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mTOR kinase inhibitors as potential cancer therapeutic drugs

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Abstract

The mammalian target of rapamycin (mTOR) plays a critical role in the positive regulation of cell growth and survival primarily through direct interaction with raptor (forming mTORC complex 1; mTORC1) or rictor (forming mTOR complex 2; mTORC2). The mTOR axis is often activated in many types of cancer and thus has become an attractive cancer therapeutic target. The modest clinical anticancer activity of conventional mTOR allosteric inhibitors, rapamycin and its analogues (rapalogs), which preferentially inhibit mTORC1, in most types of cancer, has encouraged great efforts to develop mTOR kinase inhibitors (TORKinibs) that inhibit both mTORC1 and mTORC2, in the hope of developing a novel generation of mTOR inhibitors with better therapeutic efficacy than rapalogs. Several TORKinibs have been developed and actively studied preclinically and clinically. This review will highlight recent advances in the development and research of TORKinibs and discuss some potential issues or challenges in this area.

Keywords

mTOR; kinase; inhibitors; cancer

1. Introduction

Mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family that also includes PI3K, ataxia–telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), DNA-dependent protein kinase (DNA-PK) and suppressor of morphogenesis in genitalia-1 (SMG-1) [1]. mTOR is a serine-threonine kinase that plays a central role in positively regulating cell growth, survival and other cellular functions primarily through forming two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR and other four associated proteins, raptor, mLST8, PRAS40 and DEPTOR, and controls cell growth in part by regulation of protein translation through phosphorylating S6 kinase (S6K) and eIF4E-binding protein 1 (4E-BP1). mTORC2 contains mTOR, rictor, mLST8, DEPTOR, mSin1 and protor and positively regulates cell survival and proliferation primarily by phosphorylating Akt and serum and glucocorticoid-inducible kinase (SGK) [2; 3; 4; 5]. The

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Conflict of interest None declared

mTOR axis is frequently activated in many types of human cancers, largely due to activation of upstream signaling (e.g., PI3K/Akt) that regulates the mTOR axis, and thus has emerged as a promising cancer therapeutic target [4; 6; 7].

The conventional mTOR inhibitor rapamycin (sirolimus) and its analogues (rapalogs) are specific allosteric inhibitors of mTOR which act through binding and recruiting an accessory protein named FKBP12 to the FRB domain of mTOR. They are generally thought to have weak activity against mTORC2 [8]. Some rapalogs such as CCI-779 (temsirolimus) and RAD001 (everolimus) have been actively tested in various phases of oncology clinical trials [9; 10] and have demonstrated encouraging clinical efficacy in treatment of patients with metastatic renal cell carcinoma [11; 12; 13], pancreatic neuroendocrine tumors [14], or postmenopausal hormone receptor-positive advanced breast cancer [15]. However, the single agent activity of rapalogs in most other cancer types has been modest at best [7; 16; 17]. This may be largely due to the fact that rapalogs lack effective activity against mTORC2, exert incomplete inhibitory effects on mTORC1, and induce feedback activation of Akt, Mnk/eIF4E and ERK/RSK2 survival pathways that attenuate their anticancer efficacy [8; 18; 19; 20; 21].

The discovery of mTORC2 as an Akt S473 kinase [22] and subsequent demonstration of its involvement in cancer development [23; 24] have spurred efforts to identify novel mTOR inhibitors that inhibit both mTORC1 and mTORC2 activity. As a result, several ATP-competitive inhibitors of mTOR (i.e., mTOR kinase inhibitors; TORKinibs) such as PP244, INK126, Torin 1, AZD8055 and WYE-354 have been developed and tested in clinical trials [9; 25; 26; 27]. TORKinibs have been shown to more dramatically inhibit protein synthesis, suppress Akt S473 phosphorylation and induce G1 arrest and/or apoptosis in some cancer cells than rapamycin [28; 29; 30; 31]. A robust *in vivo* anticancer activity of these inhibitors against certain types of cancers was also observed [29; 32; 33]. The current review will focus on highlighting recent advances in development of TORKinibs as potential cancer therapeutic agents and discussing some related issues. Dual PI3K/mTOR kinase inhibitors (e.g., BEZ235) will not be included in the review.

2. Preclinical studies of TORKinibs

Chemical structures of different TORKinibs are presented in Fig. 1.

2.1. PP242

The chemical name of PP242 is 2-[4-Amino-1-(1-methylethyl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl]-1H-indol-5-ol. Developed by Intellikine Inc/Takeda (La Jolla, CA), PP242 is one of the earliest TORKinibs reported and has been widely used in the laboratory as a research tool. It inhibits mTOR *in vitro* with half-maximal inhibitory concentration (IC_{50} value) of 8 nM. Testing of PP242 against 219 purified protein kinases at a concentration 100-fold higher than its mTOR IC_{50} value revealed exceptional selectivity with respect to the protein kinome; most protein kinases were unaffected by this drug, and only four, PKC- , PKC- , RET, and JAK2 (V617F), were inhibited by more than 80%. At relatively high concentration, it also inhibited DNA-PK (IC_{50} = 0.408 M) [28]. A recent study using 10 kinases present in the Invitrogen SelectScreen® PIKK panel demonstrated that PP242 exhibited a low IC_{50} value against PI3K-C2β, PI3Kδ, and DNA-PK (IC_{50} < 100 nM) and moderate IC_{50} values for PI3K-C2α, PI3Kα, PI3Kβ, and PI3Kγ (IC_{50} ~100–1000 nM) [34].

