A Novel, Selective, and Efficacious Nanomolar Pyridopyrazinone Inhibitor of V600E BRAF

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Abstract

Oncogenic BRAF is a critical driver of proliferation and survival and is thus a validated therapeutic target in cancer. We have developed a potent inhibitor, termed 1t (CCT239065), of the mutant protein kinase, V600E BRAF. 1t inhibits signaling downstream of V600E BRAF in cancer cells, blocking DNA synthesis, and inhibiting proliferation. Importantly, we show that 1t is considerably more selective for mutated BRAF cancer cell lines compared with wild-type BRAF lines. The inhibitor is well tolerated in mice and exhibits excellent oral bioavailability (F = 71%). Suppression of V600E BRAF-mediated signaling in human tumor xenografts was observed following oral administration of a single dose of 1t. As expected, the growth rate in vivo of a wild-type BRAF human tumor xenograft model is unaffected by inhibitor 1t. In contrast, 1t elicits significant therapeutic responses in mutant BRAF–driven human melanoma xenografts. Cancer Res; 70(20): 8036–44. ©2010 AACR.
there is a pressing need to develop more potent and selective cellular inhibitors of oncogenic BRAF to enable rigorous assessment of the consequences of BRAF inhibition in tumor xenografts and ultimately in patients.

An inhibitor of V600EBRAF, SB590885, was described as a potent type I (active conformation binder) inhibitor of purified V600EBRAF in vitro and to have excellent cellular activity but poor pharmacokinetic/pharmacodynamic characteristics (11). Other inhibitors include, RAF653, a pan RAF inhibitor which is in phase I/II clinical trials (http://www.clinicaltrials.gov), and PLX4720, a potent and selective type I inhibitor of mutant BRAF–driven cell proliferation in vitro and of melanoma xenograft growth in mice (12). Its close analogue, PLX4032, is currently in phase II/III clinical trials following promising phase I results (13).

Here, we describe and characterize a new pyridopyrazinone V600EBRAF inhibitor, called 1t (CCT239065). This compound is a type II inhibitor (inactive conformation binder) and we describe its activity in vitro and in vivo, and show its potential for development as a therapeutic inhibitor that targets oncogenic BRAF.

Materials and Methods

Cell culture

WM266.4, SW620, A375M, and Ba/F3 cell lines were obtained from American Type Culture Collection/LGC standards (Tedington, United Kingdom) and D35 cells were a kind gift from Dr. Nick Hayward (Queensland Institute of Medical Research, Queensland, Australia). All lines were reauthenticated by short tandem repeat and array comparative genomic hybridization analysis within 6 months prior to submission of the article. The cells were cultured in RPMI 1640 (Ba/F3) or DMEM (WM266.4, SW620, A375M, D35) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 10% CO2. The BRAF and RAS mutation status of the cell lines was determined (Supplementary Table S1). Inhibitor 1t (CCT239065) was synthesized as described (14). Drugs were dissolved in DMSO at 10 mmol/L and diluted as required.

Molecular modeling

Inhibitor 1t was docked into BRAF (PDB code: UWH) using GOLD version 3.1.1 (15). To prepare the receptor for docking, the crystal structure was protonated using the Protonate3D tool of MOE (Chemical Computing Group, Cambridge, United Kingdom), and the ligand and water molecules were then removed. The active site was defined using a radius of 10 Å from the backbone oxygen atom of Asp594 of the ATP binding pocket. Partial charges of the ligand were derived using the Charge-2 CORINA 3D package in TSAR 3.3, and their geometries optimized using the COSMIC module of TSAR. Ten docking solutions were generated per docking run with GOLD, and the best three were stored for analysis.

Cell lysis and Western blotting

Cells lysates were prepared as described (16) for Western blotting using standard approaches and quantification using an Odyssey IR scanner (Li-Cor Biosciences). The following primary antibodies were used: phosphorylated MEK1/2 (Cell Signaling Technologies), MEK1 (BD Biosciences), phosphorylated ERK1/2 (Sigma), cyclin D1, and ERK2 (Santa Cruz Biotechnology). Secondary antibodies were goat anti-mouse Alexa Fluor 680 (Invitrogen) and goat anti-rabbit 800CW (Li-Cor Biosciences).

