Anticancer Activity of BIM-46174, a New Inhibitor of the Heterotrimeric G\(\alpha\)/G\(\beta\gamma\) Protein Complex

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Abstract

A large number of hormones and local agonists activating guanine-binding protein-coupled receptors (GPCR) play a major role in cancer progression. Here, we characterize the new imidazo-pyrazine derivative BIM-46174, which acts as a selective inhibitor of heterotrimeric G-protein complex. BIM-46174 prevents the heterotrimeric G-protein signaling linked to several GPCRs mediating \((a)\) cyclic AMP generation (G\(\alpha\)s), \((b)\) calcium release (G\(\alpha\)q), and \((c)\) cancer cell invasion by Wnt-2 frizzled receptors and high-affinity neurotensin receptors (G\(\alpha\)o/i and G\(\alpha\)q). BIM-46174 inhibits the growth of a large panel of human cancer cell lines, including anticancer drug-resistant cells. Exposure of cancer cells to BIM-46174 leads to caspase-3-dependent apoptosis and poly(ADP-ribose) polymerase cleavage. National Cancer Institute COMPARE analysis for BIM-46174 supports its novel pharmacologic profile compared with 12,000 anticancer agents. The growth rate of human tumor xenografts in athymic mice is significantly reduced after administration of BIM-46174 combined with either cisplatin, farnesyltransferase inhibitor, or topoisomerase inhibitors. Our data validate the feasibility of targeting heterotrimeric G-protein functions downstream the GPCRs to improve anticancer chemotherapy. (Cancer Res 2006; 66(18): 9227-34)

Introduction

Emerging data show the involvement of guanine-binding protein-coupled receptors (GPCR) and their downstream signaling pathways in cancer progression (1–5). Growth of many cancers is stimulated by GPCR agonists, including vasoactive intestinal peptide (VIP), endothelin, neurotensin, thrombin, calcitonin gene-related peptide (CGRP), pituitary adenyl cyclase–activating peptide, or growth hormone–releasing hormone (6–8). In addition to their effect on cancer cell proliferation, several GPCR agonists also activate the mitotic and invasive potential of transformed cells (CGRP, gastrin-releasing peptide, bombesin, neurotensin, and thrombin; refs. 9, 10). More recently, aberrant expression and activation of GPCRs and/or their associated G-proteins have been reported in human cancers (11). For example, constitutively active GPCR mutants harbor ligand-independent oncogenic properties as shown for the thyroid-stimulating hormone, luteinizing hormone (12), and cholecystokinin receptors (13). Moreover, activating mutations of the G-protein subunits \(\alpha\), \(\alpha\)2, \(\alpha\)3, and \(\beta\) are reported as a putative cause of tumor formation (14, 15). Zeiger et al. (16) first showed \textit{in vivo} that constitutive activation of G\(\alpha\) by a transgene expressing the cholera toxin A1 subunit in normal cells induced the mitotic activity, infiltration of skeletal muscle, and lung metastases. Recent research shows that G\(\beta\)\(\gamma\) dimers are also involved in cancer transformation and invasive tumor growth because forced expression of G\(\beta\)\(\gamma\)-sequestering agents, such as the COOH terminus of G-protein–coupled receptor kinase 2 (ct-\(\beta\)ARK), obliterates multiple oncogenic pathways (17, 18). Conversely, overexpression of G\(\beta\)\(\gamma\) subunits and constitutively active mutants of G\(\alpha\) subunits stimulates mitogen- and stress-activated protein kinase cascade p42/p44, p38, and c-Jun NH\(_2\)-terminal kinase, which regulate promoter activity of immediate response genes involved in cell proliferation, apoptosis, and transformation (19).

The biological effect of a given GPCR agonist is tentatively inhibited by a corresponding selective antagonist targeting the receptor (20–23). However, the therapeutic effect of this strategy should appear limited because tumor growth is simultaneously driven by several GPCR agonists. Following the binding of the GPCR agonist, the signal is transduced to the heterotrimeric G-protein complex, leading to its dissociation and liberation of activated GTP-bound Ga subunit and G\(\beta\)\(\gamma\) dimers (4). Synthetic compounds and minigenes blocking heterotrimeric G-protein functions have been described previously (24–26). However, their potential therapeutic use is also limited due to their poor cell penetration and pharmacologic activity (26).