Unlike rapamycin, PP242 inhibits mTORC2, as evidenced by its ability to block Akt S473 phosphorylation and prevent its full activation including suppression of Akt T308 phosphorylation. PP242 inhibited proliferation of primary cells more completely than

rapamycin. However, mTORC2 inhibition is not the basis for this enhanced activity. In fact, PP242 is a more effective mTORC1 inhibitor than rapamycin since it effectively inhibits the phosphorylation of 4E-BP1 at T36/45 and S65, which is only modestly affected by rapamycin, and cap-dependent translation [28].

In models of acute leukemia harboring the Philadelphia chromosome (Ph) translocation, PP242, but not rapamycin, was shown to cause death of mouse and human leukemia cells. *In vivo*, PP242 delayed leukemia onset and augmented the effects of the current front-line tyrosine kinase inhibitors more effectively than rapamycin did. Moreover, PP242 had much weaker effects than rapamycin on the proliferation and function of normal lymphocytes [35]. In agreement, PP242 effectively induced apoptosis in primary leukemic samples cultured with or without stroma and demonstrated a greater anti-leukemia effect than rapamycin in an *in vivo* leukemia mouse model [36]. In multiple myeloma (MM) cells, PP242 effectively inhibited Akt S473 phosphorylation and was more effective than rapamycin in achieving cytoreduction and apoptosis. In addition, PP242 was effective against primary MM cells *in vitro* and growth of 8226 cells in mice [32]. Similarly, PP242 was shown to be more effective than rapamycin in reducing the growth and survival of colon cancer cells including rapamycin-resistant cells. Moreover, it inhibited the growth of colon cancer xenografts in mice [37].

Besides its single agent activity, PP242 can also enhance the anticancer activity of other agents. In hepatocellular carcinoma cells, PP242 was shown to significantly enhance histone deacetylase inhibitor (i.e., SAHA, LBH589)-induced apoptosis. This enhanced apoptosis-inducing effect seems to be associated with their increased effects on suppressing Akt signaling and upregulating Bim [38]. In MM cells, synergistic anti-cancer effects, including induction of apoptosis, were also observed when PP242 was combined with the proteasome inhibitor bortezomib [32]. Recently, PP242 was shown to induce ERK1/2 activation while suppressing mTOR signaling in MM and colon cancer cells [37] as rapalogs do [20; 21]. Accordingly, the combination of PP242 and U0126, a MEK inhibitor, enhanced tumor-suppressive activity both in cell cultures and in xenograft models [37].

2.2. INK128

The chemical name of INK128 (also called MLN-0128) is 3-(2-amino-5-benzoxazolyl)-1-(1-methylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. INK128 is an orally bioavailable analog of PP242 developed by Intellikine Inc/Takeda and is currently being tested in clinical trials. INK128 inhibits mTOR kinase at sub-nanomolar concentration and demonstrates a high degree of selectivity against closely related kinases as well as against a panel of more than 400 kinases with favorable pharmaceutical properties [39].

INK128 was much more active than rapamycin in inhibiting the growth of MM cells, even in the presence of cytokines or stromal cells, as was shown for the inhibition of both mTORC1 and mTORC2 through co-knockdown of rictor and raptor [40]. Similarly, INK128 was also more potent than rapamycin and PP242 in suppressing proliferation of B-cell acute lymphoblastic leukemia (B-ALL) cell lines *in vitro* and reducing colony formation by primary human leukemia cells from adult and pediatric B-ALL patients [41]. Moreover, INK128 augmented the efficacy of dasatinib, an oral multi-BCR/ABL and Src family tyrosine kinase inhibitor, in Philadelphia chromosome-positive (Ph+) tumors. In a syngeneic mouse model of lymphoid BCR-ABL+ disease, daily oral dosing of INK128 rapidly cleared leukemic outgrowth. In primary Ph+ B-ALL xenografts, INK128 significantly enhanced the efficacy of dasatinib. In non-Ph B-ALL xenografts, single agent INK128 had a cytostatic effect that was most pronounced in mice with low disease burden. Importantly, in all *in vivo* models, INK128 was well tolerated and did not suppress endogenous bone marrow proliferation [41].

In a study in breast cancer cells, the combination of lapatinib, a dual HER2/neu and EGFR tyrosine kinase inhibitor, with INK128 prevented both HER2 and HER3 phosphorylation induced by INK128 and produced synergistic induction of cell death in different HER2-positive cell lines resistant to trastuzumab and lapatinib. *In vivo*, both cell line-based and patient-derived xenografts showed exquisite sensitivity to the antitumor activity of the combination of lapatinib and INK128, which resulted in durable tumor shrinkage and exhibited no signs of toxicity in these models [42].

