RES-529 (previously named Palomid 529, P529) is a phosphoinositide 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) pathway inhibitor that interferes with the pathway through both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) dissociation. This compound is currently being developed in oncology and ophthalmology. The oncology focus is for the treatment of glioblastoma, where it has received orphan designation by the US Food and Drug Administration, and prostate cancer. We present a review of the PI3K/AKT/mTOR pathway, its role in tumorigenesis, and the potential of RES-529 in cancer treatment. RES-529 inhibits mTORC1/mTORC2 activity in various cancer cell lines, as noted by decreased phosphorylation of substrates including ribosomal protein S6, 4E-BP1, and AKT, leading to cell growth inhibition and death, with activity generally in the range of 5–15 μmol/l. In animal tumor models where the PI3K/AKT/mTOR pathway is abnormally activated (i.e. glioblastoma, prostate cancer, and breast cancer), RES-529 reduces tumor growth by as much as 78%. RES-529 treatment is synergistic with radiation therapy, chemotherapy, and hormonal therapy in reducing tumor growth, potentially by preventing PI3K/AKT/mTOR pathway activation associated with these treatments. Furthermore, this compound has shown antiangiogenic activity in several animal models. mTORC1 and mTORC2 have redundant and distinct activities that contribute toward oncogenesis. Current inhibitors of this pathway have primarily targeted mTORC1, but have shown limited clinical efficacy. Inhibitors of mTORC1 and mTORC2 such as RES-529 may therefore have the potential to overcome the deficiencies found in targeting only mTORC1.

Keywords: AKT, glioblastoma, mTOR, mTORC1, mTORC2, P529, Palomid 529, PI3K, RES-529

RES-529: a PI3K/AKT/mTOR pathway inhibitor that dissociates the mTORC1 and mTORC2 complexes

Mark A. Weinberg

Introduction

The phosphoinositide 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) pathway plays an essential role in the regulation of cell growth, survival, and proliferation in both physiological and pathological conditions [1–9]. Inhibitors of this pathway have the potential to treat diseases such as cancer, which is associated with pathway dysregulation. This review summarizes the activity and potential of one such inhibitor, RES-529, which targets both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) through complex dissociation, in the treatment of cancer. RES-529 was developed by RestorGenex Corporation. As of January 2016, RestorGenex Corporation merged with Diffusion Pharmaceuticals LLC to form Diffusion Pharmaceuticals Inc.

PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway involves the coordinated activation of multiple molecules leading to the stimulation of various cellular processes, including transcription, translation, and cell survival (Fig. 1). This pathway is activated through various receptors and agents, including receptor tyrosine kinases, G-protein-coupled receptors, and amino acid stimulation (Fig. 1) [1–4,6,7,10,11]. Stimulation of receptor tyrosine kinases and G-protein-coupled receptors leads to the activation of class IA PI3Ks. Class I PI3Ks are heterodimeric proteins that consist of a catalytic subunit and a regulatory subunit [1–5,7]. Before activation, PI3K resides in the cytoplasm. Upon cell stimulation, the regulatory subunit interacts with phosphorylated tyrosine residues on the activated receptor, resulting in a relief of its inhibition of the catalytic subunit and the positioning of the catalytic subunit at the plasma membrane, where its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), resides [1–5,12]. PI3K phosphorylates PIP2 to produce phosphatidylinositol-3,4,5-triphosphate (PIP3) [1–5]. This activity can be antagonized by the lipid phosphatase PTEN (phosphatase and TENsin homolog deleted on chromosome 10) through the hydrolysis of PIP3 to PIP2 [1–4,13–15].

PIP3 recruits proteins that contain a pleckstrin homology domain to cellular membranes, including the
serine–threonine kinase AKT and its activating kinase, 3-phosphoinositide-dependent protein kinase 1 (PDK1), leading to AKT phosphorylation and activation [1,2,5]. AKT has more than 100 substrates, including tuberous sclerosis complex 2 (TSC2) and protein-rich AKT/PKB substrate 40 kDa (PRAS40), two proteins that are associated with the regulation of mTORC1 [1,3,16,17].

mTORC1 is one of two mTOR complexes, the other being mTORC2 [1,3,18,19]. mTOR is a serine–threonine kinase that is a member of the PI3K-related kinase family [18]. mTORC1 is inhibited by the anticancer drug rapamycin, but mTORC2 is not sensitive to rapamycin, except when treated long term in a cell-type-dependent manner [18,20]. mTORC1 is composed of five proteins: mTOR, DEP domain-containing mTOR-interacting protein (Deptor), mammalian lethal with Sec13 protein 8 (mLst8), PRAS40, and regulatory-associated protein of mTOR (Raptor) [1,18]. The composition of mTORC2 is mTOR, Deptor, mLst8, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein 1 (mSin1), and protein observed with Rictor (Proctor) [18].