Phosphorylated ERK cell-based ELISA

WM266.4 cells were seeded at 3 × 104 per well of a 96-well plate, treated with an 11-point titration of compound after 24 hours and after a further 6 hours, fixed in 4% formaldehyde and 0.1% Triton in PBS. Nonspecific sites were blocked with 5% milk/PBS and incubated with an antiphosphorylated ERK antibody (Sigma) for 2 hours, washed with 0.1% Tween 20 and incubated with an anti-mouse Europium conjugated antibody for 1 hour. Time-resolved fluorescence was measured in the presence of enhancement solution (Perkin-Elmer) using a Spectramax M5 plate reader (Molecular Devices). Fluorescence values were normalized to protein concentration as determined by the bicinchoninic acid assay (Sigma). IC50 values for ERK inhibition were determined with GraphPad Prism software (GraphPad Software) and are the mean of three independent assays.

Kinase assays

V600EBRAF protein was expressed, purified, and kinase activity measured as described using 96-well format assays and DELFIA detection (17). This assay measures the direct phosphorylation of bacterially produced GST-MEK by BRAF at an ATP concentration of 100 μmol/L. Duplicate assays were performed within the linear range of the assay (45 min at 20°C), with an 11-concentration response curve to generate IC50 values using GraphPad Prism software. Each IC50 value was derived from the mean of three independent assays. Profiling of 1t against selected kinases using SelectScreen Panel technology was performed according to the commercial provider’s protocols (Invitrogen).

Proliferation assays

The growth-inhibitory activity of 1t in a panel of melanoma, colon, and breast cancer cell lines was determined using sulforhodamine B reagent following a 5-day exposure to the compound (18). Cell proliferation was also assessed using the MTS reagent (Promega). Assays were performed in quadruplicate with a 10-point dilution series and IC50 values were calculated using GraphPad Prism software (GraphPad Software). The number of cells seeded was optimized for each cell line to ensure logarithmic growth could occur over the duration of treatment. DNA synthesis was assessed by measuring tritium-labeled thymidine incorporation. Ba/F3 cells (1 × 104–5 × 104) were seeded into the wells of 96-well plates and compounds were added to the desired concentration. After 20 hours, 0.08 μCi of [3H]thymidine (GE Healthcare) was added to each well, and after a further 4 hours, the cells were captured onto Multiscreen glass fiber 96-well plates (Millipore), washed twice with PBS and twice with methanol using a vacuum manifold. Microscint 20 (25 μL; Perkin-Elmer) was added to the wells prior to counting on a TopCount NXT.
For adherent cells, $10^5$ cells were seeded into six-well plates and 0.8 $\mu$Ci of $[^3]$H]thymine added per well. Cells were harvested by trypsinization and an aliquot analyzed as above.

**Pharmacokinetics**

All procedures involving animals were performed in accordance with National Home Office regulations under the Animals (Scientific Procedures) Act 1986 and within guidelines set out by the Institute’s Animal Ethics Committee and the Committee of the National Cancer Research Institute (19).

Pharmacokinetic analyses were performed in female BALB/cAnNCrl mice >6 weeks old, dosed i.v. (2 mg/kg, 10 mL/kg, in DMSO/Tween 20/water 10:1:89 v/v) or p.o. by gavage (10 mg/kg, 10 mL/kg, in DMSO/water 1:19 v/v). At intervals of 5 (i.v. route only), 15, and 30 minutes and 1, 3, 6, and 18 hours after dosing; three mice were placed under isoflurane anesthesia and blood for plasma preparation was taken into heparinized syringes. Femoral muscle was also taken following i.v. and p.o. administration. Plasma and tissue storage, extractions, and analysis were performed as described (20, 21).