In contrast to RAD001, INK128 induces apoptosis in different cancer cell lines. In an *in vivo* mouse study, INK128 treatment resulted in a 50% decrease in prostatic intraepithelial neoplasia (PIN) lesions in $Pten^{L/L}$ mice, which was associated with decreased proliferation and increased apoptosis. The unique cytotoxic properties of INK128 treatment in $Pten^{L/L}$ mice were evidenced by a marked reduction in prostate cancer volume. RAD001 treatment mainly had cytostatic effects leading to only partial regression of PIN lesions associated with a limited decrease in cell proliferation and no significant effect on apoptosis. Remarkably, treatment with INK128 completely blocked the progression of invasive prostate cancer locally in the prostate gland, and profoundly inhibited the total number and size of distant metastases [39].

2.3. AZD8055 and AZD2014

The chemical name of AZD8055 is (5-{2,4-bis[(3*S*)-3-methylmorpholin-4-yl]pyrido[2,3-*d*]pyrimidin-7-yl}-2-methoxyphenyl)methanol. AZD8055 is an orally bioavailable TORKinib developed by AstraZeneca (Cheshire, United Kingdom). It inhibits mTOR kinase with an IC_{50} of 0.8 nM (using native mTOR enzyme complexes extracted from HeLa cells) and shows excellent selectivity (approximately 1,000-fold) against all class I PI3K isoforms and other members of the PIKK family. Furthermore, AZD8055 has no significant activity against a panel of 260 kinases at concentrations up to 10 M [33]. AZD8055 inhibits the phosphorylation of mTORC1 substrates S6K and 4E-BP1 as well as phosphorylation of the mTORC2 substrate Akt and downstream proteins. The rapamycin-resistant T37/46 phosphorylation sites on 4E-BP1 are fully inhibited by AZD8055, resulting in significant inhibition of cap-dependent translation. The cellular IC_{50}s for AZD8055 are around 24 nM for p-Akt (S473) and 27 nM for p-S6 (S235/236). AZD8055 caused a concentration-dependent decrease in NDRG1 phosphorylation [33], a useful surrogate for SGK activity and a marker of mTORC2 function [30; 43; 44].

In vitro, AZD8055 potently inhibited proliferation of several cancer cell lines, with IC_{50} values ranging 20 to 50 nM. *In vivo*, AZD8055 induced a dose-dependent pharmacodynamic effect on p-S6 and p-Akt at plasma concentrations ~12 nM, leading to tumor growth inhibition, and resulted in significant growth inhibition and/or regression of xenografts representing a broad range of human tumor types including breast, lung, colon, prostate, and uterine [33]. Moreover, AZD8055 was shown to decrease acute myeloid leukemia (AML) blast cell proliferation and cell cycle progression, reduce the clonogenic growth of leukemic progenitors and induce caspase-dependent apoptosis in leukemic cells but not in normal immature CD34+ cells. *In vivo*, AZD8055 markedly increased the survival of AML-transplanted mice through a significant reduction of tumor growth, without apparent toxicity [45].

AZD8055 demonstrated broad *in vitro* activity against a panel of pediatric cancer lines with a median relative IC_{50} of 24.7 nM. However, at the dose and schedule (daily dosing of 20 mg/kg) studied, it demonstrated limited activity *in vivo* against pediatric solid tumor and ALL panels [46]. AZD8055 significantly inhibited 4E-BP1 (T37/46), S6 (S235/236), and Akt (S473) phosphorylation following day 1 and day 4 dosing, but suppression of mTORC1 or mTORC2 signaling did not predict tumor sensitivity [46].

CD40 is a tumor necrosis factor (TNF) receptor family member that plays a critical role in both humoral and cellular immune responses. Agonistic αCD40 antibodies, a potent mimic of the natural ligand CD154, have been shown to promote T-cell–mediated immunity in the treatment of cancers in experimental animal models [47; 48]. The combination of αCD40 agonistic antibody and AZD8055 elicited synergistic antitumor responses in a model of metastatic renal cell carcinoma. In contrast to rapamycin, AZD8055 increased the infiltration, activation, and proliferation of CD8(+) T cells and natural killer cells in liver metastatic foci when combined with the CD40 agonist. AZD8055/αCD40-treated mice also displayed an increased incidence of matured macrophages and dendritic cells compared with that achieved in mice by αCD40 or AZD8055 treatment alone. The combination treatment also increased macrophage production of TNFα, which plays an indispensable role in activation of the observed antitumor immune response. Levels of Th1 cytokines, including interleukin 12, IFN-γ, TNFα, and the Th1-associated chemokines RANTES, MIG, and IP-10 were each elevated significantly in the livers of mice treated with the combinatorial therapy versus individual treatments. Notably, the AZD8055/α CD40-induced antitumor response was abolished in IFN-γ (−/−) and CD40 (−/−) mice, establishing the reliance of the combination therapy on host IFN-γ and CD40 expression [49]. These findings offer a preclinical proof of concept that a TORKinib such as AZD8055, combined with αCD40 treatment, can contribute to initiating a restructuring of the tumor immune microenvironment to induce regression of an established metastatic cancer.