TSC2 forms a complex with TSC1 and TBC1 domain family member 7 (TBC1/2) and acts as a negative regulator of mTORC1 signaling [1,3,18]. The TSC complex is a GTPase-activating protein (GAP) for the small GTPase Ras homolog enriched in the brain (Rheb). Phosphorylation of TSC2 by AKT results in the inhibition of the GAP activity of the TSC complex, leading to Rheb activation [1,18]. The activated Rheb directly binds to mTORC1 and enhances its kinase activity, along with acting as a scaffold to promote the interaction of mTOR with its substrates [1,18,21]. In addition, mTORC1 is activated by AKT through the

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**Fig. 1**

PI3K/AKT/mTOR pathway. Ligand (yellow circle) stimulation of receptor tyrosine kinases or G-protein-coupled receptors or influx of amino acids (yellow circle) leads to the activation of proteins or pathways (indicated by green boxes or lines) or the release of the inhibitory activity of proteins or pathways (indicated by blue boxes or lines, with release indicated by green lines and or green boxes) associated with PI3K/AKT/mTOR pathway activation [1–4,6,7,10,11]. RES-529 inhibits this pathway and subsequent downstream biological effects by promoting mTORC1 and mTORC2 complex dissociation. AMPK, adenosine monophosphate-activated protein kinase; Deptor, DEP domain-containing mTOR-interacting protein; ERK, extracellular-signal-regulated kinase; MEK, mitogen-activated protein kinase (MAPK)/ERK kinase; mLst8, mammalian lethal with Sec13 protein 8; mSin1, mammalian stress-activated protein kinase interacting protein 1; mTOR, mechanistic target of rapamycin; mTORC1/2, mTOR complex 1/2; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PRAS40, protein-rich AKT/PKB substrate 40 kDa; PTEN, phosphatase and TENSin homolog deleted on chromosome 10; REDD1, regulated in development and DNA damage responses; Raptor, regulatory-associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; Rheb, Ras homolog enriched in brain; RSK, ribosomal protein S6 kinase; TBC1D7, TBC1, domain family member 7; TSC1/2, tuberous sclerosis complex 1/2.
phosphorylation of PRAS40, an inhibitory component of mTORC1, leading to dissociation of PRAS40 from the complex [1,18].

Another pathway stimulated by growth factors that leads to mTORC1 activation is the Ras-extracellular-signal-regulated kinase (ERK) pathway [1,22]. The Ras–ERK pathway activates mTORC1 through at least two mechanisms: the ERK and ribosomal protein S6 kinase (RSK)-mediated phosphorylation and inactivation of TSC2 and RSK phosphorylation of Raptor.

Amino acids activate mTORC1 through Rag GTPases. Upon amino acid stimulation, a GTP–GDP exchange occurs between the Rag GTPase subunits (RagA/RagB and RagC/RagD), leading to its activation [1,18,23]. The activated Rag GTPase complex then recruits mTORC1 to the surface of lysosomes through binding to Raptor, whereupon mTORC1 is activated through its interaction with Rheb on the lysosome. Additional components of this pathway include Regulator, which interacts with Rag GTPase and promotes its GTP–GDP exchange and lysosomal association; SLC38A9, a putative lysosomal arginine sensor for the mTORC1 pathway; and vacuolar H⁺-ATP [23–27].

Another mechanism that G-protein-coupled receptors use to activate mTORC1 activity, besides stimulating PI3K, is by promoting the sequestering to the plasma membrane of the mTORC1 inhibitory protein, regulated in development and DNA damage responses 1 (REDD1) [28], and preventing its association with mTORC1. REDD1 negatively regulates mTORC1 activity in a TSC complex-dependent and 14-3-3 protein-dependent manner and is induced under hypoxic conditions [18,29].

mTORC1 function is also regulated under stress conditions (such as hypoxia, energy depletion, and DNA damage) by adenosine monophosphate-activated protein kinase (AMPK) [1,18,30]. Under stress conditions, AMPK is activated, leading to the phosphorylation of TSC2 and Raptor, which promotes Raptor-14-3-3 protein association, and subsequent mTORC1 inactivation.

mTORC1 plays an important role in regulating mRNA translation through its phosphorylation of 4E-BP1 and p70 ribosomal protein S6 kinase 1 (S6K1) [18,22,31]. 4E-BP1 is an endogenous inhibitor of eukaryotic translation initiation factor 4E (eIF4E), a protein that promotes translation initiation by binding to the 5′ cap structure of mRNA. Phosphorylation of 4E-BP1 by mTORC1 prevents its ability to bind to eIF4E and therefore enables cap-dependent translation initiation. Phosphorylation of S6K1 by mTORC1 stimulates S6K1 kinase activity, leading to the phosphorylation and activation of proteins associated with mRNA translation and splicing, including ribosomal protein S6, eukaryotic translation initiation factor 4B (eIF4B), programmed cell death-4 (PDCD4), eukaryotic translation elongation factor 2 kinase (eEF2K), and S6K1aly/REF-like target (SKAR).

Additional functions associated with mTORC1 include regulation of transcription, lipid biosynthesis, autophagy induction, and mitochondrial activity [18,31–33]. mTORC1 promotes autophagy by phosphorylating proteins such as ULK1 and autophagy-related protein 13 (Atg13), which are involved in the initial steps of autophagy induction [18,31,33]. Autophagy is a pathway for cell survival by which, under conditions of nutrient deprivation, lysosomal degradation of cellular components occurs to supply nutrients. Mitochondrial function is regulated by mTORC1 through the modulation of various mitochondrial and nuclear-encoded mitochondrial genes, including mitochondrial ribosomal proteins [31].

