While there are multiple biomarkers that have emerged for ONC201 efficacy, an interesting observation was that acquired resistance to ONC201 appeared to be associated with a *de novo* mutation in the DRD5 gene [26]. Overexpression of the mutant or wild-type DRD5 could induce partial resistance in parental ONC201-sensitive cells. Innately low DRD5 expression correlated with lower IC50s in large cancer cell line panels, yielding a DRD2+DRD5− biomarker signature to predict tumor cell sensitivity to ONC201 *in vitro*. In some unpublished studies with smaller numbers of solid tumor cell lines presented at national meetings, there has not been a correlation between the basal mRNA expression of dopamine receptors and ONC201 sensitivity. Additionally, there has not appeared to be a correlation between epigenetically altered expression of the dopamine receptors under experimental conditions where ONC201 sensitivity was enhanced. This remains an area of active investigation in the field. Evaluation of the biomarker signature across tumor types and patient-derived tissue microarrays revealed enrichment of tumor types associated with ONC201 clinical activity including high-grade glioma, endometrial cancer and adrenal tumors. However, these biomarker relationships continue to be studied in ongoing clinical trials.

ClpP activation

In addition to DRD2/3 antagonism, direct activation of ClpP by ONC201 has recently been uncovered by chemical proteomics, chemical library screening, and acquired resistance approaches [7,8,10]. ONC201 interacts with human ClpP via non-covalent allosteric interactions that result in the protein complex opening specific areas of a substrate channel and altering the conformation of its active site. This ONC201-induced conformation causes hyperactivation of proteolytic activity of ClpP, leading to increased degradation of respiratory chain complex subunits. These changes cause impaired OXPHOS that results in mitochondrial dysfunction and tumor cell death. The underlying mechanisms and connections between ClpP activation and the cellular pathways of cell death remain under investigation. Inactivating mutations in ClpP, as well as loss of ClpP expression, render AML, acute lymphoblastic leukemia (ALL) and breast cancer cells partially resistant to ONC201, indicating that activation of ClpP is functionally important for its efficacy in these tumor types with a more prominent contribution demonstrated in leukemic versus breast cancer cell lines [7]. Mechanistically, ClpP activation triggers the mitochondrial unfolded protein response that can upregulate the ATF4/CHOP axis. Furthermore, disruption of mitochondrial function (e.g. OXPHOS) in tumor cells has been recently reported for ONC201, which may be explained by ClpP agonism [29,30]. Whether ClpP expression or activity ultimately is sufficient to predict ONC201 sensitivity in humans remains under investigation. There are numerous mechanisms for resistance to cell death within tumor cells, and so while expression of the ClpP target may be necessary for ONC201 sensitivity, it may not be sufficient to predict cell death in tumor cells [31].

Downstream signaling: ISR/Akt-ERK/DR5-TRAIL/mitochondria

ONC201 was discovered as a TRAIL gene inducing compound (TIC10) using a phenotypic, cell-based screen to identify compounds that upregulate TRAIL expression that would lead to TRAIL production by tumor (and normal) cells in a p53-independent manner [32–34]. This screen was designed based on earlier studies that showed p53-dependent upregulation of the TRAIL gene. The cell-based chemical library screen

used a TRAIL gene promoter lacking p53 DNA-binding sites that was linked to a luciferase reporter gene. The initial characterization of TIC10 revealed that it inhibited ERK and Akt leading to Foxo3a translocation to the nucleus and transcriptional activation of the TRAIL gene. TIC10 demonstrated anti-tumor effects in numerous preclinical *in vivo* models. Subsequent work identified ONC201/TIC10 as an activator of the integrated stress response involving ATF4/CHOP and downstream activation of TRAIL receptor DR5. Upstream of ATF4, ONC201/TIC10 appeared to signal through kinases HRI and PKR, eIF2-alpha and ATF4. Downstream of target engagement, ONC201 treatment consistently affects at least two pathways in tumor cells: activation of the ISR pathway [35,36], which is also activated by proteasome inhibitors, and Akt/ERK inactivation [33], which is also caused by EGFR, Akt and RAF/MEK/ERK inhibitors. Thus, ISR activation is an early effect of ONC201 treatment that causes upregulation of ATF4 translation and CHOP transcription in tumor cells within a few hours [35], whereas dual inactivation of Akt and ERK is a late treatment effect, and cell death is also demonstrated to be slow, taking 2–3 days [33]. These effects ultimately converge to upregulate the pro-apoptotic ligand TRAIL through activation and nuclear translocation of the transcription factor Foxo3a, as well as its receptor DR5 that may be induced by CHOP in addition to Foxo3a (Fig. 2A). Additionally, ONC201 has been shown to degrade c-myc via a mechanism involving Akt/GSK3B, which was demonstrated in glioblastoma (GBM) cell lines [29].

ONC201's effects on c-myc, ISR and Akt have also been suggested to be linked to inhibition of mitochondrial respiration, suppression of OXPHOS and glycolysis in multiple tumor types that ultimately results in cytostatic or pro-apoptotic effects [29,30]. The disruption of mitochondrial function in tumor cells may also be directly linked to ClpP activation by ONC201 [7]. The mitochondria-mediated apoptosis of tumor cells by ONC201 may involve decreased expression of the anti-apoptotic Bcl-2 family protein, Mcl-1. High innate Bcl-2 expression has been linked to resistance to ONC201-mediated apoptosis in tumor cells [36].

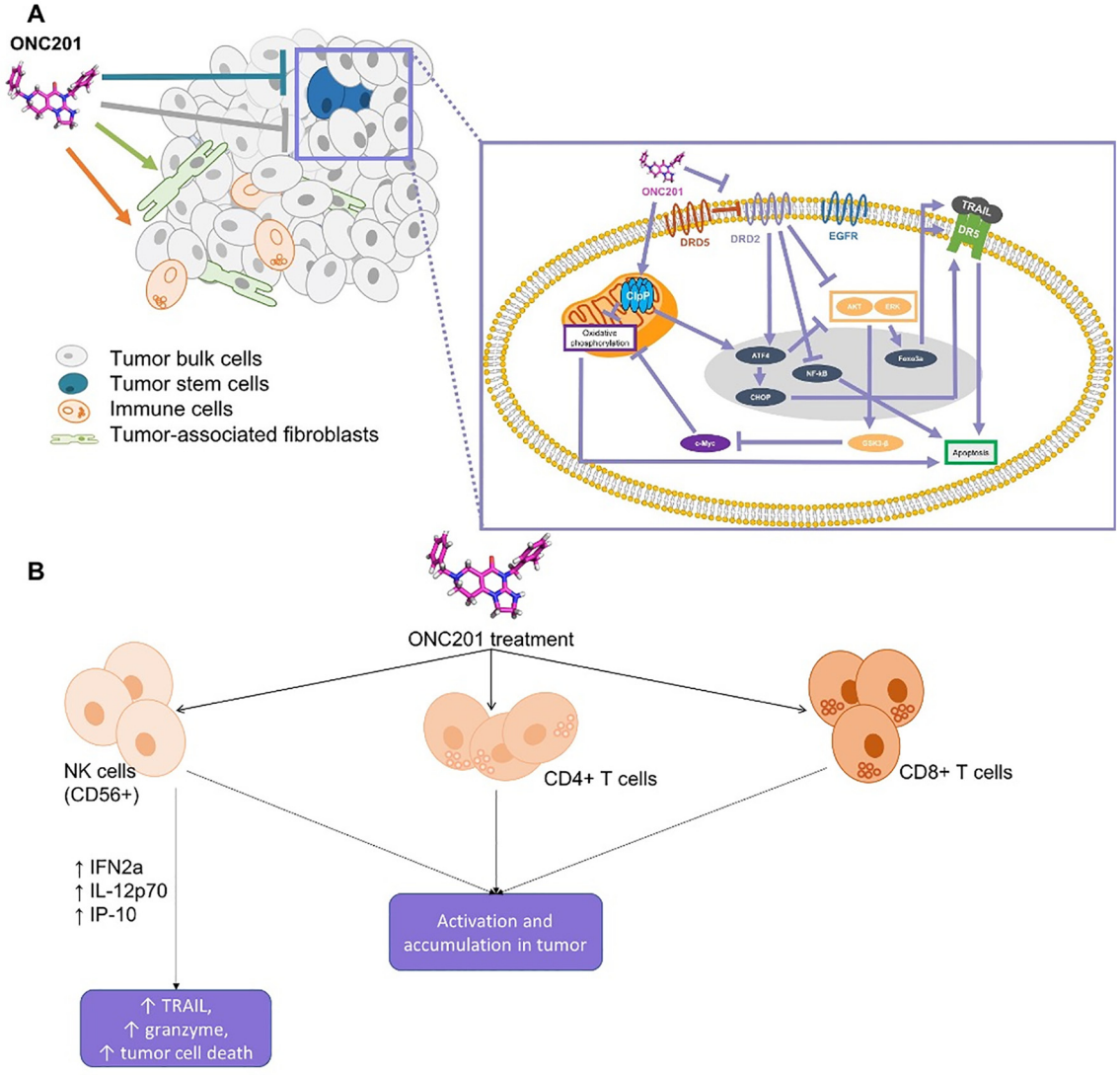

Fig. 2. ONC201 mechanism of action. (A) ONC201 affects bulk tumor cells, tumor stem cells and normal cells in the tumor microenvironment, including immune cells and fibroblasts, to elicit anti-cancer effects. DRD2 antagonism and ClpP activation by ONC201 are upstream events while downstream events include integrated stress response activation, c-myc downregulation, decreased OXPHOS, Akt/ERK inactivation, and Foxo3a activation that ultimately trigger DR5/TRAIL-mediated apoptosis. (B) ONC201 activates and increases intra-tumoral presence of NK, CD4+ and CD8+ T cells. Through an increase in IFN2α, IL-12p70 and IP-10, NK cells increase granzyme and TRAIL secretion, leading to tumor cell death [40].