**Therapy studies**

Tolerability studies were performed by dosing mice with 10 or 20 mg/kg 1t p.o. daily for 4 days and monitoring body weight for a further 27 days. Female Crl/CD1-Foxn1nu mice >6 weeks old were inoculated s.c. with a suspension of human tumor cell lines. For p.o. therapy, after inoculation of either $10^7$ A375M human melanoma cells or $7 \times 10^6$ SW620 human colorectal carcinoma cancer cells, the xenografts were allowed to grow to 50 to 150 mm$^3$. Groups of eight mice were

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Compound 1t is a potent and selective inhibitor of oncogenic BRAF. A, chemical structure of 1t/CCT239065 1-(3-tert-butyl-1-p-tolyl-1H-pyrazol-5-y1)-3-[2-(methylthio)-4-3-oxo-3,4-dihydropyrindol2,3-b]pyrazin-8-yloxy)phenyl)urea. B, inhibition of recombinant full-length $^{14}$C$^3$BRAF in vitro by 1t (○) and inhibition of ERK (measured by activation segment phosphorylation) in WM266.4 ($^{14}$C$^3$BRAF) melanoma cells following a 6-h exposure to increasing concentrations of 1t (●). C, selectivity profile of 1 $\mu$mol/L 1t tested against a panel of 80 kinases (inset). Kinases inhibited by >70% are presented in the main histogram. D, docking of 1t into the cocrystal structure of BRAF and sorafenib (pdb code UWH). The BPI pocket formed by residues Val471, Ala481, Lys483, and Ile527 is indicated by the red surface.
then allocated to treatments using stratified distribution of tumor volumes. Inhibitor 1t (20 mg/kg/d, 10 mL/kg, in DMSO/water 1:19 v/v) or control vehicle was given by gavage. Tumors were measured with calipers at least twice per week.

**Pharmacodynamics**

Mice bearing established, A375M or SW620 xenografts were prepared as for the therapy studies above. For WM266.4 tumors, 8 x 10^3 cells were inoculated. Three to four animals were dosed p.o. by gavage with 1t (20 mg/kg) and three to four animals with control vehicle. After one dose, mice were culled by cervical dislocation 4 hours postdosing. Tumors were halved and snap-frozen using liquid nitrogen. Control mice were processed similarly approximately 4 hours after dosing. Tumors were lysed in NP40 buffer and homogenized using a Precellys 24 (Stretton Scientific). Equal amounts of protein were analyzed by quantitative Western blotting as described above.

**Results**

We have developed a series of novel BRAF inhibitors (14, 22-25). One such compound called CCT290695 (henceforth referred to as 1t) with the formula 1-(3-tet-butyl-1-p-tolyl-1H-pyrazol-3-yl)-3-(2-(methylthio)-4-(3-oxo-3,4-dihydropyrido [2,3-b]pyrazin-8-yloxy)phenyl)urea (Fig. 1A) potently inhibits the kinase activity of recombinant, full-length V600E BRAF in vitro with an IC50 of 0.019 ± 0.004 μmol/L (Fig. 1B; Table 1). To demonstrate that 1t is active against oncogenic BRAF in cells, we show that it inhibits ERK1/2 phosphorylation at 0.005 ± 0.002 μmol/L in WM266.4 cells (Fig. 1B), a melanoma line in which we previously established this pathway to be driven by oncogenic V600E BRAF (5). We also show that 1t achieves high levels of selectivity in vitro and at 1 μmol/L, a concentration that is approximately 50 times higher than its IC50 value against purified V600I BRAF, it failed to inhibit most of the kinases in an 80 kinase panel that represents all branches of the human kinome (Fig. 1C; Supplementary Table S2). Profiling of 1t against 16 kinases in the SelectScreen Panel (Invitrogen) showed that the most sensitive kinases are LCK (IC50 6 nmol/L), CRAF (IC50 12 nmol/L) V600E BRAF (13 nmol/L), and SRC (23 nmol/L), but importantly, 1t is more than 6-fold less active against wild-type BRAF (81 nmol/L) and more than 50-fold less active against VEGFR2/KDR than against V600E BRAF (Table 1).