The therapeutic effects of combining the MEK inhibitor selumetinib (AZD6244) with AZD8055 were studied. Concurrent dosing in nude mouse xenograft models of human lung cancer (Calu-6, A549, H2122 and H460) and colorectal carcinoma (LoVo and HCT-116) was well tolerated and produced increased antitumor efficacy relative to the respective monotherapies. Pharmacodynamic analysis documented reciprocal pathway inhibition associated with increased apoptosis and Bim expression in tumor tissue from the combination group [50]. Similarly, the combination of AZD8055 and the histone deacetylase inhibitor SAHA almost completely inhibited tumor growth, without obvious adverse effects, by abrogating Akt and upregulating Bim; while either agent alone showed only 30% inhibition in primary hepatocellular carcinoma xenografts [38].

Several studies have shown that AZD8055 induces autophagy in cancer cells [33; 45; 51]. Moreover, AZD8055 potentiated chemotherapy-mediated autophagy [51]. AZD8055-induced autophagy is pro-survival as shown by its ability to attenuate cell death and DNA damage (p-H2AX), and to enhance clonogenic survival by cytotoxic chemotherapy. Autophagy inhibition by siRNA against Beclin 1 or LC3B, or by chloroquine, partially reversed the cytoprotective effect of AZD8055 [51]. Thus it is cautious to consider this potential negative impact of TORKinibs on the efficacy of chemotherapy in the clinic.

Further optimization of AZD8055, particularly focused on reducing turnover in human hepatocytes, has resulted in the discovery of the clinical candidate AZD2014. Its chemical name is 3-[2,4-Bis((3S)-3-methylmorpholin-4-yl)pyrido[5,6-e]pyrimidin-7-yl]-N-methylbenzamide. AZD2014 potently inhibits mTOR kinase (IC_{50} = 0.0028 μM) and displays a high level of selectivity against other members of the PIKK family (IC_{50} against PI3K isoforms α, β, γ, δ = 3.8, >30, >30 and >29 μM, respectively) and is inactive against a general panel of over 200 kinases when tested at 10 μM. This compound inhibits both mTORC1 and mTORC2 in vitro (pS6 ($S^{235/236}$) IC_{50} = 0.2 μM and pAKT (S^{473}) IC_{50} = 0.08 μM) and has shown dose-dependent tumor growth inhibition in a mouse MCF7 xenograft model alongside modulation of mTORC1 and mTORC2 biomarkers. Compared to AZD8055, AZD2014 shows improved solubility and reduced turnover rate in human hepatocyte incubations and thus has progressed to the clinical trials [52].

2.4. OSI-027

The chemical name of OSI-027 is 4,5,7-trisubstituted imidazo[5,1-*f*]triazine. This bioavailable compound is developed by OSI Pharmaceuticals (Farmingdale, NY) and is currently in clinical trials. OSI-027 inhibits both mTORC1 and mTORC2 with biochemical IC_{50} values of 22 nM and 65 nM, respectively. It shows more than 100-fold selectivity for mTOR relative to PI3Kα, PI3Kβ, PI3Kγ, and DNA-PK. When testing its selectivity against 101 kinases by using Caliper kinase profiling assays, OSI-027 did not inhibit any of those kinases by more than 50%. OSI-027 inhibited phosphorylation of the mTORC1 substrates 4E-BP1 and S6K1 as well as the mTORC2 substrate Akt in diverse cancer models *in vitro* and *in vivo* [53].

OSI-027 potently inhibited proliferation of several rapamycin-sensitive and - insensitive non-engineered and engineered cancer cell lines with IC_{50} values ranging from 0.4 to 4.5 μM. Moreover, OSI-027 induced 10% to 50% cell death in the majority of rapamycin-sensitive cell lines. OSI-027 also showed robust antitumor activity in several different human xenograft models representing various histologies including colon, ovarian, breast, lung, prostate, lymphoma, and head and neck cancer. In colon cancer xenograft models, OSI-027 showed superior efficacy compared with rapamycin [53].

OSI-027 also induced potent suppressive effects on primitive leukemic progenitors from CML patients and generated anti-leukemic responses in cells expressing the T315I-BCR-ABL mutation, which is refractory to all BCR-ABL kinase inhibitors currently in clinical use [54]. A similar suppressive effect was also observed against primitive leukemic precursors from AML patients [55]. Moreover, it has been shown that induction of apoptosis by OSI-027 appears to negatively correlate with induction of autophagy in some types of BCR-ABL transformed cells, as shown by the induction of autophagy during OSI-027-treatment and the potentiation of apoptosis by concomitant inhibition of such autophagy [54]. In another study with cell lines and clinical samples representing diverse lymphoid malignancies, OSI-027, in contrast to rapamycin, markedly diminished proliferation and induced apoptosis in a variety of lymphoid cell lines and clinical samples, including specimens of B-cell ALL, mantle cell lymphoma, marginal zone lymphoma and Sezary syndrome [56].

When combined with the EGFR inhibitor erlotinib, enhanced biochemical effects on the suppression of mTOR signaling and synergistic growth inhibition *in vitro* in head and neck cancer cells were observed. Treatment of mice bearing head and neck cancer xenografts with a combination of the FDA-approved EGFR antibody cetuximab and OSI-027 demonstrated a significant reduction of tumor volumes compared with either treatment alone [57].