Unlike mTORC1, the mechanism by which mTORC2 is activated following cellular stimulation is not as well defined. Like mTORC1, it is activated through growth factor stimulation of cells [18,34]. Recently, Liu et al. [35] identified a link between growth factor stimulation of PI3K and mTORC2 activation. They found that the PH domain of mSin1, a component of mTORC2, interacts with the kinase domain of mTOR to suppress its activity. PI3P, which is produced by activated PI3K, interacts with the PH domain of mSin1 to repress its inhibitory activity to mTOR, leading to mTORC2 activation.

mTORC2 plays an important role in regulating cell survival through the phosphorylation and activation of several AGC family kinases, including AKT, serum and glucocorticoid-induced kinase 1 (SGK1), and protein kinase C (PKC) [18,34,36]. In addition, mTORC2 regulates actin organization through the phosphorylation of PKCα and paxillin and the activation of Ras homolog gene family, member A (RhoA), and Ras-related C3 botulinum toxin substrate-1 (Rac1) [18,34]. Lipid biosynthesis also appears to be positively regulated by mTORC2, in part through the AKT-mediated activation of SREBP-1c [18,31,37]. mTORC2 also regulates mitochondrial function following its growth factor-stimulated recruitment to the mitochondrial-associated endoplasmic reticulum membrane [31,38].

The PI3K/AKT/mTOR pathway is relevant in promoting angiogenesis. Vascular endothelial growth factor (VEGF) receptor activity requires the stimulation of the PI3K/AKT/mTOR pathway [39,40]. In addition, VEGF expression is induced by the PI3K/AKT/mTOR pathway through hypoxia-inducible factor 1α (HIF-1α)-dependent
and HIF-1α-independent mechanisms, leading to increases in VEGF protein levels [41–44].

**Role of PI3K/AKT/mTOR pathway in cancer**

Genetic changes in the PI3K/AKT/mTOR pathway leading to its constitutive activation are highly prevalent in a many tumor types, including glioblastoma and prostate, breast, ovary, colon, and lung cancer [2,4,45–51]. Mutations in this pathway exist in 86% of glioblastomas, and 42% of primary and 100% of metastatic prostate cancers [45,46].

The PI3K/AKT/mTOR pathway is genetically activated through various elements in the pathway and by different mechanisms. Activating mutations of the PI3K catalytic subunit p110α gene PIK3CA occur in various cancers, including colon, brain, gastric, breast, and lung [2,4,51–53], and amplification of this subunit has also been found [47,54,55]. Mutations of the regulatory subunit of PI3K resulting in constitutive activity exist in brain, colon, and ovarian cancer [2,4,45,49,51,56,57]. Furthermore, alterations in the PI3K antagonist PTEN, including loss-of-function mutations, deletions, and epigenetic silencing of the gene, have been identified in various cancers [51,58–61].

Activating mutations and amplification of the AKT genes have also been found in different types of cancers [62–65], and PDK1 kinase domain mutations have been identified in colon cancer [64]. Mutations that increase mTORC signaling, including mTOR, TSC1, and TSC2, and Rheb mutations have been found in various cancers [66–68].

Besides mutations in the pathway itself, the overexpression and mutational alteration of upstream receptors and molecules that promote the PI3K/AKT/mTOR pathway activation, such as receptor tyrosine kinases, occur in cancer [45,50]. Thus, in summary, given the significant types and number of mutations in this pathway associated with cancer, identification of compounds that target this pathway is highly relevant.

**Rationale for mTORC1 and mTORC2 complex formation inhibitors**

mTOR inhibitors are currently approved for the treatment of renal cell carcinoma and pancreatic neuroendocrine tumors [Afinitor (everolimus; Novartis, East Hanover, New Jersey, USA); Torisel (temsirolimus; Pfizer, Philadelphia, Pennsylvania, USA)] [69,70]. However, these agents are rapamycin analogs (rapalogs) that target mTORC1, but not mTORC2, and have shown limited clinical efficacy in other tumor types, including prostate cancer and glioblastoma [71–75].

One reason for the lack of clinical efficacy of rapalogs is that they can upregulate the PI3K pathway through the induction of insulin receptor substrate-1 (IRS-1) expression, resulting in AKT activation through Ser473 phosphorylation, an mTORC2 substrate, and subsequent downstream signaling including mitogen-activated protein kinase (MAPK)/ERK activation [76–80]. Paradoxically, in various cancer cells types, mTORC1 inhibition also promotes eIF4E phosphorylation, potentially through the MAP/ERK pathway and the activation of Mnk1 (MAPK-interacting serine–threonine kinase 1) [78,81]. eIF4E plays an important role in translation initiation and phosphorylation enhances this activity.

Targeted silencing of either mTORC1 or mTORC2 by small interfering RNA (siRNA) has also shown a potential utility of targeting both kinases for cancer [82]. In a recent report by Gravina et al. [82], the silencing of mTORC2 through siRNA knockdown of Rictor led to relevant growth inhibition of human prostate 22rv1 cells, whereas siRNA knockdown of Raptor (mTORC1 silencing) had no effect. Furthermore, mTORC1 silencing led to increased expression of the androgen receptor and phosphorylation of Ser473 of AKT, whereas mTORC2 silencing decreased their levels. Overall, these results suggest that a combined mTORC1 and mTORC2 inhibitor may be more efficacious than an mTORC1 inhibitor.