Cancer stem cell sensitivity

Cancer stem cells (CSCs) are a rare subpopulation of stem-like tumor cells with the ability to repopulate all malignant lineages within the tumor from a single cell [32,37,38]. CSCs have been suspected to contribute to tumor relapse and therapy resistance in solid tumors and hematological malignancies. The efficacy of ONC201 involves targeting both bulk tumor cells and CSCs [32,37,38]. ONC201 has demonstrated effects on CSCs in colorectal cancer (CRC), prostate cancer, GBM, and AML, including in 3D sphere cultures, serial passage assays, and patient-derived models. The anti-CSC effects of ONC201 involve early changes in stem cell-related gene expression (Table 1), including modulation of stem cell pathways such as Wnt signaling and genes known to regulate self-renewal (ID1, ID2, ID3 and ALDH7A1) [38]. These effects are followed by depletion of CD133, CD44 and Aldefluor-positive CRC CSCs in an Akt/Foxo3a/DR5/TRAIL-dependent manner. Inhibition of colonosphere formation, as well as tumor growth and serial passage of CSC-initiated tumors, has been demonstrated with ONC201 in CRC [37]. In chemo-refractory AML patient samples, ONC201 induced apoptosis in leukemia stem/progenitor cells (CD45 dim+/ CD34+/ CD38-) to an extent that was equivalently observed in non-CSCs [36]. ONC201 inhibited the cellular proliferation of CSC-enriched neurosphere cultures of newly diagnosed and recurrent GBM patient samples and induced apoptosis in stem-like glioma cells [38,39]. The capability of ONC201 to be equally effective in the CSC subpopulation precludes a common mechanism of resistance and could augment its long-term therapeutic utility, especially in advanced, chemo-refractory cancers [37]. The anti-CSC efficacy of ONC201 provides a rationale for its use in neoadjuvant or adjuvant settings, as well as a PD biomarker [38].

Immune activation

ONC201 treatment stimulates intra-tumoral accumulation of activated NK and CD3+ cells (Fig. 2B) in immune-competent syngeneic mouse models [40]. NK cell depletion reduces ONC201 antitumor efficacy *in vivo*, demonstrating the relevance of immune stimulation to overall antitumor effects, along with direct tumor cell kill. The *in vivo* immune-mediated anti-tumor effect involving NK cells includes tumor cells that are relatively refractory to the pro-apoptotic effects of ONC201 in cell culture, such as Bax-null tumor cells [40]. Additionally, co-culture experiments revealed that ONC201 induces membrane-bound TRAIL on NK cells that mediate their cytotoxic effects [40]. TRAIL induction [41,42] is known to be associated with activation of NK cell cytotoxicity and sensitization of tumors cells to NK cells. Thus, ONC201-mediated immune activation provides a PD biomarker and rationale for combination with appropriate immune checkpoint modulators.

Interestingly, dopamine is a key regulator of the innate and adaptive immune system. Dopamine and dopamine receptors are secreted and expressed, respectively, by various immune cells. This includes NK and T-cells that can be targeted to modulate tumor immune response [20,43,44]. Dopamine inhibits NK cell effector function [44], and DRD2 antagonism has been shown to activate NK cells [43]. However, there has been no experimental evidence to date in any preclinical model or human tumor specimens linking either dopamine or dopamine receptors to the immune stimulatory effects of ONC201. Also, ISR activation [45–47] and PI3K/Akt inhibition [48] have been associated with activation of NK cell cytotoxicity and sensitization of tumors cells to NK cells. The relevance of these aspects of the ONC201 mechanism of action for immune activation remains to be evaluated.

Bystander effect via fibroblasts

ONC201 selectively kills cancer cells, but not normal cells, and exhibits a favorable therapeutic index *in vitro* and *in vivo*. *In vitro* co-culture studies suggest that ONC201 induces TRAIL in fibroblast cells that can contribute to tumor cell apoptosis via a bystander effect mediated by TRAIL signaling [33]. Thus, along with its direct effects on tumor cells, ONC201 targets various components of the tumor microenvironment, including CSCs, immune cells and fibroblasts to elicit its anti-cancer efficacy (Fig. 2A). It is important to note that while ONC201 can promote TRAIL production by normal cells [33], the activation of the integrated stress response appears to occur in the tumor cells [35]. Activation of TRAIL receptor DR5 in the tumor cells but not normal cells may underlie tumor selectivity and therapeutic index [49].

ONC201 preclinical efficacy and safety

Various *in vitro*, *ex vivo*, and *in vivo* experiments have demonstrated the efficacy of ONC201 across solid tumors and hematological malignancies refractory to standard of care therapies. ONC201 has shown antitumor efficacy as a single agent in over 25 *in vivo* preclinical cancer models, including subcutaneous, orthotopic and patient-derived xenografts (PDXs), and immune-competent and genetic mouse models of cancer (Fig. 3). The anti-metastatic activity of ONC201 has been documented in multiple preclinical models across tumor types, including breast, CRC and endometrial cancers [40,50,51]. However, the first demonstration was made through studies of dose intensification of ONC201 in preclinical models [40]. These findings led to an increase in the frequency of ONC201 dosing in clinical trials from every 3 weeks to every week. ONC201 inhibits cell adhesion, migration and invasion in a TRAIL-dependent manner, consistent with the literature on TRAIL and inhibition of metastasis [40,50,51]. The inhibition of metastasis was confirmed in xenograft and syngeneic models, including tail vein injection, as well as orthotopic models of metastasis [33,40,52].

Brain tumors

In GBM, DRD2 is overexpressed relative to other dopamine receptor family members and is associated with a poor prognosis [16]. ONC201 *in vitro* efficacy correlated with DRD2 mRNA expression in a small panel of GBM cell lines [26] and was independent of mutations in p53, EGFR, or IDH1/2 genes [29,33,38]. ONC201 induced TRAIL and apoptosis in various GBM cell lines and patient-derived cells in 2D and 3D cultures, including in cells resistant to the standard first-line therapies temozolomide (TMZ) and radiation, as well as glioma stem cells. A lower GI50

Table 1. Summary of ONC201-mediated anti-CSC effects. (Y = yes, N = no, ND = not determined).

Tumor type [Reference]	Patient-derived cells tested	*In Vivo* Data	CSC markers tested (flow cytometry)	CSC-related gene expression changes
GBM [38]	Y	N	ND	ABCB5, ALDH1A1, CD133, NANOG
CRC [37,38]	N	Y	Aldefluor, CD44, CD133	ID1, ID2, ID3, KLF9, TCF7L2, WNT16, ALDH1A1, ALDH7A1, ABCB5, NANOG, CD133
Prostate [38]	N	N	ND	ABCB5, ALDH1A1, ALDH7A1, CD133, NANOG, WNT16
AML [36]	Y	Y	CD45, CD34, CD38	ND

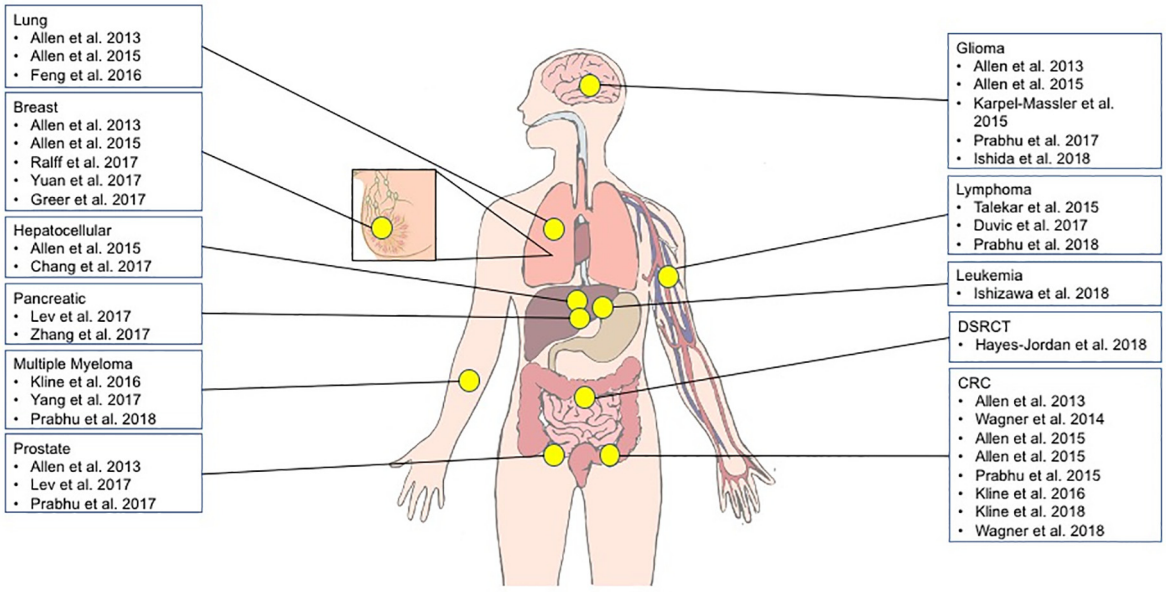

Fig. 3. Single agent ONC201 efficacy in preclinical models of solid tumors and hematological malignancies. Publications that report the single-agent efficacy of ONC201 published as of July 2020. DSRCT: desmoplastic small round cell tumor. CRC: colorectal cancer

and increased completeness of response for ONC201 was observed in gliomas with the histone H3 K27M mutation that was also associated with high DRD2 and low DRD5 expression relative to H3 wild-type or G34R/V gliomas [53]. ONC201 penetrates the intact blood–brain barrier, as shown by sustained TRAIL induction in brain tissues of mice following a single dose [33]; moreover, single agent ONC201 reduced tumor growth and improves survival in multiple high-grade glioma xenograft models. A single dose of ONC201 doubled median survival in an intracranial xenograft GBM model and induced tumor regressions detectable by bioluminescent imaging [29,33]. DRD2 is also overexpressed in medulloblastoma; ONC201 has demonstrated inhibition of cell viability in cell lines and patient-derived cells in this tumor type [26]. However, neither expression of DRD2 nor H3 K27M alone predicts clinical sensitivity or response to ONC201.

Lung cancer

ONC201 has antiproliferative, cytotoxic and pro-apoptotic efficacy in primary human lung cancer cells without imparting toxicity to normal human cells [54]. ONC201 induced TRAIL and DR5, as well as activated caspase-8 in a dose-dependent manner, suggesting extrinsic apoptosis. Furthermore, inactivation of Akt and ERK was observed, as well as nuclear translocation of Foxo3a that resulted in transcriptional induction of TRAIL and DR5. In subcutaneous xenografts of human non-small cell lung cancer (NSCLC), ONC201 decreased tumor burden and slowed tumor growth without affecting body weight [33,54]. Interestingly, previous studies have shown that DRD2 is highly expressed in human lung cancer cells and lung cancer tissues. Small cell lung cancer (SCLC) cells overexpress DRD2 and patients exhibit elevated plasma dopamine levels [21]. The role of DRD2 in the anti-cancer effects of ONC201 in lung cancer remains to be examined, and similar to other tumor types, is not expected to predict sensitivity to ONC201 on its own in the clinic.

Endometrial cancer

ONC201 inhibits endometrial cancer cell growth and proliferation in a dose-dependent manner. ONC201 inhibited adhesion, invasion and metastasis in multiple uterine serous carcinoma cell lines, as well as induced apoptosis. In addition, pre-treatment of ONC201 in uterine serous carcinoma cell lines increased their sensitivity to paclitaxel. Increased caspase-3, caspase-9, PARP, caspase-8, and DR5 expression shows that ONC201 induces both endogenous and extrinsic apoptotic pathways in uterine serous carcinoma [50]. DRD2 is overexpressed in endometrial cancer [26], however its direct role in the anti-cancer effects of ONC201 in this tumor type remains to be determined.

