PI3K inhibitors for cancer treatment: where do we stand?

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Abstract
In contrast with cytotoxic agents that do not differentiate between normal proliferating and tumour cells, targeted therapies primarily exert their actions in cancer cells. Initiation and maintenance of tumours are due to genetic alterations in specific loci. The identification of the genes in which these alterations occur has opened new opportunities for cancer treatment. The PI3K (phosphoinositide 3-kinase) pathway is often overactive in human cancers, and various genetic alterations have been found to cause this. In all cases, PI3K inhibition is considered to be one of the most promising targeted therapies for cancer treatment. The present mini-review provides an update on new PI3K inhibitors currently in or entering clinical development. Recent discoveries, challenges and future prospects will be discussed.

Introduction
Activation of the PI3K (phosphoinositide 3-kinase) pathway is a recurrent feature observed in human tumours. Findings accumulated over the past 20 years have clearly underlined the role of this pathway for the maintenance of the tumorigenic state. The high frequency of genetic alterations in the PI3K pathway has made targeting components of the pathway, including PI3K, PD1K (phosphoinositide-dependent kinase 1), PKB (protein kinase B)/Akt and FRAP [FKBP (FK506-binding protein)-rapamycin-associated protein]/mTOR (mammalian target of rapamycin), a high priority in academic and industrial laboratories [1–3]. The present mini-review summarizes current drug-discovery efforts and challenges to identify and develop PI3K and dual PI3K/mTOR inhibitors for cancer treatment.

mTOR and/or PI3K inhibition for cancer treatment
The mTORC1 [mTOR complex 1; mTOR complexed to raptor (regulatory associated protein of mTOR)] is a master regulator of cell mass and metabolism, which is in part regulated by growth factor signalling through the canonical RTK (receptor tyrosine kinase)–PI3K–Akt axis and by nutrient (through class III PI3Ks), hypoxia or AMP [through TSC (tuberous sclerosis complex) 1 and TSC2] signalling[4,5]. In this context, mTORC1 blockade is also expected to lead to significant anti-tumour effects in tumour cells in which the PI3K pathway is constitutively active. Indeed allosteric mTORC1 inhibitors (e.g. rapamycin, CCI-779, RAD001 and AP23353) have shown promising anti-tumour activities in Akt-dependent prostate cancer [6], Neu/ErbB2-dependent breast cancer [7], NF1 (neurofibromatosis 1) mutant MPNST (malignant peripheral nerve sheath tumour) models [8], VHL (von Hippel–Lindau) negative kidney cancer model [9] or PTEN (phosphatase and tensin homologue deleted on chromosome 10)-deficient tumour models [10,11]. As a single agent, the rapamycin derivatives CCI-779 and RAD001 have recently provided significant activities in the treatment of patients with metastatic real cell carcinoma [12,13]. Activities against other solid tumours, including breast cancer [14], glioblastoma [15] and neuroendocrine carcinoma [16], were, however, not as impressive. The molecular mechanisms responsible for these differences in sensitivity have not yet been clearly underlined. Evidence exists showing that mTORC1 inhibition can lead to pathway reactivation: abrogation of the negative-feedback loop which is normally initiated by the direct mTORC1 substrate p70 S6K (p70 S6 kinase) on IRS (insulin receptor substrate) proteins [17,18] can lead to strong PI3K–Akt pathway reactivation; RAD001 can cause ERK (extracellular-signal-regulated kinase) pathway reactivation in a PI3K-dependent manner [19]. Moreover, rapamycin derivatives cannot inhibit mTORC2 [mTOR complex 2; mTOR complexed to rictor (rapamycin-insensitive companion of mTOR)], which is one of the two Akt upstream kinases. Altogether, this would suggest that pathway activation and reactivation could be avoided by PI3K or concomitant PI3K and mTOR catalytic inhibition (that would target both mTORC1 and mTORC2).

The first generation of PI3K inhibitors includes the viridin soil bacteria product wortmannin, its derivative demethoxyviridin and LY29402, which is a morpholino derivative

Key words: cancer, mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), small-molecule kinase inhibitor.

Abbreviations used: DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; DNA-PK, DNA-dependent protein kinase; cell, embryonic induction factor; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated kinase; 4E-BP1, eIF4E-binding protein 1; FOG-PKT, 2-[4F]fluoro-2-deoxy-D-glucose positron-emission tomography; GI50, concentration giving half-maximal growth inhibition; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; p70S6K, p70 S6 kinase; PKB, PKA, peripheral blood mononuclear cell; PI3K, phosphoinositide 3-kinase; PIK, phosphatase and tensin homologue deleted on chromosome 10; RPS6, ribosomal protein S6; TSC, tuberous sclerosis complex; VEGF, vascular endothelial growth factor.