**Pharmacology of RES-529**

RES-529 (previously named Palomid 529, P529) is a PI3K/AKT/mTOR pathway inhibitor that targets both mTORC1 and mTORC2 through mTOR complex dissociation. It is a modification of a dibenzo[c]chromen-6-one-anti-estrogen derivative (Fig. 2) [83]. The concept behind the initial development of RES-529 was the observation that antiestrogens have antiangiogenic and antiproliferative activities that are not because of their antagonism of estrogen receptor function [84,85]. Although initially developed using an antiestrogen scaffold, RES-529 has no antiestrogenic activity. At 10 μmol/l concentrations, RES-529 reduces the binding of 0.5 nmol/l [3H]estradiol to estrogen receptor (ER)α and ERβ by 3% or less [83].

RES-529 is an orally administered compound that has good blood–brain penetration and lacks affinity to ATP-binding cassette, subfamily B, member 1 (ABCB1) and ATP-binding cassette, subfamily G, member 2 (ABCG2) drug efflux transporters [86]. RES-529 has been
evaluated in two phase I open-label trials in patients with neovascular age-related macular degeneration (NCT01271270 and NCT01033721) [87–89]. In these clinical studies, RES-529 was administered as an ocular injection. The drug was shown to be generally well tolerated and there were no drug-related systemic adverse events [87]. Development for age-related macular degeneration through subconjunctival administration is ongoing. The oral formulation of RES-529 is currently being developed for the treatment of glioblastoma, for which it has received orphan designation by the US Food and Drug Administration, and prostate cancer [90].

RES-529 was identified as a PI3K/AKT/mTOR pathway inhibitor in studies determining its mechanism of action in the antiproliferative and apoptotic activity on glioblastoma, endothelial, and prostate tumor cells along with keloid dermal fibroblasts, as described in more detail in the following section [83,91,92]. Treatment of human prostate PC-3 cells with RES-529 led to the time-dependent inhibition of ribosomal protein S6 (Ser235/236), 4E-BP1 (Thr37/46), glycogen synthase kinase-3β (GSK-3β, Ser9), forkhead box protein O1a (Foxo1a), Ser256, mouse double minute 2 homolog (MDM-2, Ser166), and p70S6K (Thr389) phosphorylation (Fig. 3a) [91]. RES-529 did not inhibit the phosphorylation of AKT (Thr308) or PDK1 (Ser241), indicating that it does not affect PDK1. Besides inhibiting the phosphorylation of mTORC1 targets, such as ribosomal protein S6 and 4E-BP1 [18,22,91], RES-529 treatment has also been shown in various studies to inhibit AKT (Ser473) phosphorylation, an mTORC2-specific substrate (Fig. 3b) [77,83,91,92].

Because the phosphorylation of GSK-3β on Ser9 is associated with GSK-3β inactivation [93], the effects of RES-529 treatment on GSK-3β activity and modulation of downstream markers were evaluated by Gravina et al. [94] in PC-3 and 22rv1 cells. RES-529 treatment significantly increased GSK-3β activity in cells (P < 0.05). Active GSK-3β is known to promote apoptosis by the reduced expression and increased nuclear translocation of survivin [94,95]. Furthermore, Gravina et al. [94] showed that radiation treatment acted synergistically with RES-529 to further increase GSK-3β activity and decrease survivin levels. Treatment of cells with the GSK-3β inhibitor SB216761 prevented the decrease in survivin levels with RES-529 and radiation, indicating that the activity of RES-529 on survivin levels was through a GSK-3β-dependent process.

RES-529 was also shown to regulate levels of components of the cell cycle, MAPK, and apoptotic pathways through PI3K/AKT/mTOR pathway inhibition [91]. RES-529 treatment generally resulted in dramatically reduced levels of the cell cycle-associated proteins cyclin B1, cyclin D1, cyclin-dependent kinase 4 (cdk4), and cdk6 in PC-3 and 22rv1 cells along with a concomitant increase in p21 and p27 levels, on the basis of western blot analysis. An increase in ERK and p38 MAPK activity and reduced c-Jun N-terminal kinase (p-JNK) activity was also observed in these cells. Finally, changes in protein levels consistent with apoptosis were observed, with increases in Bel-Xs and Bax [BCL-2 (B-cell lymphoma-2)-associated] protein levels and reduced amounts of Bel-2, B-cell lymphoma extra long (Bel-Xl), and phosphorylated BCL-2-associated death promoter (BAD).

Insight into the mechanism by which RES-529 acts as a PI3K/AKT/mTOR pathway inhibitor emerged from the work of Xue et al. [83], who showed that treatment of C6V10 rat neuroblastoma cells with 20 μmol/l RES-529 promoted the dissociation of the mTORC1 and mTORC2 on the basis of immunoprecipitation experiments from lysates of treated cells that were stimulated with insulin-like growth factor-1 (Fig. 3c). Further studies are ongoing in various laboratories to further evaluate the mechanism of RES-529 action.

**Antiangiogenic activity of RES-529**

RES-529 has shown antiangiogenic potential in both cellular and animal models. RES-529 inhibited both VEGF-stimulated and β fibroblast growth factor-stimulated human umbilical vein endothelial (HUVEC) cell proliferation with half-maximal inhibitory concentrations (IC50) of ∼10 and 30 nmol/l, respectively [83]. Treatment of HUVEC cells with RES-529 also resulted in a four-fold induction of apoptosis on the basis of DNA fragmentation [83].