Pheochromocytoma/paraganglioma (PC–PG)

PC–PG cells were highly sensitive to ONC201 *in vitro*, exhibiting apoptosis and Akt/ERK inhibition [26,55]. PC–PG are dopamine-secreting neuroendocrine tumors that are associated with the adrenal gland [56]. An analysis of RNA expression in cell lines of the TCGA database showed that DRD2 expression was highest in PC–PG relative to all other tumor types [26]. These findings were also confirmed at the protein level using tissue microarrays across various solid tumor types. DRD2 was highly overexpressed in PC–PG cells relative to normal adrenal cells [26]. The specific contribution of DRD2 to the anti-cancer effects of ONC201 in PC–PG remains to be investigated.

Colorectal cancer (CRC)

ONC201 demonstrated preclinical efficacy in multiple human CRC cell lines regardless of p53 or KRAS alterations that confer resistance to standard-of-care therapies [33]. Cytotoxic and apoptotic effects occurred in a dose- and frequency-dependent manner via inactivation of Akt and ERK, as well as downstream induction of TRAIL and DR5 expression [32,33,40,49]. Furthermore, ONC201 induced TRAIL and subsequent cell death in a fresh CRC patient specimen that was not responsive to standard-of-care 5-fluorouracil (5-FU) chemotherapy [37]. *In vivo* studies using an orthotopic mouse model of p53−/− metastatic CRC have shown that ONC201 has single agent antitumor efficacy, reducing both tumor burden and spread to metastatic sites without apparent toxicity [37]. Inhibition of metastasis was also observed in both subcutaneous and intravenous xenograft models of CRC, in which only vehicle-treated mice had metastatic populations [33,40]. These effects were associated with Akt/ERK/Foxo3a/TRAIL/DR5 signaling effects [37]. Additionally, previ-

ous studies have demonstrated that *in vivo* dosing of ONC201 activated, as well as induced TRAIL secretion by natural killer (NK) cells that further inhibited metastasis [40].

As outlined above, ONC201 targeted CSCs in CRC and other solid tumors. CSC markers such as ID1-3 and ALDH7A1 were significantly downregulated and self-renewal is decreased in CRC cell lines in response to ONC201. Such effects were observed both *in vitro* and *in vivo* through the use of colonosphere formation assays and subcutaneous CRC xenografts in mice [37,38]. Resistance to ONC201 treatment in CRC can arise through the overexpression or mutation of DRD5 or hyperactivation of mTOR [26,57].

Breast cancer

In vitro studies demonstrated that ONC201 exerted pro-apoptotic and antiproliferative effects associated with induction of TRAIL, DR5 and cleaved PARP were observed following treatment [58]. ONC201 was also able to cause tumor regression in breast cancer xenografts as a single agent [33,58].

ONC201 induced antiproliferative effects in both mesenchymal- and epithelial-like triple-negative breast cancer (TNBC) cell lines [59]. Studies have shown that the efficacy of ONC201 in breast cancer, especially TNBC, can involve a number of mechanisms. As seen in other tumor types, TRAIL-dependent induction of cell death by Akt/ERK inhibition and subsequent Foxo3a activation occurs in some cell lines [59]. ONC201 can also act via TRAIL-independent mechanisms, as demonstrated by the induction of ATF4 that was deleterious in TNBC cells [10,58,59]. ATF4 can signal TRAIL pathway dependent cell death as well as TRAIL pathway independent anti-tumor effects. Interestingly, a third mechanism of cell death has been described in TNBC cell lines *in vitro* that involves disruption of mitochondrial function. This activates AMPK, reduces oxygen consumption rates and increases extracellular acidification rates in multiple breast cancer cell lines, thereby causing structural damage while also decreasing mitochondrial DNA [30]. After 72 h of incubation with ONC201, significant mitochondrial matrix lysis was observed [30]. More recent reports suggest that these effects may be linked to activation of ClpP by the compound. *In vitro* studies using ClpP knockdown cells showed that the protein was essential for activation of ISR in these cells, as its absence eliminated ONC201-induced CHOP upregulation [10]. A potential resistance mechanism for ONC201 may be increased dependence of cells on glycolysis relative to mitochondrial respiration for ATP production, as cancer cell lines with decreased mitochondrial DNA were shown to be more resistant to ONC201 treatment [30].

Prostate cancer

ONC201 had antitumor efficacy *in vitro* and inhibited growth of subcutaneous prostate cancer tumor growth via downregulation of AR-V7, AR and phospho-AR and upregulation of the ISR-related proteins ATF4, GADD34, ATF6, XBP-1S and BIP [33]. In addition, ONC201 downregulated PSA by preventing its transcription via ATF3/4 [60]. CSC pathways were also modulated upon ONC201 treatment: components of the Wnt signaling pathway and genes known to regulate self-renewal (ALDH7A1) in prostate cancer were downregulated, and tumor sphere formation of prostate cancer cells was significantly reduced [38].

Desmoplastic small round cell tumor (DSRCT)

DSRCT is a highly aggressive, pathologically-defined soft tissue sarcoma that was found to express the pro-apoptotic TRAIL receptors DR4 and DR5, which led investigators to explore ONC201 in this tumor type [61]. ONC201 exhibited dose-dependent antiproliferative effects *in vitro* via promotion of caspase activation and subsequent induction of extrinsic apoptosis. In order to assess the *in vivo* efficacy of ONC201, the first orthotopic xenograft model of DSRCT was developed, in which cells were injected in the lower abdominal peri-testicular region resulting in intra-peritoneal, liver and pancreatic metastases. Treatment with both low and high doses of ONC201 reduced tumor growth in this model and resulted in complete regressions in some animals [61].

Hepatocellular carcinoma (HCC)

ONC201 demonstrated antiproliferative efficacy in human HCC cells that was associated with Akt and ERK inactivation in a dose-dependent manner, resulting in Foxo3a nuclear translocation and subsequent induction of caspase-8, caspase-3, TRAIL and DR5. Normal human hepatocytes were not affected under similar treatment conditions [62]. DNA-activated protein kinase catalytic subunit (DNA-PKcs) was discovered in an shRNA screen to act as an ONC201 resistance factor in HCC. Knockdown, mutation or deletion of DNA-PKcs facilitated nuclear translocation of Foxo3a, whereas its overexpression attenuated the cytotoxic efficacy of ONC201 [62].

Hematological malignancies

Whereas few hematopoietic progenitor cells and no hematopoietic stem cells express appreciable levels of dopamine receptors, DRD2 and other dopamine receptors are expressed on multiple neoplastic lymphocytes [17,63]. Moreover, a clinical trial showed the competitive DRD2 antagonist, thioridazine, in combination with cytarabine, reduced acute myeloid leukemia (AML) blast counts in a manner that correlated with DRD2 expression in patients [64]. This proof-of-concept suggests that DRD2 may be a potentially viable target in hematologic malignancies; however, dose-limiting toxicities including QTc interval prolongation and neurological events preclude further clinical investigation and use of thioridazine [64].

ONC201 efficacy has been demonstrated in various hematological indications, including cell lines and patient samples that are resistant to standard-of-care therapy. ONC201 exhibited time- and dose-dependent pro-apoptotic and antiproliferative effects in acute lymphoblastic leukemia (ALL), anaplastic large cell lymphoma (ALCL), AML, Burkitt's lymphoma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), cutaneous T-cell lymphoma (CTCL), diffuse large B-cell lymphoma (DLBCL), Hodgkin's lymphoma (nodular sclerosis), mantle cell lymphoma (MCL), and multiple myeloma (MM) cell lines in a TP53-, complex karyotype-, and, in the case of AML, Flt3-independent manner [32,36]. ONC201 demonstrated antitumor efficacy in multiple hematologic malignancy models, including both orthotopic xenograft models and Eμ-transgenic mice that spontaneously develop metastatic B-cell lymphoma [7,33,65].

ONC201 induced apoptotic or cytostatic effects in pediatric Burkitt's lymphoma and T-cell lymphoma cell lines; moreover, TRAIL and DR5 induction was observed in a dose-dependent manner [66]. Unlike solid tumors, many adult hematologic malignancy cell lines did not exhibit ONC201-induced DR5 expression or TRAIL, as well as the accompanying Akt/ERK inactivation and Foxo3a nuclear translocation in one report [36]. Consistent with findings in solid tumors, ONC201 activated the intrinsic apoptotic pathway and upregulated ISR-related genes such as CHOP, as well as inhibited mTOR [36,65,67]. ONC201 also caused upregulation of ATF4 independently of eIF2-alpha phosphorylation [36]. The mitochondrial protease ClpP has been implicated in these effects; ONC201 demonstrated antiproliferative effects and impairment of OXPHOS correlating with ClpP expression in leukemia and lymphoma

cell lines [7]. Moreover, inactivation or mutation of ClpP conferred resistance to ONC201 treatment in leukemia and lymphoma [7].

ONC201 both inhibited cell growth and induced apoptosis via activation of the ISR pathway in CTCL cells [68]. In CD4+ malignant T cells, ONC201 also inactivated the JAK/STAT and NF-kB pathways, which are known to be essential for cell survival, proliferation and resistance to apoptosis in CTCL; however, no effects were observed in normal CD4+ T cells [68]. In MM, ONC201 was shown to upregulate the pro-apoptotic protein Bim and reduce Mcl-1, Bcl-2, and Bcl-xl levels *in vitro* [36].

Combinatorial efficacy

In accordance with its unique mechanism of action, ONC201 displayed synergy when combined with radiation, chemotherapeutic drugs, targeted agents and immune-checkpoint agents used to treat a wide range of cancers (Table 2). In concert with synergistic efficacy, the wide therapeutic index and sustained antitumor efficacy with infrequent administration positions ONC201 as an ideal therapy for combination with other anti-cancer agents that work by complementary mechanisms.

Radiation

ONC201 exhibited additive or synergistic efficacy when combined with radiation *in vitro* and *in vivo* in high-grade gliomas and other solid tumors [77,79] such as breast [71], prostate [76], lung and CRC [69]. *In vitro* studies confirmed modest synergy in cell viability and clonogenicity assays and involved pre-treatment with ONC201. Mechanistically, ONC201 combined with radiation resulted in improved induction of apoptosis and ISR in GBM, diffuse intrinsic pontine glioma (DIPG), atypical teratoid rhabdoid tumor (ATRT), breast cancer and prostate cancer cell lines [79,80]. The combination resulted in induction of PKA substrate phosphorylation indicative of DRD2 antagonism and cell death [79]. Combinatorial efficacy was observed in a xenograft model of breast cancer resulting in decreased tumor growth, anti-metastatic effects, and improved survival [71]. Activation of immune cells played a key role in the synergistic antitumor effects of radiation and ONC201 in this model, as depletion of either NK or CD8 T-cells dramatically blunted the combinatorial efficacy *in vivo* [71].