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Table 1 | Current PI3K inhibitors in or expected to be in the clinic for cancer treatment

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Mode of action</th>
<th>Structure</th>
<th>Current development stage</th>
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| SF1126   | Semafore| Pan-class I PI3K, mTOR, DNA-PK| ![SF1126 Structure](image) | Entered Phase I/II clinical trials in March 2007
Targeted diseases: breast cancer, ovarian cancer, renal cell carcinoma, endometrial cancer
Starting dose: 90 mg/m²
Schedule: intravenous (1.5 h, infusion), twice per week
An update was given at the AACR Special Conference on PI3K (11–14 November 2008) [55] |
| PX-866   | Oncothyreon / ProlX | Pan-class I PI3K, others? | ![PX-866 Structure](image) | Entered Phase I trial in June 2008
Targeted diseases: advanced solid tumours
Starting dose: undisclosed
Route and schedule: oral, once per day |
| GDC-0941 | Genentech / Piramed / Roche | Pan-class I PI3K | ![GDC-0941 Structure](image) | Entered Phase I trial in April 2008
Targeted diseases: solid tumours
Starting dose: undisclosed
Route and schedule: oral, once per day |
| NVP-BEZ235 | Novartis | Pan-class I PI3K, mTOR | ![NVP-BEZ235 Structure](image) | Entered Phase I trial in December 2006
Targeted diseases: solid tumours
Starting dose: 10 mg
Route and schedule: oral, once per day |
| XL147    | Exelixis | Pan-class I PI3K | Undisclosed | Entered Phase I trial in June 2007
Targeted diseases: solid tumours
Starting dose: 25 mg
Route and schedule: oral, once per day |
| XL765    | Exelixis | Pan-class I PI3K, mTOR | Undisclosed | Entered Phase I trial in June 2007
Targeted diseases: solid tumours
Starting dose: 30 mg
Route and schedule: oral, twice per day |
| D-87503  | Æterna Zentaris | Pan-class I PI3K, ERK2 | ![D-87503 Structure](image) | Pre-clinical data presented at the 2007 AACR Annual Meeting [56]
No IND (investigational new drug) filing at the time of writing (October 2008) |
| D-106669 | Æterna Zentaris | | | |
| GSK615   | GlaxoSmithKline | Pan class I PI3K | Undisclosed | Pre-clinical profile presented at the AACR Special Conference on PI3K (11–14 November 2008) [57]
Entered Phase I trial in September 2008
Targeted diseases: solid tumours and lymphoma |
of quercetin. These compounds have been used widely as pharmaceutical tools to provide evidence for the involvement of the PI3K pathway in various biological systems.

From wortmannin and LY294002: the path towards clinical candidates for PI3K inhibition

On the basis of these original chemical scaffolds, a new generation of compounds, including the semi-synthetic viridin and wortmannin derivative PX-866 (ProX Pharmaceuticals) and the LY294002 RGDS (Arg-Gly-Asp-Ser)-conjugated pro-drug SF1126 (Semafore Pharmaceuticals), which are more stable and have better pharmacokinetic properties than wortmannin and LY294002 (Table 1) respectively, were shown to be active against ovarian (OvCar3), colon (HT29), lung (A549), glioblastoma (U87MG), prostate (PC3) and breast (MDA-MB468) subcutaneous xenografts [20–22]. Both drugs have entered Phase I clinical trials (Table 1).

Massive efforts have been undertaken in both academic laboratories and private companies over the last 5 years, to develop and implement the required tools to enable the creation of medicinal chemistry flow charts addressing PI3K inhibition. For example, the three-dimensional structure of the regulatory subunits p85α and p110α in association with its regulatory subunit p85α [23,24] has been solved, and co-crystals of PI3K in complex with inhibitors from distinct chemical scaffolds have been obtained [25,26]. These data, together with the original co-crystal structures obtained with PI3Kα [27,28], have been crucial to identify key interactions needed to improve specificity and potency against the different class I PI3Ks, and fuelled the use of structure-based drug-design approaches to identify new molecular entities. In addition, the establishment of robust biochemical and cellular assays [1,29] has been critical to determine the activity and selectivity profile of PI3K inhibitors. For instance, a thorough chemical proteomic analysis revealed an unexpected number of protein kinases that are significantly inhibited by LY294002 [30]. Representative examples of a new generation of PI3K compounds identified by the use of technological advances are discussed below.