Here, we report the identification of BIM-46174, a novel synthetic small molecule inhibiting the heterotrimeric G-protein complex. Accordingly, BIM-46174 abrogates cancer cell invasion controlled by several GPCR agonists and inhibits cellular proliferation in a series of drug-resistant human cancer cell lines through caspase-3-dependent apoptosis. In addition, BIM-46174 exerts intrinsic antitumor activities and potentiates the activity of several classic anticancer agents in athymic mice bearing human lung and pancreatic cancer xenografts. Our data provide new insights into heterotrimeric G-proteins as targets for anticancer therapy using BIM-46174 alone or in combination with clinical anticancer drugs to limit the progression of advanced cancers.

Note: C. Gespach and P.G. Kasprzyk contributed equally to this work.

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Materials and Methods

GPCR and signal transduction analysis. MCF-7 cancer cells were cultured at 37°C in 5% CO2 humidified atmosphere using 96-well plates and DMEM containing 10% FCS, 1% glutamine, and antibiotics. After 4 days, cells were preincubated for 1 hour with BIM-46174. Then, cyclic AMP (cAMP)-inducing agents were added for a further 30-minute incubation period at 37°C. Intracellular cAMP levels were measured by RIA (kit SMP01A, NEN Life Science Products, Les Ulis, France). The EC50 and maximum effect (E_max) of activators were determined by computer-assisted nonlinear regression analysis (Data analysis toolbox, MDL Information Systems, Bourg La Reine, France). Intracellular calcium accumulation was measured by fluorescence assay according to the manufacturer (kit Calcium Plus, Molecular Devices, St. Gregoire, France). Data are expressed as relative fluorescence units.

Cell proliferation assay. Cell growth was determined by the WST1 colorimetric assay (Boehringer Mannheim, Meylan, Roche) as described previously (27). Briefly, cytotoxicity experiments were carried out at least thrice, each experiment representing eight determinations per concentration over the entire range. For each drug, the values included in the linear part of the sigmoid curve in each experiment were retained and a linear regression analysis was used to estimate IC50 values.

Cell cycle and apoptosis determination. FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France) was used to assess the change of cell cycle phase distribution and apoptosis induction as described previously (28). Briefly, cells were seeded at 0.5 × 10^5/mL culture medium in 25 cm² plastic flasks 24 hours before treatment. HL60 and NCI-H69 cells were then treated with drugs, and at selected time points, cells were harvested by centrifugation and washed twice in cold PBS. The cells were counted in a Beckman Z1 Coulter (Villepinte, France), fixed for 30 minutes at 4°C in 70% ethanol-PBS buffer, incubated with 50 μg/mL RNase A and 25 μg/mL propidium iodide or 5 μL Annexin V-FITC and 10 μL propidium iodide in binding buffer, and analyzed by two-color flow cytometry using a FACSCalibur cytometer.

Measurement of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase. Meso Scale Discovery (MSD) biomarker detection assays were used to measure the amounts of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) in cells following manufacturer’s recommendations. Briefly, logarithmically growing HL60 (2.5 × 10^5 per well) and NCI-H69 (4 × 10^5 per well) cells were seeded in 12-well plate for 18 hours and then treated by BIM-46174 (30 μmol/L) for 6 and 24 hours, respectively. Whole-cell lysates (10 μg proteins) were added to MSD multiplex plate coated with an anticleaved PARP antibody and an anticleaved caspase-3 antibody. Both cleaved PARP and cleaved caspase-3 were revealed with one anti-PARP antibody and one anti-caspase-3 antibody labeled MSD Sulfo-TAG reagent.

Inside the MSD instrument, a voltage applied to the plate electrode causes the labels bound to the electrode surface to emit light. The instrument measures intensity of the emitted light to afford a quantitative measure of the proteins of interest present in the samples issued of treated or untreated cells. All data are mean ± SE from at least two experiments.

Cellular invasion assay. Cancer cell invasion assays were done using collagen type I gels and parental or stably transfected colorectal, kidney, and lung cell lines as described previously (18, 29, 30). This assay is based on counting the percentage of invasive cells in collagen type I versus total number of viable cells in the same field. For each drug or agents at the concentrations used, it was systematically checked that none of them induced a loss of cell viability by trypan blue exclusion test during the 24-hour collagen invasion assay.