Temozolomide (TMZ)

ONC201 exhibits synergy with TMZ *in vitro* in GBM, DIPG and ATRT cell lines [79]. Additionally, the triple combination of ONC201 with radiation and TMZ was also synergistic [79].

Taxanes

In uterine serous carcinoma, ONC201 demonstrated antitumor efficacy similar to that of paclitaxel when given as a single agent; however, when given in combination, paclitaxel and ONC201 synergized to inhibit cancer cell growth *in vitro* [50]. ONC201 also demonstrated synergy in

Table 2. Combinations with ONC201 with demonstrated efficacy in preclinical models. Publications from PubMed detailing the combinatorial efficacy of ONC201 as of July 2020. (Y = yes, N = No).

Tumor Type	Drug Combination	In vivo data	Publication
Lung	Taxanes (paclitaxel and docetaxel)	Y	[33]
	Radiation	N	[69]
Breast	Taxanes (paclitaxel and docetaxel)	N	[58]
	PARP inhibitors (olaparib and rucaparib)	N	[70]
	Radiation	Y	[71]
	DR5 antibody (lexatumumab)	N	[72]
Hepatocellular	Sorafenib	Y	[73]
Pancreatic	Gemcitabine	Y	[74]
Multiple Myeloma	Bortezomib or carfilzomib	N	[65]
	Bortezomib	N	[67]
	Ixazomib	N	[67]
	Dexamethasone	N	[67]
Prostate	Olaparib	N	[70]
	Everolimus	Y	[75]
	Enzalutamide	N	[71,75]
	Radiation	N	[76]
Glioma	Bevacizumab	Y	[33]
	Bcl-2 inhibitor (ABT263)	Y	[39]
	Olaparib	N	[70]
	Radiation	N	[77]
	Temozolomide, radiation	N	[69]
Lymphoma	Cytarabine	N	[66]
	Bortezomib	Y	[67]
	Cytarabine	Y	[67]
AML	Bcl-2 inhibitor (ABT-199)	N	[36]
	Cytarabine	N	[67]
	5-azacytidine	N	[67]
CRC	Bevacizumab	Y	[33]
	Bevacizumab	Y	[52]
	Anti-PD-1 therapy	Y	[40]
	Radiation	Y	[69]
Ovarian	Olaparib	N	[70]
Endometrial	Paclitaxel	N	[50]
	Lexatumumab	N	[78]

combination with both docetaxel and paclitaxel in DLD-1 and SW620 colon cancer cell lines and resulted in durable tumor regressions, including complete elimination of tumor burden, in subcutaneous xenografts of NSCLC [33]. ONC201 also synergized with both docetaxel and paclitaxel in TNBC cell lines [58].

Cytarabine

ONC201 synergized with cytarabine *in vitro* in non-Hodgkin's lymphoma (NHL), AML and ALL cells [66,67]. Combinatorial efficacy with cytarabine was confirmed in a subcutaneous cell line xenograft of Burkitt's lymphoma, demonstrating improved antitumor activity relative to either agent alone [67].

Gemcitabine

ONC201 potentiated gemcitabine cytotoxicity in pancreatic cancer cells *in vitro* [74]. Combination treatment of subcutaneous cell line xenografts in SCID mice also resulted in antitumor activity and improved survival, relative to either agent alone, in a well-tolerated manner [74].

Epigenetic modulators

ONC201 demonstrated synergy with 5-azacytidine in AML cell lines irrespective of p53 status [67]. The two agents also synergized at higher doses in GBM, HCC and breast cancer cells [81]. Consistent with these results, 5-azacytidine also combined synergistically with bortezomib in MM, indicating that ISR induction is synergistic with 5-azacytidine antitumor effects [81]. ONC201 in combination with additional epigenetic modulators, including romidepsin and vorinostat, led to synergistic efficacy in a panel of human cancer cell lines [73].

Sorafenib

ONC201 synergized with sorafenib at higher doses in HCC to reduce cell viability and induce apoptosis *in vitro* [73]. Subcutaneous cell line xenografts of HCC in athymic nude mice were used to show that ONC201 and sorafenib also cooperated *in vivo*; the combination treatment led to complete tumor regressions. The mechanism of combinatorial efficacy involved cooperative upregulation of both TRAIL and DR5, as well as inhibition of Akt. Moreover, data suggested that sorafenib may augment the susceptibility of IAP, FLIP and Bcl-2 family proteins to ONC201-induced degradation [73].

Anti-angiogenic agents

In subcutaneous cell line xenograft models of CRC as well as CRC PDXs, bevacizumab synergized with ONC201 in both inhibiting tumor growth and promoting apoptosis, with no apparent toxicities [52]. Moreover, the combinatorial treatment led to a significant decrease in Ki67 expression as well as inhibition of HUVEC migration [52]. Of note, bevacizumab did not increase ONC201-induced TRAIL or CHOP/DR5 expression [52]. In an orthotopic xenograft model of p53-deficient CRC, bevacizumab and ONC201 cooperated to reduce tumor burden as well as metastasis to the lungs, liver, lymph nodes and peritoneum, again without toxicities [33].

In addition to CRC, single dose bevacizumab combined with ONC201 in an intracranial xenograft model of GBM to triple survival [33]. The combinatorial efficacy of ONC201 with anti-angiogenic agents may be associated with OXPHOS inhibition by ONC201, as anti-angiogenic therapy is known to cause tumor cells to switch from being dependent on glycolysis to mitochondrial metabolism [82].

Bcl-2 inhibition

ONC201 has demonstrated synergy with genetic as well as pharmacological Bcl-2 inhibition in liquid and solid tumors [36,39,73]. ONC201 treatment led to downregulation of Mcl-1, an anti-apoptotic Bcl-2 family member, in GBM cell lines [39]. The combination of ONC201 with the Bcl-2/Bcl-xL inhibitor ABT-263 resulted in synergistic antiproliferative effects *in vitro* in pediatric, adult and proneural GBM. In addition, the combination treatment downregulated Bag3 and Usp9X, two regulators of Mcl-1 stability. In a subcutaneous cell line xenograft mouse model of GBM the combination reduced tumor size more than either treatment alone, without apparent toxicities. The mechanism of synergy also involved decreasing levels of phospho-ERK and AKT, as well as pro-apoptotic proteins Bax and Bak to induce intrinsic apoptosis [39].

Given that Bcl-2 overexpression reduced the efficacy of ONC201 and that Mcl-1 overexpression resulted in resistance to Bcl-2 inhibitors, AML cells were treated *in vitro* with a combination of ONC201 and the Bcl-2-specific inhibitor ABT-199. This combination treatment resulted in synergistic cytotoxicity in a p53-independent manner via mutual elimination of resistance mechanisms [36].

Proteasome inhibitors

ONC201 synergized with bortezomib in MM cell lines to induce Bim, increase PARP cleavage, and reduce cell viability [65,67]. The combination also reduced cell viability in ALCL and HCC cells, increased apoptosis in MCL cells and enhanced the efficacy of bortezomib in pediatric NHL cells [32,66,67]. Treatment of Burkitt's lymphoma subcutaneous cell line xenograft model showed that ONC201 synergized with bortezomib *in vivo* to induce CHOP and tumor necrosis without body weight loss [67].

ONC201 also synergized with ixazomib and dexamethasone to reduce viability of MM cells *in vitro,* and with carfilzomib to induce Bim and increase PARP cleavage in MM cells [65,67]. Importantly, ONC201 demonstrated synergy with proteasome inhibitors in MM regardless of previous standard-of-care therapy [65]. Thus, ONC201 synergized with multiple proteasome inhibitors across hematologic malignancies.

mTOR inhibitors

mTOR signaling can serve as an innate resistance mechanism to ONC201 in CRC, prompting exploration of mTOR inhibitor combination therapy [57,60]. The mTOR kinase inhibitor AZD-8055 was shown to synergize with ONC201 in CRC, potentiating its antiproliferative activity and enhancing its cytotoxicity in CRC cells via TRAIL induction. Moreover, the combination led to inactivation of Akt and mTOR activation *in vitro* [57].

In addition, ONC201 synergized with everolimus in prostate cancer cell lines to induce apoptosis, as well as downregulate molecules downstream of mTOR such as S6 and 4EBP1 [60]. Moreover, ONC201 and everolimus synergistically inhibited tumor growth in a subcutaneous cell line xenograft model of prostate cancer without apparent toxicity [60].

Enzalutamide

ONC201 combined with enzalutamide, an anti-androgen treatment, resulted in improved inhibition of prostate cancer stem cell sphere formation and growth relative to either agent alone [60].

PARP inhibitors

PARP-1 is highly expressed in tumor cells that are resistant to DR5-agonist antibodies, and treatment with PARP inhibitors results in increased DR5 expression [83–85]. Consequently, PARP inhibitors sensitized various solid tumors and hematological malignancies to recombinant TRAIL and DR5-agonist antibodies [83–85]. PARP inhibitor treatment resulted in PI3K/Akt pathway activation, and the combination of PARP inhibitors with PI3K blockade improved antitumor effects in various solid tumors [86–88]. Additionally, proteasome inhibition sensitized multiple myeloma cells to PARP inhibition [89].

Consistent with these findings that overlap with the mechanism of action of ONC201, synergy between ONC201 and PARP inhibitors have been noted in multiple solid tumors *in vitro* in cell viability assays [70]. In BRCA-deficient breast and ovarian cancer cells, synergy between ONC201 and PARP inhibitors olaparib and rucaparib was observed. Robust synergy for the combination of ONC201 and olaparib was also observed in prostate cancer and GBM cell lines [70].

IGF1-R inhibition

Low IGF1-R expression correlated with increased ONC201-induced apoptosis in pancreatic cancer cell lines [75]. ONC201 treatment induced IGF1-R levels and synergized with the IGF1-R inhibitor AG1024 *in vitro* in cell viability and apoptosis assays. Additionally, combinatorial efficacy was confirmed *in vivo* in a pancreatic cancer cell line xenograft model [75].

Bromodomain inhibitors

ONC201 combinatorial efficacy in gliomas was observed with the bromodomain inhibitor (BRD2-4) antagonist, OTX015 *in vitro* and *in vivo* [29]. OTX015 reduced c-myc expression, inhibited OXPHOS and enhanced ONC201-mediated apoptosis. Consequently, combined treatment with OTX015 and ONC201 enhanced tumor growth inhibition without body weight loss or organ toxicity in a subcutaneous GBM cell line xenograft model [29].