Initially discovered by Yamanouchi (now called Astellas Pharmaceuticals), pyridofuropyrimidines, such as PI103 [31], and thienopirymidines [32] were used as starting points for medicinal chemistry lead optimization. PI103 inhibits PI3K, but also mTOR and DNA-PK (DNA-dependent protein kinase) and has been shown to produce significant benefit using various in vivo models when given through the intraperitoneal route, including glioblastoma [33,34]. GDC-0941 (Genentech) has improved drug-like and pharmacokinetic properties over PI103 [35], justifying its clinical development (Table 1). XL147 and XL765 (Exelixis) entered clinical trials in June 2007 (Table 1). Little is known about these inhibitors (the chemical structures have not yet been disclosed), with the exception that XL147 does not inhibit mTOR in contrast with XL765. NVP-BEZ235 (Novartis) is an imidazoquinoline derivative that has been optimized for PI3K inhibition [36]. The pre-clinical (see below) and safety profile of this compound supported its clinical development as a ‘first in class’ PI3K inhibitor for cancer treatment.

NVP-BEZ235, a dual PI3K/mTOR inhibitor: lessons learned and challenges

In addition to PI3K, NVP-BEZ235 also blocks mTOR kinase activity in biochemical assays [IC50 = 20.7 nM; K-LISA (kinase activity ELISA)] and the mTORC1 and mTORC2 kinase activity in immune-kinase assays. Accordingly, the compound is able to significantly reduce the levels of phosphorylated RPS6 (ribosomal protein S6) in TSC1-deficient cells [1]. In contrast with what is described for available PI3K inhibitors such as wortmannin, LY294002 or ZSTK474 [37], NVP-BEZ235 produces strong antiproliferative activity, with low-nanomolar GI50 (concentration giving half-maximal growth inhibition), regardless of the genetic alterations present [1]. Similar data were obtained in a panel of breast cancer cell lines (not shown) and are reminiscent of the effects observed with rapamycin derivatives [10,38]. Seemingly, the mTOR inhibition is responsible for the strong antiproliferative activities displayed. One consequence of this could be that a monolayer proliferation assay might be misleading as a read-out. Hence, GI50 determination will not be useful to delineate and predict which tumour lines harbouring specific genetic alterations (PTEN, PIK3CA, AKT, etc.) will be likely to respond to treatment. In vivo efficacy studies might be more

**Table 1 | Continued**

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<th>Compound</th>
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<tr>
<td>CAL101</td>
<td>Calistoga</td>
<td>PI3Kα</td>
<td><img src="image" alt="Structure" /></td>
<td>Pre-clinical profile presented at the AACR Special Conference on PI3K (11-14 November 2008) [58] Entered Phase I trial in June 2008 Targeted diseases: relapsed or refractory chronic lymphocytic leukaemia, acute myeloid leukaemia, selected B-cell non-Hodgkin’s lymphoma Starting dose: 50 mg Route and schedule: oral, twice per day</td>
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useful for such correlative studies. Alternatively, and perhaps more approachable, three-dimensional culture assays might be better to establish such correlative studies. These assays have been used successfully to show the antiproliferative activities of PX-866 against cells grown in spheroids [39].

It is also interesting to note that cell death induction (whether apoptotic, autophagic or necrotic) is not always observed using these inhibitors. Similar observations were made with the PTEN-null cell lines U87MG and PC3M exposed to the dual PI3K/mTOR inhibitor NVP-BEZ235 [1]. PI3K and Akt are key mediators of cell survival, but these abilities would be unrecognised probably only under specific experimental conditions, or in tumour lines with different genetic alterations. More studies are certainly required to draw definitive conclusions, but it is tempting to speculate that different responses are likely to occur between different cancer lineages. Moreover, the effect of the compound in the tumour environment will certainly also have a strong influence on the clinical response. PI3K is an important player in VEGF (vascular endothelial growth factor) signalling, and, as such, a PI3K inhibitor might display some anti-tumour activity in vivo by inhibiting angiogenesis, even if modest effects are observed in vitro.