2.5. Torin 1 and Torin 2

The chemical name of Torin 1 is 1-[4-[4-(1-oxopropyl)-1-piperazinyl]-3-(trifluoromethyl)phenyl]-9-(3-quinolinyl)benzo[h]-1,6-naphthyridin-2(1H)-one. Torin 1 was synthesized and developed from quinoline 1, which was identified in a biochemical mTOR assay by a group of scientists at the Dana Farber Cancer Institute (Boston, MA) [58]. In *in vitro* kinase assays using immuno-purified mTORC1 or mTORC2, Torin 1 inhibits both mTOR-containing complexes with IC_{50} values between 2 and 10 nM and acts through an ATP-competitive mechanism. The IC_{50} for Torin 1 in cells is also between 2 and 10 nM. Unlike rapamycin, Torin 1 had no effect on the stability of either mTORC1 or mTORC2. Torin 1 is selective for mTOR over related kinases. IC_{50} values for Torin1 were determined using *in vitro* kinase assays for mTOR (3 nM), hVps34 (3 μM), PI3K-α (1.8 μM), DNA-PK (1.0 μM), and ATM (0.6 μM). Moreover, it exhibited 100-fold binding selectivity relative to

450 other protein kinases [31; 58]. A recent study using 10 kinases present in the Invitrogen SelectScreen® PIKK panel has shown that Torin 1 potently inhibits DNA-PK (IC_{50} ~6.3 nM) and moderately inhibits PI3K-C2α, PI3K-C2 , hVPS34, PI3Kα, PI3K , and PI3Kγ (IC_{50} ~150–600 nM) [34]. Even at the cellular level, Torin 1 potently inhibited PI3K activity (IC_{50} ~60 nM) albeit with a 12-fold higher concentration over that needed for inhibition of mTORC2 (IC_{50} ~5 nM) [34].

Torin 1 impaired cell growth and proliferation (e.g., induction cell cycle arrest) to a far greater degree than rapamycin [31]. Torin1 was efficacious at a dose of 20 mg/kg in a U87MG xenograft model and demonstrated good pharmacodynamic inhibition of downstream effectors of mTOR (e.g., p-S6 S235/236 and p-Akt S473) in tumor and peripheral tissues [58]. Interestingly, the authors suggested that these effects of Torin 1 are independent of mTORC2 inhibition and are instead because of suppression of rapamycin-resistant functions of mTORC1 that are necessary for cap-dependent translation and suppression of autophagy. These effects are at least partly mediated by suppression of mTORC1-dependent and rapamycin-resistant phosphorylation of 4E-BP1 (T37/46) [31].

Starting with Torin 1, a focused medicinal chemistry effort led to the discovery of an improved mTOR inhibitor, Torin 2. The chemical name of Torin 2 is 9-(6-amino-3-pyridinyl)-1-[3-(trifluoromethyl)phenyl]-benzo[*h*]-1,6-naphthyridin-2(1*H*)-one. Torin 2 possesses an EC_{50} of 0.25 nM in the inhibition of cellular mTOR activity. Torin 2 exhibits 800-fold selectivity over PI3K (IC_{50} ~200 nM) and over 100-fold binding selectivity relative to 440 other protein kinases. Torin 2 has significantly improved bioavailability (54%), metabolic stability, and plasma exposure relative to Torin 1 [59]. The newest study has shown that Torin 2 also exhibits potent biochemical and cellular activity against PIKK family kinases including ATM (EC_{50}, 28 nM), ATR (EC_{50}, 35 nM), and DNA-PK (EC_{50}, 118 nM). Single-agent treatment with Torin 2 *in vivo* did not yield significant efficacy against KRAS-driven lung tumors, but its combination with MEK inhibitor AZD6244 yielded a significant growth inhibition [60].

2.6. WAY-600, WYE-687, and WYE-354

The chemical names of these TORKinibs are 6-(1H-indol-5-yl)-4-morpholino-1-(1-(pyridin-3-ylmethyl)piperidin-4-yl)-1H-pyrazolo[3,4-d]pyrimidine, methyl 4-(4-morpholino-1-(1-(pyridin-3-ylmethyl)piperidin-4-yl)-1H-pyrazolo[3,4-d]pyrimidin-6-yl)phenylcarbamate, and 4-[6-[4-[(methoxycarbonyl)amino]phenyl]-4-(4-morpholinyl)-1H-pyrazolo[3,4-d] pyrimidin-1-yl]-1piperidinecarboxylic acid methyl ester, respectively. These compounds were synthesized by Wyeth Research (Pearl River, NY) based on the lead compound WAY-001, which was discovered through high-throughput screening of a chemical library against a recombinant mTOR enzyme and was 6-fold more potent against PI3Kα than against mTOR [61]. They are potent mTOR kinase inhibitors (IC_{50}, 5–9 nM) with significant selectivity over PI3K isofoms (> 100-fold). In a cell-based assay, several members of the PIKK family can be activated by the cancer drug etoposide (VP-16). VP-16–induced PIKK substrate phosphorylations of p-p53 (S15) and p-Chk1 (S345) were not blocked by these inhibitors at 3 M, indicating that the underlying PIKKs are not targeted [61].