Growth of human cancer cell tumor xenografts. Human cancer cells (10^6) were s.c. injected into the flanks of female athymic NCR-nu/nu 4- to 6-week-old mice. Once the tumors (50-100 mm³) were established, treatment was done by i.p. injections. Tumor volume measurements and animal weights were monitored two to three times weekly. Tumor volumes were calculated as described previously (31).

Results

Identification of BIM-46174 as an inhibitor of heterotrimeric G-protein complex. To identify selective inhibitors of heterotrimeric G-protein complex from our chemical library, we have developed a differential screening approach using human breast cancer MCF-7 cells as a model. In intact cells, the adenyl cyclase effector generating cAMP from ATP can be stimulated indirectly by cholaer toxin via ADP ribosylation and constitutive activation of the Gα subunit, leading to activation of the plasma membrane–bound adenyl cyclase. A second way to stimulate adenyl cyclase is to use a direct adenyl cyclase activator, the diterpene forskolin. Using this differential approach, pretreatment of cells with a potential heterotrimeric G-protein complex inhibitor should inhibit cholaer toxin–induced cAMP generation but should be ineffective when adenyl cyclase was directly activated by forskolin. We found that the imidazo-pyrazine derivative BIM-46174 (Fig. 1A) significantly decreased the potency EC50 of cholaer toxin–induced cAMP generation without affecting the E_max = 78 ± 19 pmol/10⁵ cells (Fig. 1B). Thus, BIM-46174 competitively inhibited cholaer toxin–induced cAMP accumulation with a pA2 value of 5.3 ± 0.05 (n = 5). In contrast, no effect on BIM-46174 was observed on cAMP generation promoted by the direct adenyl cyclase activator forskolin even with high concentrations (100 μmol/L; Fig. 1C). To further assess the potential role of BIM-46174 as an inhibitor of heterotrimeric G-protein complex, its pharmacologic action has been challenged against adenyl cyclase activation induced by one natural neuropeptide, VIP. The receptor of VIP is coupled to cAMP generation via the Gα/γ heterotrimeric complex in MCF-7 cells (32). As shown in Fig. 2A, preincubation of MCF-7 cells with BIM-46174 for 1 hour remarkably decreased the E_max of VIP (8 ± 1 pmol/10⁵ cells) in a concentration-dependent manner (0-100 μmol/L. BIM-46174) without affecting the VIP potency EC50 = 0.9 ± 0.1 pmol/L, showing that BIM-46174 is a noncompetitive inhibitor of VIP receptor with a pD2 value of 5.2 ± 0.04 (n = 4). In addition, the increase of preincubation time (0.5, 1, and 2.5 hours) with BIM-46174 (10 μmol/L) decreases the ratio of the E_max of VIP after preincubation over the E_max of VIP alone (63%, 45%, and 32%), respectively (data not shown). Inhibition of both cholaer toxin- and VIP-induced cAMP generation by BIM-46174 was reversible on drug removal from the MCF-7 medium because cAMP levels could be recovered by 80% to 100% within 3 to 4 hours following a washout step (data not shown).

Because several GPCR agonists involved in cancer progression are linked to intracellular calcium translocation via the heterotrimeric protein complex Gαq/Gβγ, we next determined the effect of BIM-46174 on this cascade. As expected, BIM-46174 significantly reduced intracellular calcium translocation induced by the GPCR agonist endothelin-1 (ET-1) in human melanoma cells A2058 (Fig. 2B; ref. 33). Interestingly, when the intracellular calcium accumulation is induced by the G-protein–independent calcium ionophore A23187, the release was not affected by BIM-46174 (Fig. 2B).