The antiangiogenic activity of RES-529 was shown in two different mouse models [83]. In a neonatal P7 mouse model in which pathologic retinal angiogenesis was induced by placing the mice into hyperoxia conditions, followed by normal conditions, RES-529 (RES-529 150 mg/kg/day, 5 days, intraperitoneal) inhibited pathologic angiogenesis, as indicated by a decrease in the number of glomeruloid tufts by ∼50% [83]. In a model where angiogenesis was induced by VEGF-A produced by an injection of an adenovirus vector containing the VEGF-A gene into the mouse ear, treatment of mice with RES-529 (200 mg/kg/2 days, intraperitoneal) before the adenovirus injection resulted in a dose-dependent reduced number and size of blood vessels as well as decreased vessel permeability. At the higher dose of RES-529, angiogenesis and vascular permeability were reduced by ∼50% compared with the control on the basis of Evans blue staining. Furthermore, PI3K/AKT/mTOR pathway signaling was shown to be reduced in this model [83].
Antitumor activity of RES-529: cellular models

Growth inhibition was observed with RES-529 treatment in various cancer cell lines from the National Cancer Institute-60 (NCI-60) tumor panel, with IC₅₀ ranges of 5–15 μmol/l for central nervous system cancer cells and 5–30 μmol/l for prostate cancer cells (Fig. 4) [96]. Growth-inhibitory activity of RES-529 treatment was also observed in both PTEN-positive and PTEN-negative prostate cancer cell lines, although PTEN-negative cells appeared to be more sensitive [91].

Growth inhibition was also accompanied by cell death. In the studies by Gravina et al. [91], RES-529 treatment of PC-3 and 22rv1 cells resulted in a notable induction of apoptosis, as determined by an increase in caspase-3 activity and annexin V staining.

Antitumor activity of RES-529: animal models

RES-529 has shown antitumor activity in a variety of mouse models, including those for glioblastoma [83], and prostate [91] and breast cancer [97]. In a C6V10 glioblastoma subcutaneous xenograft model, mice pretreated with RES-529 (200 mg/kg/2 days, intraperitoneal) 1 week before and for 3 weeks after a tumor cell injection showed an ∼70% decrease in tumor volume compared with the control (Fig. 5) [83]. In another glioblastoma tumor model using human U87 cells, mice treated with micronized RES-529 3 days after a tumor cell injection showed a reduction in tumor growth by ∼78 and 29% with 50 and 25 mg/kg/2 days, intraperitoneal, RES-529, respectively, after 24 days compared with the control [83]. Furthermore, no noticeable toxicity was observed with this treatment [83]. The antitumor activity in the U87 glioblastoma mouse xenograft model was confirmed in a recent report by Lin et al. [86], in which a significant decrease in tumor volume was observed in mice treated with 54 mg/kg, intraperitoneal, micronized RES-529 compared with the control (P < 0.05) starting at 11 days of treatment to the end of the study (18 days), with tumor...
volume in the RES-529-treated mice being ~52% lower than the control.

In PC-3 and 22rv1 mouse prostate xenograft models, a significant reduction in tumor mass was observed with RES-529 treatment (P < 0.001; Fig. 6) [91]. In the PC-3 xenograft model, a 10, 47.6, and 59.3% tumor volume reduction was observed with RES-529 50, 100, and 200 mg/kg, oral, treatment, respectively, with similar results found in the 22rv1 xenograft model. These reductions in tumor mass were accompanied by tumor cell apoptosis in both models. Furthermore, a potent effect on angiogenic neovascularization was observed in the PC-3 xenograft model, as shown by a marked decrease in the number, size, and stability of the blood vessel bed (as indicated by decreased staining for fibrin aggregates and CD31-positive microvessels) and a decrease in VEGF-positive tumor cells (Fig. 6).

RES-529 was also evaluated in breast cancer using two mouse xenograft models [97]. One model utilized a human MCF-7 breast cancer cell line and the second used embryonic fibroblast cells from mice expressing a truncated Brca1 allele lacking the BRCT repeats (Brca1tr/tr) and with higher levels of p-AKT than wild-type mouse embryonic fibroblasts [97]. RES-529 was shown to significantly inhibit tumor growth in both models (P < 0.001) as well as decrease AKT and ribosomal S6 phosphorylation.

**Synergistic activity of RES-529: cellular and animal models**

Various anticancer therapies, including radiation therapy, chemotherapy, and hormonal therapy, have been shown to activate the PI3K/AKT/mTOR pathway [91,96].