Unlike the rapalogs, these inhibitors acutely block substrate phosphorylation (e.g., p-4E-BP1 T37/46 and p-Akt S473) by mTORC1 and mTORC2 *in vitro* and in cells in response to growth factor, amino acids, and hyperactive PI3K/Akt. Unlike the inhibitors of PI3K or dual-pan PI3K/mTOR, cellular inhibition of p-S6K1 (T389) and p-Akt (S473) by these agents occurs at significantly lower concentrations than that of p-Akt (T308) (PI3K-PDK1 readout), showing mTOR selectivity in the cellular se tting. These agents reduced Akt downstream function and inhibited proliferation of diverse cancer cell lines with IC_{50} values

generally in the sub-micromolar to single-digit micromolar range. These effects correlated with a strong G_1 cell cycle arrest in both rapamycin-sensitive and rapamycin-resistant cells, selective induction of apoptosis, repression of global protein synthesis, and down-regulation of angiogenic factors. When injected into tumor-bearing mice, WYE-354 inhibited mTORC1 and mTORC2 and displayed robust antitumor activity in PTEN-null tumors [61].

2.7. Ku-0063794

The chemical name of Ku-0063794 is (5-(2-((2R,6S)-2,6-dimethylmorpholino)-4-morpholinopyrido[2,3-d]pyrimidin-7-yl)-2-methoxyphenyl)methanol. This is also one of the earliest TORKinibs reported. Ku-0063794 inhibits both mTORC1 and mTORC2 with an IC_{50} of approximately 10 nM, but does not suppress the activity of 76 other protein kinases or seven lipid kinases, including Class 1 PI3Ks at 1000-fold higher concentrations. Ku-0063794 suppresses activation and hydrophobic motif phosphorylation of Akt, S6K and SGK, but not of ribosomal S6 kinase (RSK), an AGC kinase not regulated by mTOR. Ku-0063794 also inhibits Akt T308 phosphorylation by PDK1. This is likely secondary to phosphorylation of Akt S473 that promotes phosphorylation of T308 and/or induces a conformational change that protects T308 from dephosphorylation. In contrast, Ku-0063794 does not affect T308 phosphorylation in fibroblasts lacking essential mTORC2 subunits, suggesting that signaling processes have adapted to enable T308 phosphorylation to occur in the absence of S473 phosphorylation. Like other TORKinibs, Ku-0063794 induced a much greater dephosphorylation of 4E-BP1 than rapamycin, even in mTORC2-deficient cells, suggesting a form of mTOR distinct from mTORC1, or mTORC2 phosphorylation of 4E-BP1 [30]. Ku-0063794 suppressed cell growth and induced a G1 cell-cycle arrest. However, information is lacking regarding its effects on cancer cell growth *in vitro* and *in vivo*.

3. Clinical studies of TORKinibs

To date, a few TORKinibs (e.g., INK128, OSI-027, AZD8055 and AZD2014) have been tested in phase I clinical trials (Table 1), most in patients with advanced solid malignancies [8; 62]. Except for AZD8055, clinical trials with other TORKinibs have not been reported. In a recently completed phase 1 clinical trial that recruited 49 patients with advanced solid malignancies or lymphomas who took AZD8055 orally twice-daily (BID) at doses starting at 10 mg, dose-limiting toxicities were found within the 40 mg to 120 mg (BID) range; all were grade 3 rises in transaminases, reversible in all patients, apart from one who had liver metastases. The maximum tolerated dose (MTD) was defined as 90 mg BID. The most frequent adverse events assessed to be related to AZD8055 were increased alanine aminotransferase (22%), increased aspartate aminotransferase (22%) and fatigue (16%). AZD8055 was rapidly absorbed (median t_{max} 0.5 h) and exposure increased with increasing doses. Seven patients had stable disease for ≥ 4 months. Partial metabolic responses, assessed by fluorodeoxyglucose positron emission tomography, were observed at ≥ 40 mg BID (n = 8 at day 35). Overall, apart from elevated transaminases, which occurred at most dose levels, the drug had an acceptable toxicity profile; however, no RECIST responses were seen [63].

A similar study in Japanese patients with advanced solid tumors was also reported recently. It was suggested that the tolerability (e.g., MTD) and pharmacokinetic profiles of AZD8055 in Japanese patients are similar to those reported in Western patients. Among 17 patients in the trial, no responses were reported, but two patients had stable disease although mean p-Akt and p-4E-BP1 levels decreased in most cohorts [64].

4. Impact of genetic alterations on cancer cell responses to TORKinibs

Certain genetic alterations such as frequent mutations in PIK3CA, loss of expression of PTEN and/or over-expression of receptor tyrosine kinases can result in hyper-activation of the PI3K/mTOR axis and may confer sensitivity to agents that target this axis. In a study of a panel of 31 breast cancer cell lines, it was shown that breast cancer cells harboring PIK3CA mutations are selectively sensitive to RAD001 and PP242. However, cells with PTEN loss of function were not sensitive to these drugs, suggesting that the functional consequences of these two mechanisms of activation of the mTOR pathway are quite distinct. In addition, a subset of HER2-amplified cell lines showed increased sensitivity to PP242, but not to RAD001, irrespective of the PIK3CA/PTEN status. These selective sensitivities were confirmed in more physiologically relevant three-dimensional cell culture models [65].