We have shown that close analogues of compound 1t are type II inhibitors and so bind to the inactive conformation of BRAF (24, 25). Docking studies suggest that 1t also binds to the inactive conformation of BRAF, with the pyridopyrazin-3 (4H)-one moiety forming two hydrogen bonds with the backbone of Cys532 of the hinge region (Fig. 1D). Three more H bonds are predicted to be formed by the urea moiety of the inhibitor, two between the NH groups and the Glu501 side chain and one between the carbonyl moiety and the backbone of Asp594 of the DFG motif. The tert-butyl pyrazole of the terminal pyrazole ring of 1t resides in a kinase pocket beyond the gatekeeper residue, termed the BPII pocket by Liao (26). Importantly, the thiomethyl group of the middle aromatic ring elaborates into the BPI pocket (26) and forms van der Waals contact with the aliphatic side chains of Ile527, Val471, Lys483, Ala481, and Thr529. We previously reported how elaboration into the BPI pocket improves the selectivity of BRAF inhibitors (22, 24), so the thiomethyl group is likely to contribute to both potency and selectivity of 1t.

In accordance with its in vitro selectivity, 1t inhibits the growth of cancer cell lines harboring V600E/D BRAF mutations, but is relatively ineffective in cell lines in which BRAF is wild-type (Fig. 2A). Concordant with this, 1t induces a profound inhibition of DNA synthesis in mutant V600E BRAF cells but not in mutant KRAS cells (Fig. 2B). To further characterize the BRAF-selective activity of 1t, we generated a mutant of V600E BRAF in which the gatekeeper threonine at position 529 is mutated to asparagine (T529N/V600E BRAF). This mutant is resistant to 1t in vitro and is not inhibited by this compound at up to 10 μmol/L (Fig. 2C). Ba/F3 cells normally grow in an IL-3–dependent manner, but their growth can be rendered IL-3–independent by enforced expression of V600E BRAF or T529N/V600E BRAF (27). Notably, ERK phosphorylation is considerably more sensitive to 1t in V600E BRAF-expressing Ba/F3 cells than in the T529N/V600E-BRAF-expressing Ba/F3 cells (Fig. 2D) and this is reflected in their growth, with the V600E-BRAF-expressing Ba/F3 cells being inhibited by 96% following a

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<th>Kinase</th>
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NOTE: The potency of 1t was assessed by performing a 10-point titration of the compound in the presence of 100 μmol/L of ATP and each individual kinase using the Z’-LYTE system (Invitrogen). Note, when analyzed using our in-house DELFI/A assay format, we observe an IC50 for V600E BRAF of 0.019 μmol/L.
24-hour treatment with 1 μmol/L of 1t compared with only 21% in the T29N,V600E BRAF-expressing cells (Fig. 2D).

To demonstrate its selectivity further, we show that after 24 hours, 1t potently inhibits MEK and ERK phosphorylation in V600D BRAF WM266.4 cells (10–100 nmol/L) and this is accompanied by decreased expression of cyclin D1 (Fig. 3A), the transcription of which is regulated by the mitogen-activated protein kinase pathway (28). In contrast, no such responses are observed in BRAF wild-type D35 melanoma cells at concentrations up to 10 μmol/L (Fig. 3B). Furthermore, in KRAS mutant SW620 colorectal carcinoma cells, 1t induces a profound increase in MEK and ERK phosphorylation and this is accompanied by increased cyclin D1 expression (Fig. 3C). We attribute this effect to the transactivation of CRAF by BRAF through a mechanism involving RAS-dependent BRAF/CRAF heterodimerization, which promotes activation of the downstream signaling cascade, as we and others recently reported (29, 30). Notably, the increase in pathway activation is accompanied by a small increase in proliferation driven by 1t in SW620 cells (Fig. 3D).