2-Deoxyglucose (2-DG)

Aerobic respiration to generate ATP is a key bioenergetic mechanism for tumor and normal cells that relies on two distinct processes, glycolysis or OXPHOS [90]. ONC201 inhibited OXPHOS and combined synergistically with glycolysis inhibitor 2-DG to elicit anti-cancer effects in GBM. The combination treatment enhanced inhibition of GBM cell viability, colony formation, migration, and ATP content. Additionally, the combined treatment reduced tumor growth in the chorioallantoic membrane assay and did not cause toxicity in normal human cells or chicken embryos [90].

TRAIL receptor-targeted therapy

In most TNBC cells, ONC201 has an antiproliferative rather than apoptotic effect, which was hypothesized to be caused by insufficient TRAIL induction [58]. Combination of ONC201 with recombinant human TRAIL (rhTRAIL) converted the TNBC cell response from an antiproliferative to apoptotic one, and led to cleavage of PARP, caspase-3, caspase-9, and caspase-8. The apoptotic effect of ONC201 in combination with rhTRAIL was also observed in GBM, CRC, lung cancer and pancreatic cancer cell lines, but normal cells were not affected. Similarly, addition of a DR5 agonistic antibody (lexatumumab) to ONC201-pretreated cells induced an apoptotic, rather than antiproliferative effect, in non-TNBC cells [72]. Pretreatment of ONC201 followed by treatment with either TRAIL or a DR5 agonist also led to cell death in endometrial cancer cell lines [78].

Anti-PD-1 therapy

The biology and downstream effects of immune checkpoint blockade provide rationale for combination therapy with ONC201 in multiple tumor types. PD-1 blockade has been shown to synergize with MAPK and PI3K-Akt inhibition in melanoma and lung cancer; moreover, TRAIL-mediated cancer cell death enhanced the efficacy of checkpoint inhibition [91–93]. In addition to downstream effects that overlap with those of ONC201 treatment, the effect of ONC201 on CSCs could improve anti-PD-1 therapy, as CSCs evade T-cell-mediated immunity in both GBM and head and neck cancer [94,95]. Lastly, Anti-PD-1 therapies activate T-cell-mediated killing mechanisms, thereby adding to NK cell-mediated ONC201 effects.

Considering that ONC201 predominantly activates the NK cell axis of the immune system, combinatorial efficacy with T-cell-targeting anti-PD-1 therapy was explored. Treatment with ONC201 in combination with anti-PD-1 therapy in a syngeneic model of CRC resulted in improved tumor suppression [40].

KDM4 inhibition

The chromatin regulator KDM4A acts to silence expression of TRAIL and DR5 by tumor cells [96]. Inhibition of KDM4A/B with the small molecule C-4 induced strong upregulation of TRAIL and DR5 in prostate, lung and breast cancer cells through KDM4A silencing, as well as upregulation of CHOP gene expression. Treatment of subcutaneous breast and lung cancer cell line xenografts in athymic nude mice with C-4 (20 mg/kg, 5x per week) sensitized tumors to ONC201 (25 mg/kg, qW) via increased TRAIL sensitivity [96].

Nonclinical toxicology, pharmacokinetics (PK) and biodistribution

ONC201 reduced the viability of tumor cells without affecting normal cells and did not induce DNA damage *in vitro* [32,33,49]. ONC201 has demonstrated robust tolerability *in vivo*. CRC xenograft mouse models have established an ONC201 efficacious dose of 25 mg/kg; however, doses up to 100 mg/kg in mice were not toxic [40]. At higher, more frequent dosing, there were sustained PD effects and anti-metastasis effects [40]. At efficacious doses of ONC201, normal tissues in mouse models were not adversely affected despite PD effects in select tissues [33]. The safety profile of ONC201 was further established in GLP toxicology studies in rats and dogs [49]. In Sprague Dawley rats and beagle dogs, GLP toxicology studies have not shown any mortalities with oral ONC201 and a maximum tolerated dose (MTD) was not achieved at doses up to 225 mg/kg in rats and 120 mg/kg in dogs. The only findings that were observed in both rats and dogs were decreased activity and decreased food consumption (weight loss only seen in rats), which were mild and reversible. No biologically relevant adverse effects of oral ONC201 were observed in respiratory, cardiovascular or central nervous system safety pharmacology studies in rats and dogs. The no-observed-adverse-effect-level (NOAEL), defined as the highest dose level at which no adverse effects are observed [97], for a single oral dose of ONC201 in rats and dogs was 125 mg/kg and 42 mg/kg, respectively, which translate to a human equivalent dose of ~1.25 g assuming allometric scaling and an average adult human body weight of 60 kg. This NOAEL dose is 10-fold above the efficacious dose level in xenograft mouse models. These results provided the rationale for the 125 mg starting dose that was used in dose escalation clinical trials in adult patients with advanced cancer [49].

PK analysis in C57/B6 mice revealed ONC201 has a plasma terminal half-life of ~6.5 h [33]. In rats, exposure to ONC201 was dose-dependent and approximately dose-proportional. Exposure to ONC201 was slightly greater in female rats after a single oral gavage dose. The plasma terminal half-life ranged from 2.3 to 8.4 h and clearance ranged from 7.5 to 23.5 L/h/kg. Volume of distribution ranged from ~49 to ~103 L. Exposure to ONC201 in dogs was dose-dependent and increased with greater ONC201 dose levels. Exposure to ONC201 was similar in male and female dogs with the observation that all mean male C_{max} and AUC values were slightly greater than those corresponding female values. Elimination of ONC201 from plasma was similar between the mid and high dose levels; mean plasma terminal half-life ranged from 4.6 to 7.8 h [33].

A biodistribution study showed that ONC201 achieved ~5-fold higher concentrations in the brain and spinal cord relative to plasma in non-tumor-bearing Sprague-Dawley rats that was indicative of robust blood–brain barrier penetrance [98]. Other tissues with ONC201 that were ~5-fold or higher than plasma included liver, kidney, neck lymph,

abdominal cavity lymph. Skin, bone marrow and spleen had ~3 to 4-fold higher concentrations relative to plasma. Peak plasma concentration was observed at 0.5 h [98].

Clinical trials

Based on the broad spectrum of efficacy observed across solid tumors and hematological malignancies in preclinical models, ONC201 has been evaluated in 17 Phase I and/or Phase II clinical trials either as a single agent or in combination and many of these studies are ongoing (Table 3). Tumor types, as well as single agent versus combinatorial treatment strategies were determined based on activity in initial first-in-human studies, and more recently based on emerging knowledge of DRD2/ClpP expression that is potentially relevant although not predictive of drug response. Other determining factors included dysregulation of key downstream pathways related to the mechanism of action or synergy, biodistribution, efficacy data in preclinical models, and the unmet clinical need.

Dose and schedule

Preclinical studies using ONC201 involved dosing via oral, intravenous and intraperitoneal routes of administration that each demonstrated efficacy. Oral administration was chosen for clinical dosing based on bioavailability and facile administration. ONC201 was introduced into the clinic with once every three-week dosing based on the prolonged intratumoral PD effects that outlasted systemic presence and the sustained antitumor efficacy observed in single dose *in vivo* xenograft tumor experiments [33]. Subsequently, *in vivo* studies in xenograft tumor models demonstrated improvement in antitumor efficacy including suppression of metastasis with once weekly administration relative to every three-week dosing [40]. Additionally, enhanced immune stimulatory activity was observed with weekly dosing relative to every three-week dosing [99]. Considering these observations, its benign safety profile, and wide therapeutic index, ONC201 was subsequently administered once weekly in clinical trials.

Based on the wide therapeutic index and saturable efficacy of ONC201 in preclinical studies, the first-in-human clinical study of ONC201 was conducted using a single patient cohort dose escalation to identify a biologically optimal dose as the recommended Phase II dose (RP2D), rather than an MTD [99,100]. As outlined above, the starting dose in humans of 125 mg was equivalent to the efficacious dose level of 25 mg/kg in xenograft mouse models. The RP2D was established as 625 mg, which yielded plasma concentrations that exceeded micromolar therapeutic thresholds, induced surrogate systemic biomarkers such as prolactin, and triggered intratumoral hallmarks of ISR activation and apoptosis while being well tolerated [99,100].

Safety and pharmacokinetics

ONC201 was well tolerated at various dose levels and administration schedules in Phase I [99,100], Phase I/II [101], Phase II clinical trials [98,102] and expanded access protocols [103] reported thus far. Dose-limiting toxicities or treatment discontinuation due to drug-related toxicity were not observed across the various dose levels ranging from 125 mg to 625 mg administered once every one or three weeks. A range of mild or moderate and low frequency adverse events that are typical of a Phase I advanced solid tumor population were attributed as possibly related to ONC201: pyrexia, fatigue, nausea, emesis, diarrhea, anorexia, stomach pain, cognitive disturbance, confusion, dizziness, gait disturbance, tinnitus, dysgeusia, neutropenia, allergic reaction, hypophosphatemia, platelet count decrease, and elevated amylase [99,100]. Based on the safety profile of weekly dosing, further dose intensification with ONC201 is warranted in certain tumor types with limited single agent efficacy and will be explored in future trials, including twice weekly dosing on two consecutive days.

The first-in-human phase I trial used an accelerated titration to enroll patients with advanced solid tumors resistant to standard treatments [100]. Patients received ONC201 orally once every 3 weeks at an assigned dose ranging between 125 mg and 625 mg, which was selected as the RP2D as detailed above. A mean plasma terminal half-life of 11.3 h was observed in patients who received the 625 mg RP2D. The mean C_{max} of 3.6 μg/mL (~9.3 μmol/L) was achieved at 1.8 h post-administration (T_{max}); mean AUC was 37.7 h·μg/mL. A generally consistent mean CL/F of 25.2 L/hour was observed across all dose groups [100].

This trial also evaluated the safety of weekly administration using a 3 + 3 design at doses of 375 or 625 mg in the same population [99]. Patients dosed with weekly ONC201 exhibited a 9.4 h plasma terminal half-life, 1.4 h T_{max}, 4.3 μg/mL C_{max} and 34.3 h·μg/mL AUC. Weekly dosing of ONC201 did not appear to differ from the previously reported PK profile

Table 3. Ongoing or completed ONC201 clinical trials by tumor type. As of July 2020. ORR = overall response rate, PFS = progression free survival, RP2D = recommended phase II dose.