**PI3K inhibition in humans: how do we measure and quantify it?**

Tumour pharmacodynamic markers for PI3K inhibition are, from the perspective of pathway modulation, a straightforward approach. Post-treatment tumour biopsies, prepared for immunohistochemistry staining, are expected to show some level of inhibition in pSer473-Akt and/or pSer235/pSer236-RPS6 levels. However, technical hurdles might arise when trying to establish phosphoprotein basal levels, which is a prerequisite for H-score (histoscore) calculation. The sensitivity of the method is dependent on the preparation of the samples and the specificity of the antibodies. Because the availability of pre- and post-treatment paired tumour biopsies is unlikely during Phase I dose-escalation trials, the detection and quantification of response biomarkers by the collection of surrogate tissues such as skin will certainly extend the sampling panoply and reduce the variability owing to the samples and the specificity of the antibodies. Because the availability of pre- and post-treatment paired tumour biopsies is unlikely during Phase I dose-escalation trials, the detection and quantification of response biomarkers by the collection of surrogate tissues such as skin will certainly extend the sampling panoply and reduce the variability owing to the heterogeneity generally observed and inherent to tumour masses. Immunohistochemistry studies performed in skin from mice have revealed the presence of pSer473-Akt staining, but only in the external root sheath keratinocyte layer of the hair follicle. Accordingly, the PI3K pathway has recently been shown to be an important mediator of keratinocyte plasticity, through a thrombin-dependent mechanism [40]. The PI3K inhibitor PX-866 was found to reduce pSer473-Akt levels in this region of the hair follicle upon administration. Moreover, human hair with intact roots (i.e. containing the follicles) was also shown to give positive staining for pSer473-Akt [41]. This approach is minimally invasive and could nicely complement skin biopsies.

PI3K inhibitor trials might, once more, benefit from the pre-clinical and clinical pharmacodynamic studies that have been performed with mTORC1 allosteric inhibitors. A pharmacodynamic study performed with tumour and skin biopsies from patients treated with RAD001 at different doses and schedules (20, 50 and 70 mg weekly or 10 and 20 mg daily), revealed strong reduction of pSer235/pSer236-RPS6 and pSer1108-eIF (eukaryotic initiation factor) 4G levels similar in both tissues [42]. Interestingly, pThr37/46-4E-BP1 (eIF4E-binding protein 1) levels were more strongly reduced in skin than in tumours. The activated levels of this mTOR direct effector has been shown to be well correlated with the grade of breast tumour malignancy, in contrast with RPS6 and p70S6K [43]. Hence, constant reduction with time of some key biomarkers might not predict the anti-tumour activity; other tumour response markers [Ki67, PCNA (proliferating-cell nuclear antigen)] should be tested in parallel.

The preparation of PBMCs (peripheral blood mononuclear cells) is an interesting alternative as an easy-to-obtain surrogate tissue. PBMCs from A20948 tumour-bearing rats were shown to have reduced pThr245-4E-BP1 levels upon treatment with RAD001, and they could also be used to monitor the activity of p70S6K in a regular immune kinase assay [44]. These pharmacokinetic/pharmacodynamic studies have even allowed the establishment of the OBD (optimal biological dose) for RAD001 (everolimus) in humans [38,45]. Data presented at the ASCO 2008 annual meeting have revealed the ability of XL765 to reduce pThr451-PRAS40 (proline-rich Akt substrate of 40 kDa) and pThr37/46-4E-BP1 levels in PBMCs from treated patients. No data were, however, reported for pSer473-Akt, pThr308-Akt or pSer235/pSer236-RPS6 levels. One might speculate that the basal levels for these markers are below the limit of detection. In agreement with this, rat PBMCs have undetectable pSer235/pSer236-RPS6 levels [44].

Perhaps even more easy to measure are blood glucose and insulin concentrations. It is well established that PI3K, notably the α paralogue, is a major player in glucose uptake in insulin-dependent tissues [25,46,47]. As such, its inhibition might have an impact on glucose homoeostasis. Indeed, this is the case in mice upon treatment with PX-866, an effect that could be silenced with the PPARγ (peroxisome-proliferator-activated receptor γ) inhibitor pioglitazone [21]. It is, however, too early to speculate whether these effects could be recapitulated in humans, as these two parameters are largely influenced by the general poor health of patients enrolled in Phase I studies, and by the fact that an increase in insulin and glucose will certainly depend on the pharmacokinetic profile of the compound (e.g. tissue distribution, clearance, accumulation). To avoid artefactual results arising from food intake, a well-controlled glucose-tolerance test should be performed on patients that have fasted for a minimal period of time. Tumour cells tend to use only glycolysis and to not employ the mitochondrial oxidative pathway for ATP production. Consequently, fewer ATP molecules are produced per molecule of glucose. This effect, also known as the anaerobic glycolysis or ‘Warburg effect’, has recently been demonstrated to be the consequence of the expression of the fetal (PKM2), rather than the adult (PKM1), form of pyruvate kinase [48,49]. Tumour cells compensate by having a higher glucose uptake. The use of FDG-PET...
Figure 1 | Effects of PI3K inhibitors on VEGF-induced neovascularization and vasculature permeability in vivo