We next examined the efficacy of 1t in vivo. When administered by i.v. injection, 1t shows a very low plasma clearance (0.4 mL/h) consistent with the absence of metabolism and a terminal half-life of 6.8 hours (Fig. 4A; Supplementary Table S3). Plasma concentrations of 1t achieve over 100-fold greater than the average GI50 value we observe for BRAF mutant cancer cell lines in vitro (Fig. 4A; Supplementary Table S3) and are sustained above the average GI50 in plasma and muscle (used as a tumor tissue surrogate) for over 18 hours. 1t has excellent oral bioavailability of 71% and a single oral dose of 10 mg/kg maintained plasma and muscle concentrations above 19 and 3 μmol/L, respectively, for at least 18 hours (Fig. 4A; Supplementary Table S3). Given these excellent pharmacokinetic properties, we assessed 1t for biomarker modulation in vivo to show the on-target activity of the compound. A single p.o. dose of 20 mg/kg suppresses the phosphorylation of MEK by >50% in mutant BRAF human WM266.4 melanoma xenografts, relative to vehicle-treated mice (Fig. 4B). We therefore determined the tolerability of 1t following multiple oral dosing of 10 and 20 mg/kg/d in mice for 4 days and measured the effect on body weight (Fig. 4C). No adverse effects were observed. The growth of established V600E BRAF A375M melanoma xenografts is reduced by p.o. administration of 1t (20 mg/kg every day)
for 24 days, with a significant growth inhibition of 50% ($P < 0.05$) upon completion of the experiment (Fig. 5A). Inhibition of MEK phosphorylation following a single dose of I is also observed in this tumor model (Fig. 5B). To show the dependency on BRAF inhibition for antitumor efficacy of I, we also treated mice bearing the $^{G12V}$KRAS mutant human colorectal carcinoma SW620 xenografts for 23 days. No inhibition of tumor growth is observed in this model, consistent with the in vitro data for this cell line (Fig. 5C). Curiously, we also do not see enhanced tumor growth in this model, despite the increase in MEK phosphorylation induced in these tumors (Fig. 5D). Importantly, I is well tolerated, as judged by the observation that the continuous daily dosing used in these therapy experiments does not cause any deaths and causes <10% body weight loss over the course of the treatment (Supplementary Fig. S1).

Discussion

Herein we describe the activity of a novel highly selective small molecule inhibitor of oncogenic BRAF. In vitro, this compound does not inhibit the majority of kinases in a panel of 80 receptor and non–receptor kinases and selectively inhibits the proliferation of cancer cell lines harboring oncogenic mutations in BRAF. In silico docking shows that the thiomethyl group on the central ring of I (CCT239065) extends into the BPI cavity of BRAF and may therefore contribute to I selectivity. We previously showed that oncogenic RAS signals exclusively through CRAF and may therefore contribute to BRAF inhibition (31), and notably, I is also relatively ineffective against cancer lines harboring mutations in RAS genes, as observed for other selective BRAF inhibitors (11, 12). Interestingly, given the equipotent activity of I against $^{V600E}$BRAF and CRAF in vitro, it is surprising that CRAF inhibition is not achieved in RAS mutant cells. However, like many other RAF inhibitors, I is ATP-competitive and it has recently been shown that $^{V600E}$BRAF has considerably lower affinity for ATP than wild-type BRAF or wild-type CRAF, providing an elegant explanation of why wild-type BRAF and CRAF may not be efficiently inhibited by I in cells (29).

Our data also reveal that sensitivity to BRAF drugs may not be determined by BRAF mutation status alone. For example, $^{V600E}$BRAF mutant HT29 cells were less sensitive...
to \( \text{It} \) than the majority of the other BRAF mutant cell lines, whereas SKMEL23 cells were considerably more sensitive to \( \text{It} \) than the other BRAF/RAS wild-type cells. Similar responses have been previously reported in these lines using another BRAF inhibitor, GDC-0879 (32). It has been suggested that HT29 cells are resistant to drugs of this class because they express high levels of glucuronosyltransferase that could metabolize these drugs (33). Conversely, it is feasible that SKMEL23 cells have, as yet unidentified, genetic alterations that confer sensitivity to this class of drug. These observations highlight the fact that sensitivity to specific drugs may not always be determined by a single mutation, and that other genetic aberrations in specific cancer cells could modify cell responses (29). Nevertheless, together, our data suggest that in the cellular context, \( \text{It} \) selectively inhibits oncogenic BRAF over CRAF or the other kinases that are critical for proliferation of BRAF wild-type or RAS mutant cells.