Tumor Type	NCT Number	Phase	Combination	Target Enrollment	Primary Endpoint	Status
Acute Leukemia	NCT02392572	I/II	Venetoclax	33	RP2D, ORR	Recruiting
	NCT03932643	I	–	20	RP2D	Recruiting
Breast	NCT03394027	II	–	90	PFS8, ORR	Recruiting
	NCT03733119	II	–	112	ORR	Recruiting
Colorectal	NCT03791398	Ib/II	Nivolumab	34	RP2D, PFS	Recruiting
Endometrial	NCT03099499	II	–	36	PFS3, ORR	Recruiting
	NCT03394027	II	–	90	ORR	Recruiting
	NCT03485729	II	–	42	PFS2	Recruiting
High-Grade Gliomas	NCT03416530	I	Radiation	90	RP2D	Recruiting
	NCT03295396	II	–	95	ORR	Recruiting
	NCT02525692	II	–	76	PFS6	Recruiting
Multiple Myeloma	NCT02863991	I/II	Dexamethasone	21	RP2D, ORR	Closed
	NCT03492138	I/II	Dexamethasone & ixazomib	42	RP2D	Closed
Neuroendocrine	NCT03034200	II	–	24	ORR	Recruiting
Non-Hodgkin's Lymphoma	NCT02420795	I/II	–	60	RP2D, ORR	Closed
Ovarian	NCT04055649	II	Paclitaxel	62	RP2D, ORR, PFS	Recruiting
Solid Tumors	NCT02250781/ NCT02324621	I	–	58	RP2D	Completed
	NCT02609230	I	–	54	RP2D	Completed

via changes in systemic accumulation, nor did it alter metabolism based on the PK profile observed in the second cycle of treatment [99].

Pharmacodynamics

PD assays in patients with solid tumors and hematological malignancies revealed engagement of a number of biomarkers that demonstrated the biological activity of the RP2D (Fig. 2A). The first-in-human study of oral ONC201 administered once every 3 weeks in patients with refractory solid tumors assessed tumor cell apoptosis via levels of caspase-cleaved cytokeratin 18 (cCK18) using serum M30 and M65 assays [99,100]. Increases in the M30 assay (cCK18), but not the M65 assay (total CK18), were observed in the patients who remained on study for 9 cycles, whereas the opposite was observed in a patient with rapid disease progression. Additionally, serum TRAIL levels were determined as measures of TRAIL induction. An approximately 20% increase in serum TRAIL that peaked at about 24 h post-administration was observed in half of the patients on trial. A majority of the patients evaluated exhibited prolactin induction, a biomarker of DRD2 antagonism in the pituitary gland, with a mean of 2.4-fold over baseline and peak induction occurring at either 6 hours, 14 days or 21 days post-dose [100]. Serum prolactin induction was also observed in recurrent GBM patients treated with ONC201 every three weeks [102]. In all cases, prolactin levels did not surpass hyperprolactinemia thresholds.

Consistent with these data, serum prolactin levels measured with weekly ONC201 in the Phase I trial were elevated >2-fold over baseline in the majority of patients; additionally, 65% of patients exhibited induction of cCK18 [99,100]. These results were consistent across the dose levels evaluated. Moreover, IHC analysis of pre- and post-treatment tissues for intra-tumoral CHOP, DR5 and TUNEL staining confirmed ONC201-mediated target engagement [99].

Similarly, induction of DR5, ATF4, and TUNEL staining was observed in a separate study of 6 patients treated with weekly ONC201 undergoing surgical resection of recurrent GBM in a window-of-opportunity cohort [98]. Intratumoral PD activity of ONC201 was more pronounced in GBM patients with low DRD5 expression determined by IHC, which is consistent with preclinical findings outlined above [98].

ONC201 induced ISR activation in tumor cells with the DRD2+DRD5− biomarker signature and caused a delayed, yet sustained, immune cell infiltration in samples from an MCL patient [101]. Significant increases in CHOP, CD45+ lymphocytes and CD8+ T cells were observed for samples obtained as far out as 6 months following the end of treatment, suggesting a sustained immune response [101]. Immune activation, including an increase in circulating NK cells, induction of immune cytokines and effector molecules such as TRAIL, increased tumor infiltration of granzyme B+ and CD56+ cells, and induction of serum perforin (found in cytotoxic T lymphocyte and NK cell granules) was also observed in response to ONC201 weekly oral administration across a cohort of advanced solid tumor patients [40,99].

Efficacy

A subset of the prostate and endometrial cancer patients in the first-in-human study described above experienced tumor regressions in individual metastatic lesions upon ONC201 treatment as a single agent, including a chemotherapy-resistant clear cell endometrial cancer patient with a mixed response (including reduction in pulmonary metastatic and lymph node disease) and two elderly (>90 years old) patients (one with type II endometrial cancer and one with castrate-resistant prostate cancer) with reduction in bone metastatic disease and subsequent stable disease for > 9 cycles [100].

In an expansion of this study, weekly ONC201 administered at 375 or 625 mg in 20 adults with advanced refractory tumors also yielded clinical benefit [99]. A disease control rate of 42.9% was achieved across patients, and 25% of patients exhibited prolonged stable disease for >6 months by RECIST criteria. Additionally, prostate and endometrial cancer patients both experienced tumor regressions and one endometrial cancer patient remained progression-free for >70 weeks. Prostate cancer patients had a PFS >30 weeks ($n = 4$) and one patient had significant tumor shrinkage in both the primary tumor and bone metastasis.

The first MCL patient to receive ONC201 in a Phase I/II study for non-Hodgkin's lymphoma was treated with 125 mg orally once every 3 weeks, and was noted to have new disease per biopsy after 7 cycles, upon which the treatment was discontinued but other therapies were not pursued [101]. Six months after ONC201 discontinuation, extensive re-evaluation revealed only a small lymphoid infiltrate in one rectal biopsy that was accompanied by immune cell infiltration as described above [101].

The Phase II clinical trial of ONC201 enrolled a cohort of 17 adult patients with recurrent IDH1/2-wild type bevacizumab-naÝve GBM to receive ONC201 at 625 mg once every three weeks [26]. Patients had previously received at least TMZ and radiotherapy, had unequivocal evidence of progressive disease, and were ≥12 weeks out from radiotherapy. The median overall survival of these patients was 41.6 weeks, with OS6 of 71% and OS9 of 53%. Two out of 17 patients achieved PFS at 6 months, giving a PFS6 of 11.8%. One patient in this cohort whose tumor was subsequently found to harbor the H3 K27M mutation achieved an overall 96% reduction in tumor size and a complete regression of the primary thalamic lesion. Per investigator assessment, this response was durable for >27 months after first being achieved at 4.9 months following ONC201 initiation. Another patient who initiated ONC201 after salvage surgery remains disease-free and has continued ONC201 for over 4 years [102]. Low DRD5 expression in archival tumor specimens was associated with relatively prolonged PFS and OS in this cohort [26].

An additional cohort in this Phase II GBM trial evaluated the biological activity of 625 mg weekly ONC201 in adult patients with recurrent GBM. Among a 20-patient cohort, PFS6 was 5% and mOS was 7.5 months [98]. One patient, a 74-year old with sub-centimeter, multifocal, H3 K27M-mutant recurrent GBM experienced a complete regression of enhancing lesions that remained durable for over 1.5 years [98].

Gliomas that harbor the H3 K27M mutation have been identified as a molecular subset of brain tumors with a DRD2+DRD5− expression signature that are responsive to ONC201, which was uncovered following the first outlier response described above [53]. H3 K27M is a missense mutation that occurs in one of several genes encoding for the histone H3 protein. Tumor cells harboring this mutation exhibit a histone hypomethylation profile that causes epigenetic dysregulation of the expression of many genes associated with cancer [104,105]. The H3 K27M mutation defines a distinct form of Grade IV glioma codified in the 2016 World Health Organization (WHO) classification of CNS tumors [106,107]. This disease entity is characterized by a poor prognosis and a high prevalence in midline gliomas that predominantly afflict children and young adults [108]. Clinical studies report a dismal prognosis for pediatric patients with H3 K27M-mutant midline gliomas, with a median overall survival of <12 months [107,109,110]. The median overall survival of adult patients with H3 K27M-mutant gliomas is approximately 16 months [111]. The H3 K27M mutation occurs in a unique spatiotemporal pattern, with midline gliomas involving the pons (i.e. DIPG) tending to occur in pediatric patients (<18 years of age), and midline gliomas involving the thalamus and spinal cord tending to occur in young adult patients [112,113].

Since the midline region of the brain is involved in critical physiological functions, many of these tumors are not operable, especially in the

brainstem where the pons is located. Standard therapy for midline gliomas involves neurosurgery, if feasible, followed by fractionated external beam radiotherapy. Radiotherapy remains the sole standard-of-care alone that is considered palliative, as relapse is inevitable and it only transiently improves symptoms and tumor burden and the 2-year survival rate in some forms of this disease is <10% [112,113].

Based on durable tumor responses reported in adult recurrent H3 K27M-mutant glioma patients with single-agent ONC201, multiple clinical trials are under way to further explore the efficacy of ONC201 in this indication. Dedicated and multi-arm Phase II trials (NCT03295396, NCT0252569), as well as expanded access protocols (NCT03134131), have been enrolling adults with H3 K27M-mutant gliomas for ONC201 treatment (625 mg weekly). Based on a data cutoff of July 31, 2019, 20 patients received ONC201 who meet specific eligibility criteria designed for an integrated ORR analysis by RANO-HGG criteria for patients with recurrent, contrast-enhancing H3 K27M-mutant glioma who received single agent ONC201. An objective response rate of 27% was observed by blinded independent review [114].

Additionally, an analysis of ONC201 in thalamic H3 K27M-mutant glioma was conducted, as DRD2 expression is relatively high in the thalamus of non-malignant brain tissue. As of May 2019, 10 of 20 recurrent patients and 9 of 11 patients who initiated ONC201 prior to recurrence remain on treatment and a median PFS has not yet been reached for either cohort with a median follow up of 21.9 months from diagnosis. Moreover, several complete or partial responses have been observed in either treatment setting [115].

ONC201 is also being evaluated in pediatric patients who have newly diagnosed DIPG or recurrent/refractory H3 K27M-mutant glioma in a multi-center, open-label, dose-escalation and dose-expansion Phase I trial (NCT02316530) [116]. The safety experience has been similar to that of adults, as has the PK profile: $T_{max} \sim 1.9$ h; $T_{1/2} \sim 8$ h; $C_{max} \sim 2.1$ μg/mL; AUC ~ 2.3 h*μg/mL [116].

In addition to clinical trials, the clinical experience with ONC201 has been reported for H3 K27M-mutant glioma or DIPG patients who could not access a clinical trial and were treated on single patient expanded access protocols. These patients received ONC201 once a week at 625 mg, which was scaled by body weight if <18 years. In this report, five of the 18 total patients treated continued on treatment progression-free for a median of 53.14 weeks, whereas 13 patients discontinued due to disease progression. Multiple patients experienced clinical benefit with ONC201 treatment [103]. One adult patient with recurrent H3 K27M-mutant diffuse midline glioma, who had previously been treated with TMZ and radiation, experienced a complete response of the disease in the thalamus and other sites after 10 months of treatment. Improvements in disease-related symptoms were reported and patient remained on therapy for >17 months. A second adult with the same disease and prior therapy showed reduction in tumor size by RANO criteria and remained on therapy for >12 months, with eventual stable disease and improvement in the ability to partake in day-to-day activities [103].