5. Selectivity and potency of TORKinibs

Since TORKinibs are ATP-competitive kinase inhibitors, selectivity and potential off-target effects are always an unavoidable issue. Recently a systematic kinome-wide effort to profile the selectivity and potency of some TORKinibs (Torin 1, PP242, Ku-0063794 and WYE354) using chemical proteomics and assays for enzymatic activity, protein binding, and disruption of cellular signaling was conducted [34]. Enzymatic and cellular assays revealed that all four compounds are potent inhibitors of mTORC1 and mTORC2, with Torin1 exhibiting ~20-fold greater potency in the inhibition of S6K T389 phosphorylation (EC_{50} = 2 nM) relative to other inhibitors. *In vitro* biochemical profiling at 10 μM revealed binding of PP242 to numerous kinases, although WYE354 and Ku-0063794 bound only to p38 kinases and PI3K isoforms and Torin1 to ATM and Rad3-related protein, and DNA-PK. Analysis of these protein targets in cellular assays did not reveal any off-target activities for Torin1, WYE354, and Ku-0063794 at concentrations below 1 μM but did show that PP242 efficiently inhibited the RET receptor (EC_{50}, 42 nM) and JAK1/2/3 kinases (EC_{50}, 780 nM) [34].

Hence, the concentration is a critical factor when considering the selectivity and potency of a given TORKinib. In general, selectivity will be reduced as the concentration increases, although the potency will be increased. Many studies have claimed that TORKinibs are more potent than rapalogs (e.g., rapamycin) in suppressing growth and inducing apoptosis of cancer cells. However these studies used much higher concentrations (e.g., M ranges) of TORKinibs compared with lower concentrations of rapalogs (e.g., nM ranges). For example, PP242 inhibits mTOR at low nM ranges [28; 34]; however, up to 40-fold higher concentrations of PP242 (up to 2.5 M) than rapamycin (up to 62 nM) were used to indicate that PP242 is more potent than rapamycin in suppressing mTOR signaling (e.g., p-4E-BP1 T36/45 and p-Akt) [28]. OSI-027 inhibits both mTORC1 and mTORC2 with biochemical IC_{50} values of 22 nM and 65 nM, respectively [53]. However 10-20 M OSI-027 was used to compare with 10-20 nM rapamycin to conclude that OSI-027 is much more potent than rapamycin in inhibiting mTOR signaling, suppressing cell growth and inducing apoptosis in cancer cells [54; 55; 56].

Rapalogs typically induce cell cycle arrest with minimal effect on induction of apoptosis. Some studies have shown that TORKinibs (e.g., OSI-027) induce apoptosis of cancer cells. However very high concentrations (10-20 M) of TORkinibs were used to show this induction of apoptosis [53; 54; 55; 56], and it seems unlikely that such a biological activity of TORKinibs is purely due to inhibition of mTOR.

6. Feedback activation of survival signaling pathway

It is well known that rapalogs induce feedback activation of Akt, ERK1/2 and eIF4E survival signaling while inhibiting the mTOR axis, which in turn attenuates the anticancer efficacy of rapalogs [66]. In a recent study, AZD8055 was shown to inhibit mTORC2 and Akt S473 phosphorylation, which led to Akt T308 dephosphorylation and suppression of Akt activity and downstream signaling. However, these latter effects were transient. Inhibition of mTOR kinase also relieves feedback inhibition of receptor tyrosine kinases (RTK), leading to subsequent PI3K activation and rephosphorylation of Akt T308 sufficient to reactivate Akt activity and signaling. Thus, catalytic inhibition of mTOR kinase leads to a new steady state characterized by profound suppression of mTORC1 and accumulation of activated Akt phosphorylated on T308, but not S473. Accordingly, combined inhibition of mTOR kinase (e.g., with AZD8055) and the induced RTKs (e.g., with laptinib) fully abolished Akt signaling and resulted in substantial cell death and tumor regression *in vivo* [67].

In addition, PP242 was shown to induce ERK activation in MM cell lines as well as primary cells, which was not correlated with S6K inhibition nor was it prevented by PI3K inhibition. ERK activation can be prevented by MEK inhibitors and is associated with concurrent stimulation of Raf kinase activity but not Ras activation. Knockdown studies confirmed mTORC1 inhibition was the key proximal event that resulted in ERK activation. Furthermore, ectopic expression of eIF4E blunted PP242-induced ERK phosphorylation. Since PP242 was more potent than rapamycin in causing sequestering of eIF4E, an mTORC1/4E-BP1/eIF-4E-mediated mechanism of ERK activation could explain the greater effectiveness of PP242. Use of MEK inhibitors confirmed that ERK activation served as a mechanism of resistance to the lethal effects of PP242. Thus, although TORKinibs overcome Akt activation often seen with rapalog therapy, feedback ERK activation is still a problem of resistance and is more severe than that seen with use of rapalogs [68]. In breast cancer cells, PP242 was shown to increase p-MAPK levels and the combination of PP242 and U0126 generated enhanced inhibitory effects on the growth of breast cancer cells both *in vitro* and *in vivo* [37]. Similarly, Ku-0063794 was shown to activate MAPK in endothelial cells (e.g., HUVEC); this effect is likely due to inhibition of mTORC2 because knockdown of raptor (mTORC1) but not rictor (mTORC2) had similar effects. Accordingly treatment of endothelial cells with Ku-0063794 in combination with U0126 reduced endothelial cell survival, proliferation, migration and tube formation more significantly than either inhibitor alone. Similarly, in a tumor xenograft model, the anti-angiogenic efficacy of TORKinibs was enhanced by the pharmacological blockade of MAPK [69].