Consistent with the selective nature of \( \text{It} \), there is a close correlation between the inhibition of ERK phosphorylation and the inhibition of growth in \( \text{V}^{600\text{D/E}} \) BRAF mutant cells and analysis of the ERK pathway gives direct evidence of \( \text{V}^{600\text{D/E}} \) BRAF inhibition, resulting in loss of MEK and ERK phosphorylation and loss of cyclin D1 expression. \( \text{It} \) therefore induces collapse of signaling downstream of oncogenic BRAF and importantly this leads to an inhibition of DNA synthesis and growth arrest. It is interesting to note that the cellular potency of \( \text{It} \) is approximately 4-fold greater than the ability of \( \text{It} \) to inhibit recombinant \( \text{V}^{600\text{D/E}} \) BRAF \textit{in vitro}. The reasons for this are unclear but may reflect the complex nature of the interactions between BRAF and other proteins in the cell, such as the molecular chaperone HSP90, which may improve drug access to BRAF in cells, but not \textit{in vitro}. Alternatively, it is possible that the drug accumulates in cells. To address this, and show that the therapeutic activity of \( \text{It} \) is dependent on its ability to target mutant BRAF, we generated a gatekeeper mutant of \( \text{V}^{600\text{E}} \) BRAF that is resistant to \( \text{It} \). This was used to transform Ba/F3 cells and we show that \( \text{T}^{529\text{N},\text{V}^{600\text{E}}} \) BRAF resistance to \( \text{It} \) translates into a dramatic reduction in antiproliferative activity. These data show that off-target effects, such as those against SRC, LCK, or p38α that were suggested by the \textit{in vitro} kinase screens do not seem to contribute to the compound’s activity in BRAF mutant cell lines. Clearly, however, we cannot completely exclude the possibility that in some genetic backgrounds, such as is present in SKMEL23 cells, other kinases/proteins could be targeted by \( \text{It} \).

\( \text{It} \) shows excellent oral bioavailability of 71% and dosing via this route led to a 50% inhibition of MEK phosphorylation in tumors following a single dose, confirming that \( \text{It} \) targets oncogenic BRAF \textit{in vivo}. Notably, daily p.o. dosing of \( \text{It} \) elicits a therapeutic response in \( \text{V}^{600\text{E}} \) BRAF human A375 melanoma tumor xenografts. Furthermore, \( \text{It} \) does not affect the growth of \( \text{G}^{12\text{V}} \) KRAS mutant SW620 tumors, consistent with mutant BRAF being the primary target of the compound. Interestingly, treatment of KRAS mutant tumors with \( \text{It} \) causes a 2-fold increase in MEK phosphorylation, which we attribute to enhanced activation of CRAF in response to selective BRAF inhibition (29, 30). Importantly, we do not, however, observe drug-induced accelerated tumor growth \textit{in vivo} in contrast to observations made with GDC-0879 (29). \( \text{It} \) is also well tolerated, with no adverse effects.
observed following daily drug treatment for extended periods, and we also did not observe any skin lesions of the type described with another BRAF inhibitor, GDC-0879 (29). This also shows that off-target activity against kinases such as SRC, LCK, or p38α inhibition was not inherently toxic.

Our modeling data suggest that it binds to the inactive conformation (type II inhibitor) of BRAF. In this, it is similar to sorafenib and RAF265, but distinct from agents such as SB590885 and PLX4720/PLX4032 that bind to the active or "active-like" conformation (type I inhibitors; refs. 11, 12, 34, 35). From a clinical perspective, it is likely to be important to have drugs that bind to BRAF through distinct mechanisms. Clinical experience with kinase inhibitors shows that clinical resistance often emerges through the acquisition of secondary mutations within the catalytic cleft of the target that prevents drug binding, including but not limited to gatekeeper mutations (36–38). In these cases, the availability of drugs with different binding modes provides an important alternative treatment option for patients and we have recently shown that type I binders are more sensitive to gatekeeper changes than type II binders (27). Given the potency, selectivity, and efficacy of it both in cell culture models and in human tumor xenograft models, our aim now is to assess the potential of agents such as it in melanoma patients whose tumors are driven by oncogenic BRAF.

**Disclosure of Potential Conflicts of Interest**

Authors who are, or have been, employed by The Institute of Cancer Research are subject to a "Rewards to Inventors Scheme," which may reward contributors to a programme that is subsequently licensed.

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