A 10-year-old patient with biopsy-proven H3 K27M-mutant DIPG was treated with ONC201 (500 mg weekly, scaled by body weight) following completion of radiotherapy [103,117]. Subjective improvement in hearing was reported two months into therapy; after four months, near-complete resolution of the patient's facial palsy and ability to completely close both eyes were observed along with resolution of cerebellar dysmetria, ataxia and gait disturbance. Such clinical improvements were observed for 12 months after initiation of treatment, upon which new lesions were discovered and the patient was given dexamethasone and bevacizumab to regain stability [103,117]. A second pediatric patient was also treated with ONC201 upon MRI findings consistent with DIPG as well as a biopsy revealing an H3.3 K27M-mutant diffuse midline glioma [103]. The 33-month-old patient received field irradiation followed by ONC201 on weight-based dosing; subsequent radiographic improvements in FLAIR as well as stable tumor size were observed [103].

The FDA has granted Fast Track designation to ONC201 for the treatment of adult recurrent H3 K27M-mutant high-grade glioma and recurrent H3 K27M-mutant glioma is the targeted registration population.

Combination therapy

Based on preclinical rationales for synergistic anti-cancer effects, clinical trials are also exploring the combinatorial safety and efficacy of ONC201 with radiation in newly diagnosed DIPG (NCT03416530), nivolumab in microsatellite stable metastatic CRC (NCT03791398), paclitaxel in platinum-resistant refractory or recurrent ovarian cancer (NCT04055649), ixazomib, dexamethasone in relapsed/refractory MM (NCT03492138) and venetoclax in relapsed or refractory AML, ALL, or myelodysplastic syndrome (MDS) (NCT02392572).

Imipridone chemical scaffold and ONC201 analogs

a. Structure activity relationship

Based on the favorable characteristics of ONC201 spanning safety, PK, PD, bioavailability, solubility, chemical stability, and its novel tri-heterocyclic pharmacophore, a medicinal chemistry effort was undertaken to define a structure–activity relationship (SAR) for its novel chemical scaffold [118]. The SAR led to the identification of new imipridones that retained the core structure of ONC201 while exhibiting differential engagement of distinct GPCR and ClpP targets, differentiated target pharmacology, altered spectrums of activity in cancer, and altered potency [10,51,119]. The medicinal chemistry was not designed to optimize any target engagement property of the analogs.

b. ONC206

The initial ONC201 synthetic analog synthesis effort identified ONC206 as the most potent nanomolar DRD2 antagonist that exhibited enhanced non-competitive DRD2 antagonism while maintaining selectivity [120]. However, ONC206 was not synthesized based on any attempt to optimize any structural or functional feature of ONC201 or any target interaction. This was later rationalized by shotgun mutagenesis assays that revealed the requirement of additional allosteric DRD2 residues relative to ONC201 and other DRD2 antagonists [120]. It is speculated that the receptor pharmacology could enable distinct functional effects on the dopamine receptor and also yields increased potency. It is hypothesized that the latter could enable ONC206 to address certain DRD2-dysregulated tumors located in tissues where ONC201 does not fully engage the target due its biodistribution and potency.

The downstream signaling of ONC206 is consistent with ONC201, which involves ISR activation, Akt/ERK inactivation and TRAIL/DR5 induction [51]. The downstream effects of ONC206 result in tumor-specific apoptosis, while not imparting cytotoxicity to normal cells [51]. The antitumor efficacy of ONC206 has been demonstrated in preclinical models across multiple tumor types [29,51,120]. In addition to inhibiting colony growth of CRC and melanoma cell lines, ONC206 was able to induce apoptosis or cell cycle arrest, and inhibit tumor cell migration and invasion *in vitro* [51]. ONC206 demonstrated increased efficacy relative to ONC201 in serous endometrial cancer in vitro and in vivo. DRD2 knockdown in serous endometrial cancer cells impaired ONC206-mediated inhibition of cell viability [121]. Moreover, GBM cells with relatively high innate expression of c-myc exhibit increased levels

of apoptosis with ONC206 treatment, suggesting that this may be a predictive biomarker [29]. ONC206 administered at 50 mg/kg twice a week intraperitoneally reduced the tumor size of GBM patient-derived xenograft (PDX) GBM models [29]. PK studies in Sprague-Dawley rats demonstrated that oral ONC206 has a half-life of 6 hours and a C_{max} of 12 μM [122]. The IND for ONC206 was accepted by FDA and the first-in-human clinical trial for patients with recurrent and rare primary CNS tumors has been initiated.

c. ONC212

Another ONC201 analog synthesized was ONC212, a fluorinated ONC201-derivative which was found to be the most potent anti-cancer imipridone family member to date, exhibiting cytotoxicity at low nanomolar concentrations. The anti-cancer efficacy of ONC212 may be driven by agonism of the orphan GPCR GPR132 and hyperactivation of the mitochondrial protease ClpP, leading to induction of tumor-selective apoptosis [7,10,123,124]. GPCR profiling of ONC212 revealed no interaction with DRD2 or any other GPCRs with known ligands. Instead, a potent nanomolar agonism of the tumor suppressor orphan GPCR GPR132 was identified [123–125].

Knockout mouse models have established a putative tumor suppressive role of GPR132 in leukemia and analysis of primary leukemic samples has revealed high GPR132 expression [123,126]. High GPR132 expression is inconsistent with a tumor suppressor role and may not necessarily be associated with functional activation. GPR132 signals pro-survival pathway activation as well and its function in human cancer beyond leukemia remains unclear. A tumor suppressive activity of GPR132 involves cell cycle arrest and apoptosis and regulation of key pathways such as Ras signaling [127,128]. The highest innate GPR132 expression was observed in leukemia and lymphoma, compared to all other tumor types in TCGA [124]. GPR132 overexpression in tumor cells diminished growth and induced apoptosis [127,129]. ONC212 treatment also significantly increased GPR132 mRNA expression in AML cell lines, further potentiating GPR132 tumor suppressive effects. However, the involvement of GPR132 in the mechanism of tumor suppression following ONC212 treatment has not been rigorously investigated in other tumor types. Interestingly, high GPR132 expression significantly correlated with ONC212 efficacy in GDSC human cancer cell lines and in a subset of AML cell lines, as measured by AUC. GPR132 heterozygous knockout decreased ONC212-mediated apoptosis, thereby establishing the relevance of GPR132 for ONC212 anti-cancer efficacy [124]. However, some unpublished data in pancreatic cancer has suggested that knockdown of GPR132 does not lead to reduction in ONC212 cytotoxicity.

GPR132 expression was highest in pancreatic cancer across all solid tumor types in TCGA, which is concordant with the efficacy of ONC212 in pancreatic cancer models *in vitro* and *in vivo* [75]. Growth inhibition of pancreatic cancer cell lines and PDX cell lines was achieved at nanomolar concentrations, and ONC212 induced cell-surface TRAIL expression and apoptosis. Treatment of pancreatic cancer xenografts in mice with ONC212 led to significant growth inhibition of tumors and reduced tumor cell proliferation, as evidenced by reduced Ki67 expression. These anti-cancer effects were shown to be a result of activation of the ISR pathway and subsequent apoptosis. In addition to its efficacy as a single agent, ONC212 demonstrated synergistic potential with the IGF1-R inhibitor AG1024, chemotherapeutic drugs (5-fluorouracil, oxaliplatin, and irinotecan) and crizotinib in pancreatic cancer cell lines [75].

ONC212 activates ClpP and induces casein cleavage independent of ClpX, a regulatory chaperone protein needed for ClpP-mediated cleavage under basal conditions [51]. ONC212 exhibits increased potency and enhanced complementarity for ClpP relative to ONC201, likely due to the highly electronegative CF_3 substituent that reaches into an apolar pocket of ClpP [7]. Similar to ONC201 and ONC206, ONC212 activates the ISR pathway, causing upregulation of TRAIL and DR5, and downregulates phosphorylation of Akt and ERK [51].

BioID is a proximity-dependent labeling system that utilizes promiscuous labeling enzymes; such an enzyme is fused to a protein of interest (e.g. ClpP), and all proteins that interact with the protein of interest are biotinylated and identified via mass spectrometry. Use of BioID screening to identify proteins in T-REx 293 human embryonic kidney cells that interact with ClpP revealed respiratory chain subunits SDHB and NDUFA12 that are degraded in response to ONC212 [7]. Importantly, this and the apoptotic effect of ONC212 were blocked by overexpressing a ClpP construct harboring an inactivating D190A mutation, which was identified in AML cell lines with acquired resistance to ONC212. ClpP was shown to be relevant to the *in vivo* antitumor efficacy of ONC212: MCL xenograft models with wild-type ClpP had significantly reduced tumor burden post-treatment unlike xenografts with D190A-mutated ClpP MCL. Additionally, pretreatment of AML PDX cells with 250 nM ONC212 for 36 h significantly extended the survival of xenografted mice [7].

ONC212 has broad-spectrum activity *in vitro* across solid tumors and hematological malignancies in the Genomic of Drug Sensitivity in Cancer (GDSC) panel of >1000 cell lines and efficacy in xenograft mouse models for AML, GBM, pancreatic, CRC, liver and skin cancer [7,29,51,75,124]. ONC212 reduced both the growth and viability of leukemia, lymphoma, colon cancer, cervical cancer, breast cancer, and ovarian cancer cell lines; moreover, it induced apoptosis in patient-derived AML cell lines. ONC212 was the most potent of all imipridones tested in reducing proliferation of both patient-derived and stem-like GBM cells both *in vitro* and *in vivo* [29]. Studies of tumor cell energy metabolism suggested that these effects were due to suppression of glycolysis and OXPHOS. Innate c-myc expression in GBM was shown to be predictive of ONC212 efficacy. ONC212 dosed at 100 mg/kg twice weekly significantly extended survival in orthotopic GBM cell line xenograft models [29]. Efficacy was also demonstrated in cell line xenografts of melanoma and HCC, wherein ONC212 decreased tumor volume, reduced tumor cell proliferation, and induced apoptosis [51].

The compound exhibits a wide therapeutic index *in vitro*. Toxicity assessments in C57/BL6 mice demonstrated that ONC212 was well tolerated at doses of up to 250 mg/kg intraperitoneally or orally. ONC212 exhibits a 4.3-hour plasma terminal half-life and a 1.4 μg/mL C_{max} [51]. IND-enabling studies with ONC212 are ongoing.