Effect of BIM-46174 on cancer cell invasion induced by GPCR agonists and Gα/βγ subunits. Deregulated tumor cell growth, survival, and acquisition of the invasive phenotype are the hallmark of cancer progression. It was therefore of interest to determine whether BIM-46174 could inhibit cancer cell invasion induced by the Wnt oncogenic pathway that is directly connected with the heterotrimeric G-proteins Gαq/i and Gβγ (30). Treatment of HCT8/S11-Wnt-2 cells with either BIM-46174, the Gαq/i inhibitor pertussis toxin, the Gβγ-sequestering agent ct-JARK, or the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin significantly abrogated the invasive potential of these
Wnt-2-transformed human colon cancer cells in collagen type I (Fig. 2C). We also activated HCT8/S11 invasiveness by external addition of the Wnt pathway agonist Wnt-3a and observed similar reversion by BIM-46174 and pertussis toxin (data not shown). In addition to these Wnt agonists (30), autocrine and paracrine mechanisms involving other GPCRs modulate the cell migration (34). To lend further support to our functional studies, we focused on the proinvasive loop determined by neurotensin in the metastatic human lung cancer cell line LNM35. Both neurotensin receptor antagonist SR48692 and BIM-46174 (IC\textsubscript{50} 0.7 μmol/L) completely reversed this constitutive invasive phenotype (Fig. 3A). A neurotensin neutralizing antibody produced the same inhibitory effect (data not shown). On the other hand, collagen invasion by human colon cancer cells HT29 can be induced by addition of exogenous neurotensin (EC\textsubscript{50} 8 nmol/L; Fig. 3B). As expected, such invasion is significantly blocked by BIM-46174. This pharmacologic response correlated with the ability of BIM-46174 and the nonhydrolysable GTP analogue GppNHp to displace the binding of labeled neurotensin to HT29 cell membranes, a property commonly described for GPCR coupled with G\alpha subunit (data not shown). In such 24-hour cell invasion assay, the concentrations of BIM-46174 used are not associated with alterations of cell viability by the trypan blue exclusion test. In agreement, the effective concentrations of BIM-46174 to reverse collagen invasion were much lower than the concentrations needed to induce significant apoptosis after 72-hour drug treatment (see below).

**BIM-46174 has no effect on cancer cell invasion induced by individual subunits of heterotrimeric G-protein complex:** G\alpha\textsubscript{12}, G\alpha\textsubscript{13}, or G\gamma\textsubscript{12}y2 dimer. BIM-46174 has no effect on the invasive potential of MDCK\textsubscript{ts}.src cells (35) stably transfected by constitutively activated forms of either G\alpha\textsubscript{12} or G\alpha\textsubscript{13} subunits (GTPase-deficient mutants) or by forced expression of the G\gamma\textsubscript{12}y2 dimer (Fig. 3C; ref. 18). As well, BIM-46174 was ineffective against invasion induced by the G-protein–dependent constitutively activated lipid protein kinase P3K p110α (p110\gamma). Next, we examined the effect of BIM-46174 on HCT8/S11 colon cancer cell invasion induced by aluminium fluoride (AlF\textsubscript{4}\textsuperscript{−}). AlF\textsubscript{4}− is an activator of the complex formed by the GDP-bound G\alpha subunit associated with G-protein βγ subunits. By mimicking the γ-phosphate of Go-GTP, AlF\textsubscript{4}− initiates a cascade leading to G\alpha subunit activation and intracellular calcium elevation (36). AlF\textsubscript{4}− caused marked collagen invasion by HCT8/S11 cells (Fig. 3D), a cellular response that is completely reversed by BIM-46174.

**Inhibitory effect of BIM-46174 on the in vitro proliferation of human cancer cells.** The antiproliferative activity of BIM-46174 has been measured after a protracted exposure (96 hours) of human cancer cell lines established from nine tumor sites. The potency of BIM-46174 (IC\textsubscript{50} values) ranged from 0.6 to 25 μmol/L (Table 1). The antiproliferative activities of BIM-46174 are depending on the time treatment of exposure. For example, on MIA PaCa-2 cells, the IC\textsubscript{50} values, measured at 72 hours after the initiation of the treatment, are 34, 22, 14, and 10 μmol/L when BIM-46174 is applied for 3, 6, 9, or 72 hours. It is noteworthy that BIM-46174 was also active on several drug-resistant cell lines irrespective of the resistance mechanism involved, including drug extrusion (HL60DNR and HL60ADR) and alteration of the specific targets: topoisomerases (TOPO-II in HL60/MX2, TOPO-I in CCRF-CEM/C2) and glutathione S-transferase (HIC-H69/CPR). Most interesting, the resistance factors were much lower for BIM-46174 versus the reference drugs Adriamycin, daunorubicin, etoposide (VP-16), mitoxantrone (DHAD), and cisplatin (Table 1).