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**Fig. 4**

| Cell line | Growth inhibition50 (μmol/l) |
|-----------|----------------------------|
| BT-549    | 17.0                       |
| HS 578T   | 13.3                       |
| MCF-7     | 6.0                        |
| MDA-MB-231/ATCC | 14.7                    |
| MDA-MB-435 | 2.6                       |
| NCI/ADR-RES | 12.6                     |
| COLO 205  | 12.1                       |
| HCC-2998  | 10.8                       |
| HCT-116   | 10.9                       |
| HCT-15    | 12.9                       |
| HT29      | 6.0                        |
| KM12      | 14.2                       |
| SW-620    | 14.3                       |
| SF-28     | 6.1                        |
| SF-295    | 12.2                       |
| SF-539    | 14.7                       |
| SNB-19    | 11.2                       |
| SNB-75    | 5.2                        |
| U251      | 12.0                       |
| HL-60 (TB)| 2.3                        |
| K-562     | 4.1                        |
| MOLT-4    | 21.5                       |
| RPMI-8226 | 32.7                       |
| LOX IMVI  | 2.1                        |
| M14       | 22.5                       |
| MALME-3M  | 16.4                       |
| SK-MEL-2  | 17.2                       |
| SK-MEL-5  | 18.6                       |
| SK-MEL-28 | 9.1                        |
| UACC-62   | 14.5                       |
| UACC-257  | 15.4                       |
| A549/ATCC | 13.1                       |
| EKVX      | 11.2                       |
| HOP-82    | 22.3                       |
| NCI-H226  | 15.1                       |
| NCI-H226M | 21.2                       |
| NCI-H460  | 11.4                       |
| NCI-H922  | 8.8                        |
| ICR-04    | 14.9                       |
| OVCAR-3   | 12.8                       |
| OVCAR-4   | 12.8                       |
| OVCAR-8   | 24.1                       |
| SK-OV-3   | 13.6                       |
| 22rv1     | 20.5                       |
| BFHI      | >30.0                      |
| C4-28     | 12.3                       |
| DU-145    | 18.6                       |
| LAPC-4    | 10.5                       |
| LnCaPa*   | 12.4                       |
| PC-3      | 5.1                        |
| 786-O     | 16.2                       |
| A499      | 15.9                       |
| ACHN      | 13.3                       |
| CAMK-1    | 11.2                       |
| RXF 383   | 5.2                        |
| SN 12C    | 17.0                       |
| TK-10     | 18.3                       |
| UO-31     | 15.5                       |

Cell growth inhibition of NCI-60 tumor cell line panel by RES-529 [91,96]. CNS, central nervous system; NSCLC, non-small-cell lung cancer. *Gravina et al. [91].

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**Fig. 5**

RES-529 activity in mouse glioblastoma xenograft models [83]. Growth of C6V10 glioblastoma tumor in mice treated with RES-529 (P529, 200 mg/kg/2 days, intraperitoneal). Reproduced from Xue et al. with permission from AACR [83].
Therefore, studies have been performed in cell and animal models to determine whether inhibition of this pathway through treatment with RES-529 can have synergistic activity with these treatments.

The synergistic action of RES-529 with radiation treatment has been shown in a number of prostate cell and tumor models [94,96]. In PC-3 cells, 2 μmol/l RES-529 along with 2 Gy radiation reduced cell survival by 70% compared with 15% for radiation alone (P < 0.001) [96]. A reduction in the clonogenic capacity of PC-3 cells was also shown to be greater with RES-529 when used in combination with 2 or 4 Gy radiation compared with radiation alone (P < 0.05 and < 0.01, respectively). This effect was at least partially mediated by the complete inhibition by RES-529 of the more than 10-fold radiation-induced phosphorylation of AKT. In addition, RES-529 treatment reduced the radiation-induced expression of inhibitor of differentiation-1 (id1), a molecule associated with radioresistance [98]; VEGF; matrix metalloproteinase (MMP)9; and MMP2.

The synergistic activity of RES-529 with radiation in prostate cancer was further expanded in a recent paper by Gravina et al. [94]. In this study, the decrease in the clonogenic capacity of prostate cancer cell lines LAPC-4, LnCaP, 22rv1, C4-2B, PC-3, and DU145 by radiation was further enhanced with 1 μmol/l RES-529. This was also accompanied by a significant enhancement of tumor autophagy compared with individual treatments (P < 0.05), as measured by higher Beclin-1 protein expression, and increased apoptosis, on the basis of increased cleaved caspase-3 activity. In addition, there was an increase in tumor cell senescence, which was related to tumor autophagy, and a significant increase in the percentage of DNA damage (P < 0.05) when RES-529 was combined with radiation treatment compared with radiation treatment alone. This increase in DNA damage was believed to be associated with negative effects on the homologous repair and non-homologous end-joining DNA repair pathways through the reduced expression of Rad51, Ku70, and p-DNA-PKCs by RES-529 treatment. The increased efficacy in cell growth inhibition with this combination was considered to be associated with a combined inhibitory effect on c-Myc levels as well as the ability of RES-529 to inhibit the expression of radiation-induced cyclin D1.

The synergistic effects observed in prostate cell culture models with RES-529 and radiation therapy were also observed in animal models [94,96]. RES-529 (20 mg/kg, q3d) and radiation (single 6 Gy dose 1 week after injection) treatment in a mouse PC-3 tumor model reduced tumor volume by 77% compared with the control, and treatment with the individual agents reduced growth by 43–53% after 4 weeks [96]. In histological examination, tumors from mice treated with RES-529 and radiation...
showed more extensive tumor tissue damage compared with single therapy, including tumor cell loss, cells with pyknotic nuclei, and extensive fibrosis. Treatment with RES-529 and radiation also resulted in a significant reduction in proliferating cell nuclear antigen-positive cells, indicative of apoptosis, compared with the control (17.1 ± 12.2 vs. 40.9 ± 5.5%, P < 0.01). This significant increase in apoptosis with proliferating cell nuclear antigen staining was correlated with caspase activity changes, with 8.7% caspase-3 positive cells present with the combination treatment compared with the ~5.7 and 3.3% positive cells for the individual and control treatments (P < 0.01 and < 0.001, respectively).