7. Conclusions and perspectives

The identification of TORKinibs not only provides us with valuable research tools for fully understanding the biological functions of mTORCs essential for regulation of cell proliferation and survival, but also promises the development of new and efficacious anticancer drugs. Thus, the efforts made in this regard are absolutely important and appreciated. Various preclinical studies using different cancer models have generated encouraging results. However, eventual success relies on clinical testing and outcomes of these agents. Although most clinical trials with TORKinibs have not been completed, the phase 1 results with AZD8055 have not demonstrated major responses thus far [63; 64].

Most studies have claimed that the different TORKinibs potently inhibit mTORC2 activity, generally with suppression of p-Akt S473 as a readout. However, some studies have suggested that TORKinibs exert their growth inhibitory effects or anticancer effects through strong inhibition of mTORC1 signaling, particularly p-4E-BP1 T37/46, rather than through

inhibition of mTORC2 [28; 31]. Moreover, suppression of mTORC1 or mTORC2 signaling does not predict tumor sensitivity as suggested in a recent study [46]. Therefore, it is still critical to further elucidate the involvement of mTORC2 in the regulation of cell growth and cancer development and to identify real or additional pharmacodynamic biomarkers that can more precisely predict tumor response; these efforts will certainly accelerate the success of TORKinibs as cancer therapeutic agents.

Like other successful targeted cancer therapies, selection of the patient population who may benefit most from TORKinib-targeted therapy is a critical issue we should consider. There are few reports in this regard although an effort has been made in this direction [65]. Hence, the identification of potential predictive biomarkers including genetic alteration of certain critical oncogenes or tumor suppressor genes that can be used to select patients with different types of cancers for TORKinib-based treatment should be actively pursued.

Similar to rapalogs, recent studies have suggested that mTORKinibs can initiate feedback activation of certain cell survival signaling pathways (e.g., Akt and ERK), which in turn results in attenuation of TORKinibs' therapeutic efficacies and likely development of resistance to TORKinibs [67; 68]. Strategically, interruption or blockage of these feedback activations will not only enhance the therapeutic efficacy of TORKinibs, but also prevent development of eventual resistance to TORKinibs. Thus, rational combinations will be an effective strategy to enhance TORKinib-based cancer therapy.

Nonetheless, considerable effort has been made thus far in the development of TORKinibs. Further efforts to overcome certain limitations as we discussed will certainly accelerate the success of TORKinibs as efficacious anticancer drugs. Albeit with the strong rationale of targeting both mTORC1 and mTORC2, it is still too early to tell whether TORKinibs are indeed superior to rapalogs.

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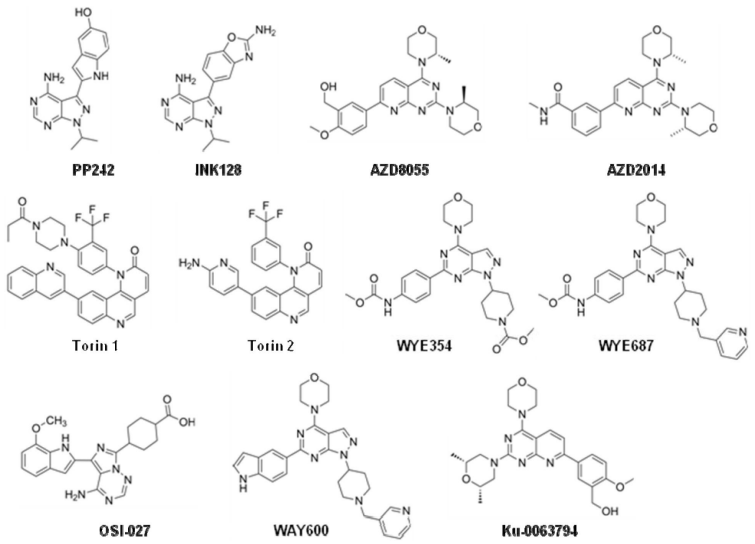

Figure 1.
Chemical structures of TORKinibs.

Table 1

TORkinibs in clinical trials

Agent	Company	Clinical trial	Status
AZD8055	AstraZeneca	Phase 1/II	Finished or discontinued
AZD2014	AstraZeneca	Phase I	Ongoing
OSI-027	OSI Pharmaceuticals	Phase I	Ongoing
INK128/ MLN-0128	Intellikine Inc/Takeda	Phase I	Ongoing