**BIM-46174 classified as National Cancer Institute COMPARE-negative compound.** To ensure the novelty of the proposed mechanism of action leading to cancer cell growth inhibition, BIM-46174 has been evaluated in the National Cancer Institute (NCI) COMPARE assay (37, 38). The NCI screens compounds for their ability to inhibit the growth of 60 human cancer cell lines in culture. Compounds with different mechanism of cell growth inhibition show different patterns of activity in the NCI screen. BIM-46174 (NSC727584) exhibited antiproliferative activities at the micromolar range on all 60 cell lines (data not shown). No
correlation was found in COMPARE analysis between BIM-46174 and 12,000 active compounds (Pearson correlation coefficient, >0.5). This database includes compounds acting through different mechanisms, such as DNA-damaging drugs, antimetabolites, tubulin- and cytoskeleton-interacting molecules, as well as signal transduction modulators. This lack of correlation suggests that BIM-46174 is acting through a novel inhibitory mechanism on the growth of several human cancer cell lines originating from nine different tumor sites.

**Effect of BIM-46174 on the cell cycle and the apoptosis.** No major change was observed in the percentage of HL60 and NCI-H69 cancer cells at the different phases of the cell cycle after 72 hours of treatment with 30 μmol/L BIM-46174 (Fig. 4A). In contrast, cisplatin treatment for 72 hours induced a clear increase of the percentage of lung NCI-H69 cells at the G2 phase of the cell proliferation cycle. However, shorter exposure (24 and 48 hours) with BIM-46174 should lead to an increase of the G2 phase (data not shown). Using Annexin V staining and flow cytometry, we assessed whether the antiproliferative activity of BIM-46174 is associated with alterations in HL60 and NCI-H69 cancer cell survival. Treatment with 30 μmol/L BIM-46174 for 72 hours resulted in a significant 5-fold increase in apoptosis in both cancer cell types (Fig. 4B). In contrast, cisplatin treatment for 72 hours did not induced significant apoptosis. Such cellular response to BIM-46174 is associated with a caspase-3 activation (Fig. 4C) and an appearance of cleaved PARP in both cell lines (Fig. 4C). Activation of caspase-8 and caspase-9 in HL60 cells is observed in the presence of BIM-46174 (Fig. 4C).

**Figure 2.** Effect of BIM-46174 on GPCR agonist-dependent signalization: cAMP synthesis, intracellular calcium release, and cell migration. A, increasing concentrations of VIP showing a noncompetitive inhibition. Statistical analyses have been done by comparison of groups in relation to the control group (Control) with a one-way ANOVA followed by Dunnett's tests. B, BIM-46174 (30 μmol/L) selectively inhibits Ca2+ release induced by the GPCR agonists: ET-1 (10 nmol/L) in melanoma cells A2058. In contrast, the same concentration of BIM-46174 was ineffective on Ca2+ release induced by the G-protein–independent Ca2+ ionophore A23187 (1 μmol/L) in the same cells A2058. Statistical analyses have been done by comparison of the mean in relation to the constant 100 with a Student's t test with a Benjamini-Hochberg adjustment for multiple comparisons. C, induction of collagen type I invasion by the forced induction of Wnt-2 in human colon cancer cells HCT8/S11-Wnt-2 (clones 3 and 8) and its reversion by the Gαi/o subunits inhibitor pertussis toxin (PTX; 200 ng/mL), the Gαq/11 scavenger, BIM-46174 (5 μmol/L), and the PI3K inhibitor wortmannin (10 nmol/L). The percentage of cells that invaded type I collagen gels is measured after a 24-hour period following the addition of the indicated effectors. Statistical analyses have been done by comparison of groups in relation to the control group (Control) with a one-way ANOVA followed by Dunnett's tests.
inhibitor BIM-46256 (40) against SCLC xenografts or the topoisomerase inhibitor BN80927 (28) against pancreatic MiaPaCa-2 xenografts are shown in Fig. 5C and D, respectively. In both cases, BIM-46174 in combination with farnesyltransferase inhibitor (P = 0.006 at day 21) or topoisomerase inhibitor (P < 0.001 at day 55) show significant antitumor activity. For all combinations, a limited body weight loss was observed in all groups. In terms of preliminary toxicity assessment, no significant changes of the blood markers, including urea, creatinine, glycemia, γ-glutamyl transpeptidase, aspartate aminotransferase, and alanine aminotransferase were observed in rats receiving a continuous infusion of BIM-46174 (2 mg/kg/h for 6 days) delivered by Alzet (L’Aberesle, France) osmotic minipumps (data not shown). This regimen can be considered as the highest dose before observing a significant weight loss.