Conclusion and future directions

ONC201 is an anti-cancer small molecule that appears to selectively target DRD2 as a unique competitive and non-competitive antagonist, as well as ClpP as an allosteric agonist. Downstream of target engagement, the molecule causes tumor cells to activate the ISR, inhibit OXPHOS, inactivate Akt/ERK signaling, degrade c-myc, and induce DR5/TRAIL. These effects trigger antiproliferative, and/or pro-apoptotic phenotypes in tumor cells with high DRD2 (Fig. 1) and/or ClpP expression or a dependency on other elements of the mechanism of action (e.g. c-myc or OXPHOS) (Fig. 2A) in a cellular context-dependent manner. Neither DRD2 nor ClpP alone can predict response to ONC201 in all preclinical models. ONC201 treatment results in inhibition of CSC self-renewal (Table 1), NK cell activation (Fig. 2B) and fibroblast mediated bystander effects within the tumor microenvironment. In accordance with the broad relevance of the mechanism of action of ONC201 in many types of cancer, the preclinical activity of ONC201 has been demonstrated in a variety of advanced solid tumors and hematological malignancies as a single agent (Fig. 3) and in synergistic combinations with radiation, chemotherapy, targeted therapy, and immunotherapy (Table 2).

The downstream signaling effects of ONC201 are persistent after drug washout *in vitro* and *in vivo*, which appears to be related to inherent properties of downstream pathways such as ISR rather than target interaction kinetics (e.g. irreversible binding). The sustained PD of ONC201 supports its infrequent administration that yields saturable efficacy in preclinical models or in the clinic. In certain tumor types and locations, dose intensification might be necessary to achieve therapeutic concentrations that continues to be explored in clinical trials. In Phase I clinical trials, dose-limiting toxicities were not observed and 625 mg ONC201 administered to adults once weekly or once every 3 weeks was selected as a biologically optimal dose based on PK and PD profiles. There are 17 ongoing or completed Phase I, Phase I/II, and/or Phase II clinical studies with ONC201 as a single agent or in combination with other anti-cancer therapies (Table 3) that evaluate a wide spectrum of tumor types. These include 4 clinical studies that enroll pediatric and adult high-grade glioma patients, and other clinical trials that enroll cancer patients with tumor types that highly express potentially predictive biomarkers such as DRD2, ClpP and c-myc (Fig. 4). The latter includes neuroendocrine tumors, EC, breast cancer, MM, AML, and NHL. Notably, it is hypothesized that while these are the tumor types for which there is published evidence of enrichment of high DRD2, ClpP or c-myc expression, the applicability of known biomarkers is likely much broader, and investigation of additional tumor types is ongoing based on this information. There is a post-treatment immune signature that has correlated with response to ONC201 for example in patients with prostate cancer [99]. Ongoing exploration of the combinatorial expression of predictive biomarkers is underway, which may provide insight into subtypes or additional tumor types with enhanced imipridone sensitivity. It is possible that either the DRD2 or ClpP binding target may be more relevant depending on the tumor type [7,8,10]. It is also possible that ultimately the immediate drug binding targets may not predict response due to the numerous factors in cells that regulate cell death [31]. Of note, in some studies, DRD2 knockdown or knockout does not impact antitumor efficacy. This could be explained by the significance of ClpP and leads to the belief that a combination of biomarkers is important for ascertaining tumor cell sensitivity across the spectrum of cancers. It is unclear if dopamine receptor-independent ONC201 effects will ultimately be explained by a role or requirement for ClpP in the anti-tumor mechanism. The involvement of the ISR, including ATF4, and the TRAIL pathway may also not be universally required nor predictive of drug effects. While the activity of ONC201 across an array of cancers continues to be evaluated as a single agent and/or in combination with other anti-cancer therapies, molecular subsets of high grade gliomas, such as H3 K27M-mutant glioma, have been prioritized as the lead indication due to the emergence of durable objective responses as a single agent in clinical trials in the recurrent setting. It remains unclear why some patients whose tumors have H3 K27M mutations have durable responses while others do not respond.

ONC201 has the potential to scale broadly across solid tumors and hematological malignancies as a single agent or in combination considering its benign safety profile, highly synergistic mechanism of action, and infrequent oral administration. However, the early clinical studies suggest that it is largely inactive as a monotherapy in most solid tumors. As such, great effort has been directed at combinatorial strategies that elicit potent anti-tumor pro-apoptotic effects (Table 2). The ongoing Phase II trial in neuroendocrine tumors (NCT03034200) may explore combinatorial treatment in the future. In addition to recurrent/refractory advanced cancer patients, ONC201 may be well-suited for administration in the newly diagnosed setting in combination with standard of care, as well as in the adjuvant, maintenance or neoadjuvant setting due to its ability to target cancer stem cells and elicit immune activation. Accordingly, ONC201 is being explored as a maintenance therapy in AML and MDS in the post-transplant setting (NCT03932643). In this regard, given its lack of significant toxicity, ONC201 may be a good candidate for future development as a chemopreventive agent.

Similar to H3 K27M, which drives an epigenetic profile that renders tumor cells more sensitive to ONC201, additional clinical biomarkers such as dopamine receptors, c-myc and ClpP expression may be predictive of tumor cell sensitivity to ONC201 in specific indications (Fig. 4), and this may enable indication prioritization and patient selection. However, further insight into what determines ultimate response in patients is

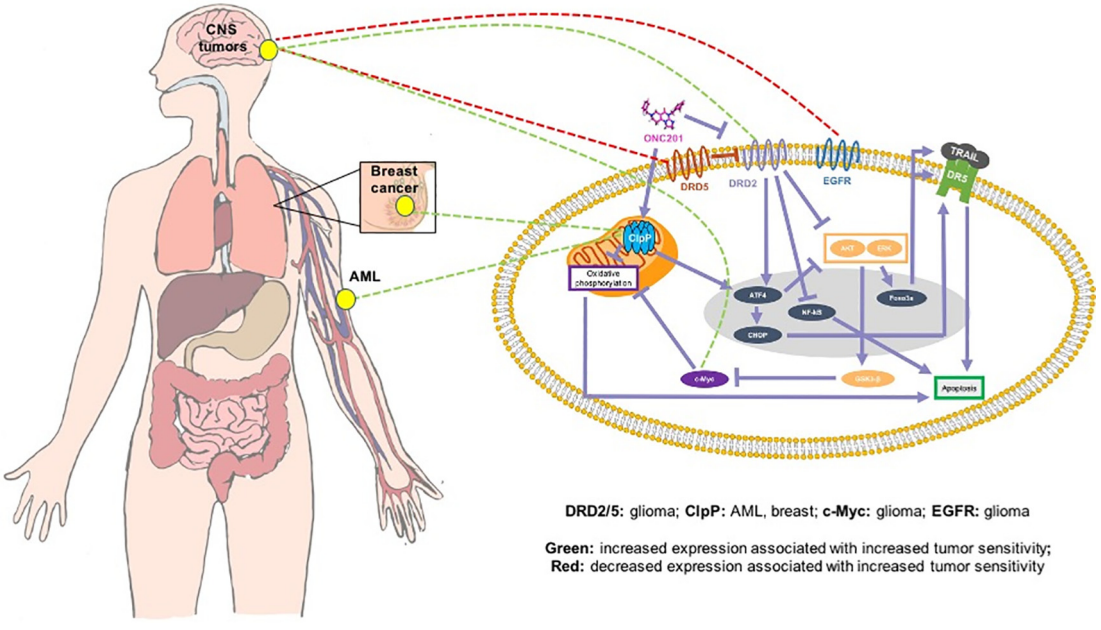

Fig. 4. Candidate predictive biomarkers for ONC201 by tumor location. DRD2/5: glioma; ClpP: AML, breast cancer; c-Myc: glioma; EGFR: glioma. Green: increased expression associated with increased tumor sensitivity to ONC201. Red: decreased expression associated with increased tumor sensitivity to ONC201.

needed to determine whether these biomarkers are suitable for patient selection. Other biomarkers similar to H3 K27M that epigenetically dysregulate gene silencing in a similar manner, such as EZHIP, in indications such as posterior fossa type A ependymoma, remain to be explored in future studies [130,131]. GBM cells that do not exhibit EGFR dysregulation appear to exhibit elevated DRD2 expression, dopamine secretion and increased sensitivity to DRD2 antagonism, providing rationale for a future biomarker-informed clinical trial with ONC201 [132]. These associations require further study to clarify any mechanistic relationships with ONC201 sensitivity or potential for clinical response in patients. Mechanisms of acquired resistance via DRD5 or ClpP mutations have been identified for ONC201 and could be addressed in future studies with combinatorial screening approaches or other imipridones [7,26,73,124]. Many other resistance mechanisms may emerge through future research including analysis of tumor specimens from clinical trials.

The unique tri-heterocyclic core structure of ONC201 and the underexploited nature of its molecular targets in oncology prompted chemical derivatization of the scaffold, leading to the creation of the imipridone class of small molecules following the successful translation of ONC201 to the clinic [118]. New imipridones were created with altered spectrums of anti-cancer activity and potency, as well as differential engagement of targets and target pharmacology [7,10,124,133]. The next imipridone to enter clinical development is ONC206, which is a DRD2 antagonist that has anti-cancer efficacy at nanomolar concentrations [29]. As the third imipridone planned for clinical introduction, ONC212 is an agonist of ClpP [7,10] and may interact with the orphan GPCR tumor suppressor GPR132 [124]. While specific targets of imipridones differ among the GPCR superfamily, it is interesting that this family of compounds consistently induces anti-cancer effects that involve similar downstream signaling such as the inactivation of Akt/ERK, ISR activation, and cell death activation. This common downstream signaling, despite engagement of distinct targets, could be due to the overlapping G proteins that control signaling downstream of the GPCRs and ClpP as a potentially shared target [3,134]. These relationships remain to be explored. Thus, the imipridone chemical scaffold has the potential to selectively and diversely targeting distinct GPCRs and ClpP while possessing a number of conserved characteristics, such as engagement of GPCR and ClpP with unique pharmacology, bioavailability, and chemical stability. Future studies optimizing the selectivity of the imipridone chemical scaffold for either GPCRs or human/bacterial ClpP could provide therapeutic opportunities for many human cancers as well as other diseases. Finally, the immune stimulatory effects and cancer stem cell targeting properties add to the versatility of imipridones and their potential for use in combination therapies to treat cancer.

Author contributions

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: VVP, SM, ARK, LS, MS, RST, and JEA are shareholders and/or employees of Oncoceutics that is developing ONC201 and other imipridones. WSED, LS, WO are co-founders and shareholders of Oncoceutics.

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