Similar results were observed in the study by Gravina et al. [94], where RES-529 enhanced the antitumor activity of radiation in mouse PC-3 and 22rv1 human prostate xenograft models (Table 1). A significant reduction in tumor volume was observed when RES-529 100 mg/kg, oral, 5 days/week, was combined with radiation (4 Gy) compared with the individual treatments alone (P < 0.05). In addition, the number of mice with tumor progression was significantly fewer with combination therapy (P < 0.05). A significant delay in the median time to progression was observed with RES-529 and radiation treatment compared with either treatment alone (P < 0.001). This delay correlated with a significant decrease in the proliferation index and an increase in the number of apoptotic cells (P < 0.01 for monotherapies vs. combination). In addition, in the PC-3 model, the mean absolute number of vessels in the tumor decreased significantly with the combination therapy (P < 0.01 for monotherapies vs. combination).

Synergy of RES-529 treatment with radiation therapy was also observed in glioblastoma xenograft and intracranial orthotopic mouse models using the human cell line U251 (Fig. 7) [99]. RES-529 plus radiation delayed the growth of xenografted U251 tumors compared with radiation alone (4 Gy) by 2.2 and 4 days with 25 and 50 mg/kg treatment, respectively. In addition, there was a significant increase in the survival of mice implanted intracranially with U251 cells with the combination of RES-529 50 mg/kg and radiation (4 Gy) treatment compared with RES-529 (P = 0.016) or radiation (P = 0.021) alone.

In addition to radiation treatment, RES-529 was also shown to have synergistic activity with cisplatin and docetaxel in 22rv1 and PC-3 cellular and mouse xenograft models [91] and hormonal therapy in 22rv1 mouse xenograft models [82]. Treatment of cells with RES-529 and either cisplatin or docetaxel, administered either in combination or sequentially, enhanced apoptosis compared with the individual administration of these agents. In mouse xenograft model studies where synergy was evaluated by calculating the combination index (CI) [100], RES-529 (100 mg/kg, oral) in combination with cisplatin (5 mg/kg, intraperitoneal) was synergistic in 22rv1 xenografts (CI = 0.69) and additive for PC-3 xenografts (CI = 1.13). RES-529 (100 mg/kg, oral) in combination with docetaxel (20 mg/kg, intraperitoneal) was synergistic in both 22rv1 and PC-3 xenograft models (CI = 0.50 and 0.34, respectively). This synergistic/additive effect was also observed when evaluated by the log cell kill. Furthermore, combination therapy significantly increased the time to progression compared with individual therapies (P < 0.0001). The combination of RES-529 with either cisplatin or docetaxel decreased the number of mice with tumors in progression from 100% with the individual treatments to 67 and 17%, respectively, in the 22rv1 xenograft model and 58 and 50%, respectively, in the PC-3 tumor xenograft model (Table 1). Recently,

**Table 1** Synergistic activity of RES-529 with radiation, cisplatin, or docetaxel in mouse prostate and glioblastoma xenograft models [91,94]

| Xenograft models | Treatment | Tumor weight (% of saline control) | Proliferation index (% of saline control) | Apoptosis (% tunnel + cells) | Vessel number | Mice with tumor progression |
|------------------|-----------|-----------------------------------|------------------------------------------|----------------------------|---------------|-----------------------------|
| **PC-3 [94]**    | Saline    | 100                               | 100                                      | < 2                        | 22.0          | 12/12                       |
|                  | RES-529 100 mg/kg | 60                            | 34                                       | 6.0                        | 17.5          | 12/12                       |
|                  | Radiation therapy 4.0 Gy | 51                            | 31                                       | 8.4                        | 16.4          | 12/12                       |
|                  | RES-529 + radiation therapy | 15*                           | 3*                                       | 21*                        | 5.5*          | 8/12                        |
| **22rv1 [94]**   | Saline    | 100                               | 100                                      | < 2                        | 38.5          | 12/12                       |
|                  | RES-529 100 mg/kg | 73                            | 37                                       | 12                         | 32.5          | 12/12                       |
|                  | Radiation therapy 4.0 Gy | 62                            | 56                                       | 10                         | 27.5          | 12/12                       |
|                  | RES-529 + radiation therapy | 19*                           | 20*                                      | 31*                        | 5.5*          | 12/12                       |
| **PC-3 [91]**    | Saline    | 100                               | 100                                      | < 2                        | 22.0          | 12/12                       |
|                  | RES-529 100 mg/kg | 51                            | 31                                       | 8.4                        | 16.4          | 12/12                       |
|                  | Cisplatin 5 mg/kg | 43                            | 50                                       | 18.2                      | 15.5          | 12/12                       |
|                  | RES-529 + cisplatin | 30                           | 18**                                     | 24.3*                     | 11.4**        | 7/12                        |
|                  | Docetaxel 20 mg/kg | 66                            | 83                                       | 7.2                        | 17.5          | 12/12                       |
|                  | RES-529 + docetaxel | 18**                          | 3**                                      | 28.2**                    | 12.5          | 6/12                        |
| **22rv1 [91]**   | Saline    | 100                               | 100                                      | < 2                        | 38.5          | 12/12                       |
|                  | RES-529 100 mg/kg | 62                            | 54                                       | 10.2                      | 27.5          | 12/12                       |
|                  | Cisplatin 5 mg/kg | 81                            | 88                                       | < 2                        | 31.5          | 12/12                       |
|                  | RES-529 + cisplatin | 23**                          | 27**                                     | 24.4**                    | 9.4**         | 8/12                        |
|                  | Docetaxel 20 mg/kg | 38                            | 44                                       | 15.1                      | 15.6          | 12/12                       |
|                  | RES-529 + docetaxel | 12**                          | 29**                                     | 34.3**                    | 7.5           | 2/12                        |