Discussion

We report the novel and atypical pharmacologic profile of BIM-46174, an imidazo-pyrazine derivative inhibiting heterotrimeric G-protein complex. Addition of BIM-46174 to tumor cells inhibits critical functions involved in cancer progression, including cell proliferation, survival, and invasion. Accordingly, in drug combination, BIM-46174 causes growth rate reduction of human lung and pancreatic cancer xenografts. Such atypical mechanism of action is well supported by the NCI COMPARE analysis showing a cancer cell sensitivity profile never observed before with 12,000 anticancer agents evaluated in the NCI chemical library. About the molecular site of action for BIM-46174, we present here convergent evidences that this new compound targets heterotrimeric G-protein signaling by inhibiting the formation and/or dissociation of the Gα/βγ heterotrimeric complex as shown by several assays and tools (i.e., intracellular cAMP production, calcium translocations, cholina toxin, and AlF4⁻). In agreement with this interpretation, BIM-46174 was ineffective in controlling the invasive phenotype induced by individually activated GTase-deficient Gα12 and Gα13 subunits and forced expression of Gβ1γ dimers. To substantiate further the selective activity of BIM-46174 on large G-proteins, its activity was tested on non-GPCR signaling pathways, including Rho-like small GTases, angiogenic tyrosine kinases, and other molecular intermediates acting downstream of GPCR-Gαβγ complexes. In that respect, BIM-46174 was ineffective on (a) molecular interventions directly activating adenylate cyclase (forskolin; Fig. 1), (b) intracellular calcium levels induced by calcium ionophore A23187 (Fig. 2), (c) the constitutively active PI3K protein lipid kinase (p110αγ; Fig. 3), (d) the GTP-bound status of the active forms of the Rac1 and RhoA small GTases (data not shown), and (e) the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) tyrosine kinase receptors driven angiogenesis in human umbilical vascular endothelial cell (HUVEC; data not shown).

Deregulation of cell proliferation and survival is commonly accepted as an initial transforming mechanism leading to the neoplastic progression at the proliferation/differentiation interface. Here, the antiproliferative activities of BIM-46174 are observed in several cancer types in vitro, including five drug-resistant models. This activity against drug-resistant cancer cell lines is an important property because drug resistance clearly remains one of the primary causes of suboptimal outcomes in cancer therapy. As BIM-46174 is characterized in the NCI COMPARE assay with an original antiproliferative profile, this supports the thought that BIM-46174 is acting on proliferative/survival signaling pathways through a
selective and original mechanism not targeted by 12,000 reference drugs (41). Interestingly, we found that the IC50 values of this compound on cell growth and invasion (0.6-25 μmol/L) are lower than the concentrations needed to block G-protein–activated cAMP accumulation and calcium release (30-100 μmol/L). Consistent with this observation, we clearly showed that BIM-46174 is acting on GPCR signaling controlled by several GPCR agonists mediating cell proliferation, survival, and invasion signals. In support with this mechanism, it is not surprising that this multitargeted GPCR drug exerts cumulative and potent action on integrated cellular functions regulated by several heterotrimeric G-protein complex and transduction systems (cAMP, cyclic guanosine 3′,5′-monophosphate, calcium translocations, etc.). The difference between IC50s observed in G-protein signaling assays and cell proliferation is explained by the fact that the G-protein signaling assays are done on cells exposed to BIM-46174 for only a short period (0.5-1 hour), whereas the antiproliferation assays are done after a longer 96-hour exposure. Our findings on the cell cycle suggest that the anticancer efficacy of BIM-46174 is not only mediated via a G2 arrest because this blockade is no longer observed with protracted treatment. Biochemical analyses revealed that BIM-46174 induced cancer cell death through caspase-3-dependent apoptotic pathway leading to a PARP cleavage with an absence of caspase-8 and caspase-9 activation. Further in-depth characterization of the apoptotic pathways targeted by BIM-46174 should be pursued to complete this first approach because GPCRs are involved in cell survival and cytoprotection either directly or indirectly through transactivation or reciprocal cross talk with receptor and nonreceptor kinases (42). However, it is noteworthy that the experimental variables (time and concentration) to observe apoptosis

