BI 885578, a Novel IGF1R/INSR Tyrosine Kinase Inhibitor with Pharmacokinetic Properties That Dissociate Antitumor Efficacy and Perturbation of Glucose Homeostasis


Abstract

Inhibition of the IGF1R, INSRA, and INSRB receptor tyrosine kinases represents an attractive approach of pharmacologic intervention in cancer, owing to the roles of the IGF1R and INSRA in promoting cell proliferation and survival. However, the central role of the INSR isoform in glucose homeostasis suggests that prolonged inhibition of this kinase could result in metabolic toxicity. We describe here the prolonged inhibition of a novel compound BI 885578, a potent and selective ATP-competitive IGF1R/INSR tyrosine kinase inhibitor distinguished by rapid intestinal absorption and a short in vivo half-life as a result of rapid metabolic clearance. BI 885578 administered daily per os, displayed an acceptable tolerability profile in mice at doses that significantly reduced the growth of xenografted human GEO and CL-14 colon carcinoma tumors. We found that treatment with BI 885578 is accompanied by increases in circulating glucose and insulin levels, which in turn leads to compensatory hyperphosphorylation of muscle INSRs and subsequent normalization of blood glucose within a few hours. In contrast, the normalization of IGF1R and INSR phosphorylation in GEO tumors occurs at a much slower rate. In accordance with this, BI 885578 led to a prolonged inhibition of cell proliferation and induction of apoptosis in GEO tumors. We propose that the remarkable therapeutic window observed for BI 885578 is achieved by virtue of the distinctive pharmacokinetic properties of the compound, capitalizing on the physiologic mechanisms of glucose homeostasis and differential levels of IGF1R and INSR expression in tumors and normal tissues.

Introduction

The insulin like growth factor-1 receptor (IGF1R) and insulin receptor (INSR) isoforms A and B are structurally related proteins consisting of an extracellular α subunit disulphide linked to a transmembrane β subunit with an intracellular tyrosine kinase domain. Dimerization via disulphide bridging between the β subunits of two protein leads to the formation of receptors with an α-β-β-α arrangement. In cells coexpressing all three proteins, the formation of six different receptor dimers is enabled: IGF1R:INSRA, INSRA:INSRA, and INSRB:INSRB homodimers and “hybrids” thereof (e.g., IGF1R:INSRA; ref. 2).

Signal transduction from the receptors is dependent on the binding of the ligands IGF1, IGF2, or insulin. The affinity of each ligand for the receptors has been extensively investigated (3–7). Briefly, IGF1 binds with high affinity to receptors containing an IGF1R protein. IGF2 displays a similar pattern of receptor binding; however, its high affinity to the INSRA homodimer and INSRA:B hybrid. Conversely, insulin only binds the INSRA and INSRB homodimers and INSRA:B hybrid. Upon ligand binding, the receptors undergo a structural rearrangement leading to activation of the kinase domains and autophosphorylation of intracellular tyrosines (8). Adapter proteins including Shc, IRS1, and -2 dock on the phospho tyrosines and transduce signaling via the MAPK and PI3K cascades.

The IGF1R is ubiquitously expressed in adult and embryonic tissues and in multiple cancers (8). Genetic evidence in rodents and humans suggest that the IGF1R and its ligands are essential for the genesis and homeostasis of multiple tissues (9, 10). Meanwhile, in diverse cancers, IGF1R signaling promotes oncogenic proliferation and survival (1, 8). The INSR displays restricted expression during early embryonic development but is aberrantly re-expressed in diverse cancers (6, 11). INSR signaling, particularly in cancer cells expressing autocrine IGF2, promotes
oncogenic cellular responses in a similar manner to the IGF1R (12–15). As such, the IGF1R and INSR both represent attractive targets for pharmacologic intervention in cancer. Conversely, insulin-INSR signaling plays a central role in glucose disposal in adult cell types such as hepatocytes, skeletal muscle, and adipocytes (16–19).

Three predominant therapeutic strategies of inhibiting signaling via the receptors and ligands (herein referred to as the ‘IGF-pathway’) have been explored in human clinical trials: IGF1R antibodies (20), IGF1/2 antibodies (21), and ATP-competitive IGF1R/INSR tyrosine kinase inhibitors (TKI; ref. 22). Multiple phase II and III clinical trials of IGF1R antibodies have yielded disappointing efficacy outcomes (23, 24). Several preclinical studies have suggested this may be due to incomplete pathway inhibition as a result of ‘sparring’ of INSR signaling (14, 25, 26).