*P < 0.01, **P < 0.001 versus respective agent (radiation, cisplatin, or docetaxel) alone.
Gravina et al. [82] reported synergy of RES-529 with the 5α-reductase inhibitor dutasteride, the androgen synthesis inhibitor abiraterone, and the androgen receptor inhibitor bicalutamide in mouse 22rv1 xenograft models.

Basis for clinical evaluation of RES-529 in glioblastoma

For the clinical development of RES-529 in oncology, the initial focus is to target relevant tumors for which there is a high unmet medical need, such as glioblastoma. The current median survival of patients with glioblastoma is 9.7 months, with the existing treatment options limited to surgery, radiotherapy, and chemotherapy, such as temozolomide [101,102].

The potential of treating glioblastoma with inhibitors of the PI3K/AKT/mTOR pathway has been shown through the identification of pathway-activating mutations in patients with glioblastoma and activity of pathway inhibitors in preclinical glioblastoma models, such as those presented for RES-529. Activating mutations in the PI3K/AKT/mTOR pathway are found in a majority of patients with glioblastoma [45,51,103]. In an analysis of 206 glioblastomas, 86% of the samples had at least one genetic event in the receptor tyrosine kinase/PI3K pathway [45]. In addition, mutations and deletions of PTEN or mutations/amplification of epidermal growth factor receptor, both of which are frequent in glioblastoma, lead to the dysregulation of the PI3K pathway [104–106].

In addition to the studies described above for RES-529, other mTOR inhibitors that target mTORC1 and mTORC2 have shown efficacy in mouse glioblastoma xenograft models [107,108]. In mouse orthotopic xenograft models using the glioblastoma cell line CD133+ GMBJ1, treatment with AZD2014, an ATP-competitive mTOR inhibitor that targets mTORC1 and mTORC2, along with radiation resulted in a significant increase in survival compared with either control or radiation alone (P = 0.014 and 0.03, respectively) [107]. AZD8055, another mTOR inhibitor targeting mTORC1 and mTORC2, inhibited tumor growth in subcutaneous human brain tumor-initiating cell mouse xenografts and mTORC1 and mTORC2 signaling [108]. Although no detailed safety results have been reported for these mouse glioblastoma xenograft studies, Xue et al. [83] observed no toxicity with RES-529.

Currently, at least 17 PI3K/AKT/mTOR pathway inhibitors are being evaluated in clinical trials for glioblastoma. However, other than RES-529, none are believed to work through the dissociation of both mTOR complexes (mTORC1 and mTORC2). Furthermore, inhibitors that target only mTORC1 have shown poor efficacy in clinical trials [73–75].

Conclusion

PI3K/AKT/mTOR inhibitors have the potential to treat various tumor types, including glioblastoma and prostate and breast cancer. On the basis of past experience with mTORC1 inhibitors, there is a need for a dual mTORC1
and mTORC2 inhibitor using various approaches, including compounds that promote complex dissociation or ATP-competitive inhibition. As a dual mTORC1 and mTORC2 inhibitor, RES-529 potentially has a number of advantages over mTORC1-specific inhibitors. mTORC1-specific inhibitors have shown efficacy that is essentially limited to renal cell carcinoma and pancreatic neuroendocrine tumors, whereas RES-529 has shown efficacy in animal tumor models for glioblastoma and prostate cancer, two cancers resistant to mTORC1 inhibitors [71–75].

Anticancer therapies, including radiation therapy, chemotherapy, and hormonal therapy, activate the PI3K/AKT/mTOR pathway and, in particular, mTORC2, as noted by increased AKT (Ser473) phosphorylation [91,96]. RES-529 inhibits anticancer therapy-induced AKT activation and acts synergistically with these agents in animal tumor models. One mechanism of resistance to mTORC1 inhibitors occurs through the upregulation of receptor tyrosine kinase signaling adapter proteins, leading to AKT activation through Ser473 phosphorylation [76], which could be circumvented by inhibiting mTORC2. However, another mechanism of resistance to mTORC1 inhibitors through MAPK pathway activation is not prevented by genetic inactivation of mTORC2 [79]. Thus, there is the potential of resistance to RES-529 through MAPK pathway activation. MAPK pathway activation has been observed previously in PC-3 and 22rv1 cells treated with RES-529 [91]. A potential disadvantage of RES-529 compared with mTORC1-specific inhibitors is the potential for increased adverse events because of inhibition of both mTORC1 and mTORC2 activity. Therefore, it will be important to determine the clinical efficacy of RES-529 in relevant cancers such as glioblastoma and prostate cancer, and how it compares with other mTOR inhibitors, both mTORC1-specific and ATP-competitive mTORC1–mTORC2 inhibitors, with respect to safety and potency.

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Conflicts of interest
M.W. was an employee of RestorGenex Corporation during the development of the manuscript.

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