Table 1. Antiproliferative activity of BIM-46174 on a series of human cancer cells in culture, including drug-resistant tumor cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cell types</th>
<th>IC50 of BIM-46174 (μmol/L)</th>
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<tr>
<td>A-427</td>
<td>Lung</td>
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<td>LNCaP</td>
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<td>CCRF-CEM</td>
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<tr>
<td>Mia PaCa-2</td>
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Drug-resistant cells | Drugs | Resistance factor |
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</tr>
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NOTE: The inhibitory potency IC50 of BIM-46174 on cell proliferation was determined after a 96-hour protracted infusion of BIM-46174 during the exponential phase of growth. For drug-resistant cancer cells, the resistance factor is calculated by dividing the IC50 of BIM-46174 on the drug-resistant variant by the IC50 of BIM-46174 on parental cells. This ratio is compared with the resistance factors observed for reference anticancer drugs.

Figure 4. Effect of BIM-46174 on the cell proliferation cycle, cell survival, caspase-3 activity, and PARP cleavage in HL60 and NCI-H69 cancer cells. A, assessed by fluorescence-activated cell sorting (FACS) analysis, BIM-46174 (30 μmol/L; 72 hours of exposure) had no major effect on cancer cell distribution in the different phases of the cell cycle. Statistical analyses have been done by comparison of groups in relation to the control group (Control) with a one-way ANOVA followed by Dunnett’s tests. B, apoptosis is induced in both cancer cell lines by BIM-46174 assessed by FACS analysis and Annexin V labeling. In contrast, cisplatin increased the percentage of cancer cells in G2 phase without effect on apoptosis. Statistical analyses have been done by comparison in relation to the control group (Vehicle) with a paired Student’s t test. C, BIM-46174 (30 μmol/L) increases cleaved caspase-3 and cleaved PARP in both cell lines. Columns, mean of three experiments for 6-hour exposure of BIM-46174; bars, SE.
are in line with the time required to observe cell killing in the time \times \text{concentration} \text{assay}. This pharmacologic signature is compatible with our data, showing that BIM-46174 may improve the therapeuticeffectofanticanceragentsagainsthumanlungandpancreaticcancercellscharacterizedbyvariousformsofdrugresistance.

More advanced cancers are characterized by local malignant recurrences after surgery and adjuvant therapy and synchronous and metachronous metastases in different organs. A myriad of GPCR agonists, such as cyclooxygenase-2-derived eicosanoids, CXC chemokines, hormones, and neuropeptides, play a key role in cancercellmotilityandinvasive behavior, tumorangiogenesis, and homing survival of metastatic cancer cells in distant organs. In addition to their direct mitogenic and invasion-promoting effects, GPCR agonists, including bombesin, endothelin, and thrombin protease-activated receptor-1 receptors, also indirectly promote metalloproteinase-mediated cleavage of epidermal growth factor receptor (EGFR) ligand precursors and EGFR transactivation (43, 44). Although BIM-46174 has no effect on the angiogenic responses induced by the VEGF and FGF tyrosine kinase receptors in normal primary endothelial cells HUVEC (data not shown), its potential effect on tumor angiogenesis promoted by GPCR agonists should be still considered in vivo. Our data show that BIM-46174 inhibits G-protein signaling and the invasive phenotype determined in cancer cells by several GPCRs.

Combination therapy using anticancer agents acting through different mechanisms is a current strategy in clinical chemotherapy to identify optimal regimens in terms of patient survival and relapse-free disease. Both pancreatic and lung cancers (SCLC and NSCLC), leading causes of mortality worldwide, are considered as particularly aggressive and respond poorly to standard treatments. The survival rate for these malignancies is very low because less than 4% to 10% survival benefit occurred at 5 years after adjuvant therapy. Our data show that BIM-46174 collaborates with cisplatin, farnesytransferase, or topoisoasemerase inhibitors in delaying the growth of human lung and pancreatic tumor xenografts in athymic mice. Although the molecular mechanisms involved in such interactions have not been completely identified, a clear benefit has been observed in these preclinical models. Additional types of tumor sites have to be evaluated with BIM-46174, especially hormonotherapy refractory prostate cancers (45) and neuroendocrine tumors characterized by the production of peptide hormones, bioamines, and neurotransmitters acting through GPCR agonist-mediated autocrine loops.

Taken together, our data bring robust evidence that targeting heterotrimeric G-protein signaling represents a promising new strategy for anticancer therapy. The design and characterization of BIM-46174 as a first generation of heterotrimeric signaling G-protein inhibitors validate such an approach.

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References


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