(A) FVB mice implanted with Teflon agar chamber loaded with agar alone (agar) or with 2 μg/ml VEGF165 (agar+VEGF) were treated orally at the indicated dose levels and regimen either with or without the vehicle control (Vehicle) for 4 days. Animals were killed 24 h after the last dose, for quantification of the amount of VEGF-induced neovascularized tissue (left panel), and Tie-2 (angiopoietin receptor) levels by ELISA (right-hand panel). *P ≤ 0.05 (ANOVA and Dunnett’s test) compared with VEGF-treated controls. (B) FVB mice pre-treated with NVP-BEZ235 (45 mg/kg), ZSTK474 (100 mg/kg, orally), or with the vehicle control, were injected intravenously with Evans Blue and challenged 30 min later with a VEGF injection in the ear. Mice were then killed, the dye extravasation area was photographed (left-hand panel), and extracts from lungs were analysed by Western blotting (right-hand panel) for the proteins indicated.

(2-[18F]fluoro-2-deoxy-D-glucose positron-emission tomography) imaging could therefore be used to monitor PI3K inhibition non-invasively. Pre-clinical validation in rodents is currently ongoing with PI3K inhibitors.

PI3K inhibition and vasculature permeability

VEGF is a growth factor polypeptide that specifically binds and activates VEGFR (VEGF receptor) 1 and 2, which are exclusively expressed at the surface of endothelial cells. VEGF is a master regulator of neo-angiogenesis, but it was first described as VPF (vascular permeability factor), as it is the factor responsible for vessel permeability regulation, i.e. the propensity of the vessels to allow the diffusion of liquids and molecules from the blood into the surrounding tissues. Biochemical and genetic studies have allowed the identification of eNOS (endothelial nitric oxide synthase) as the VEGF key downstream mediator of vessel leakage. Moreover, eNOS has now been validated as an important factor for tumour initiation and maintenance [50]. Hence, through blockade of the VEGF signalling pathway, PI3K inhibitors are expected to display anti-angiogenic properties. Mice expressing only one allele of the p85α subunit in the endothelium do present vasculature defects when challenged with high VEGF levels, resulting in reduced tumour growth, following tumour cells [51]. Accordingly, NVP-BEZ235 was shown to strongly block VEGF-induced HUVEC (human umbilical vein endothelial cell) proliferation in vitro, and
to reduce the VEGF neo-angiogenic process in vivo [52]. Moreover, NVP-BEZ235 treatment led to a strong and rapid decrease of tumour permeability and subsequent tumour intrafluid pressure. This phenomenon correlates well with the reduction of pSer1977-eNOS levels, and could not be observed for the TORC1 inhibitor RAD001, demonstrating that this is certainly due to the inhibition of PI3K. Confirming this statement, similar results were obtained when tumour-bearing animals were treated with the pan-class I PI3K inhibitor ZSTK474 (Figure 1). Vasculature permeability can be recorded non-invasively, using specific contrast agents such as Vistarem® in DCE-MRI (dynamic contrast-enhanced magnetic resonance imaging) studies. This technology was used to demonstrate the ability of the HIF1α (hypoxia-inducible factor 1α) inhibitor PX-478 to reduce tumour permeability [53]. Studies performed in BN472-tumour-bearing rats have also shown striking long-lasting decreases when animals were treated with NVP-BEZ235 [52]. Preliminary data showed similar effects in HT29-tumour-bearing animals treated with PX-866 [54]. Incorporation of such imaging technologies in Phase I/II studies will certainly provide strong evidence to demonstrate the value of PI3K inhibition in humans.

Conclusion
Massive efforts have been deployed by the scientific community to better understand the connectivity between the players involved in the PI3K pathway. For instance, the precise definition of the feedback loops emerging from mTOR have delineated the advantages and disadvantages of blocking the pathway solely at the mTOR level, and highlighted the fact that dual PI3K and mTOR blockade might overcome some of the effects associated with pure mTOR inhibition. Multiple compounds targeting PI3K or PI3K and mTOR have now entered clinical trials. On the basis of the preclinical data generated in mouse and rat models, a therapeutic window for PI3K treatment exists, but only Phase I dose-escalation trials will tell whether efficacious dose levels can be reached in a tolerable manner in humans. Understanding the nature of any such toxicity will be important. For instance, if the toxicological profiles were demonstrated to be related to the lack of target specificity, the development of second-generation paralogue-specific PI3K inhibitors would be warranted. Finally, in the very near future, the validation of predictive biomarkers for target modulation and/or anti-tumour activity associated with PI3K inhibition will be available. The imaging technologies (DCE-MRI, FDG-PET) that could be used to follow up PI3K inhibition in a non-invasive manner will certainly be indispensable tools for rapid clinical development of the long-awaited PI3K modulators.

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