IGF1R/INSR TKIs equipotently inhibit the IGF1R, INSR, and INSRB kinases, as a consequence of complete identity of the receptor ATP-binding pockets, and as such block the IGF pathway to a broader extent than IGF1R antibodies (27–29). In keeping with this, IGF1R/INSR TKIs deliver superior efficacy compared to IGF1R antibodies in various preclinical cancer models (25, 26, 30). However, inhibition of INSR-mediated glucose disposal represents a tolerability liability of IGF1R/INSR TKIs. Indeed, hyperglycemia was recently reported as a common adverse event in a phase I trial of the IGF1R/INSR TKI linistinib (22). This toxicity hurdle could potentially limit the dosing of IGF1R/INSR TKIs to levels too low to provide meaningful efficacy.

Whereas signal transduction inhibitors used in cancer therapy are generally dosed to provide maximal target inhibition during the entire dosing interval, we hypothesized that an IGF1R/INSR TKI that only transiently inhibits its targets could provide an improved therapeutic window. This hypothesis assumes that upon inhibitor dissociation, INSRB-mediated glucose homeostasis, a highly adaptable and tightly regulated process (31), would recover at a faster rate than IGF1R- and INSR-mediated tumor proliferation and survival. As such, we optimized IGF1R/INSR TKIs to display, in addition to high target potency and selectivity, short target residence times and a highly adaptable and tightly regulated process (31), would recover at a faster rate than IGF1R- and INSR-mediated tumor proliferation and survival. As such, we optimized IGF1R/INSR TKIs to display, in addition to high target potency and selectivity, short target residence times and in vivo half-life. This led to the discovery of the novel compound BI 885578. We demonstrate that BI 885578 delivers significant efficacy in mouse xenograft tumor models at doses associated with only transient and therefore well-tolerated inhibition of glucose metabolism and propose a mechanistic model explaining the observed therapeutic window based on compound characteristics, receptor expression, and feedback regulation of signal transduction.

Materials and Methods

Compounds

BI 885578 and afatinib were synthesized at Boehringer Ingelheim. PHA665752 was purchased from Sigma Aldrich.

In vitro biochemical and cellular assays

The DELFIA (Perkin Elmer) kinase assay using IGF1R-GST has been described previously (32). For DELFIA INSR kinase assays, an identical assay setup was performed using INSR-GST protein. All other kinase assays were performed at Life Technologies using the SelectScreen platform.

IGF1R null mouse embryonic fibroblasts (MEF) stably expressing human IGF1R or INSRB were a kind gift from R. Vigneri (University of Catania, Italy, obtained April 26, 2008) and were cultured in DMEM with 10% FCS, 5% sodium pyruvate, 5% nonessential amino acids, 5% glucose, and 0.3 µg/mL puromycin. Protocols for assessment of IGF1R and INSRB phosphorylation in these MEFs were described previously (33). The human GEO cell line was generously provided by S. Pepe (Università degli Studi di Napoli, Italy, obtained April 15, 2008) and was cultured in McCoy 5a medium with 10% FCS and 20 mmol/L HEPES. These cell lines were not authenticated by STR, owing to the lack of published reference signatures. The following cell lines were cultured according to the manufacturer’s instruction and authenticated by STR analysis at Boehringer Ingelheim on the dates indicated. HCT 116 (obtained June 17, 2011; STR April 17, 2012) and Calu-6 (obtained January 17, 2000; STR June 24, 2011) were from the ATCC. TC71 (obtained April 4, 2008; STR May 4, 2011), and CL-14 (obtained January 20, 2013; STR April 20, 2013) were from the DSMZ.

For proliferation assays, cells were cultured in BI 885578 for 72 hours then incubated with AlamarBlue (Serotec Ltd.) for 7 hours. Fluorescence (extinction wavelength 544 nm, emission 590 nm) was measured and IC50 values generated using GraphPad Prism.

For assessment of protein phosphorylation, GEO cells incubated with compound or DMSO (control), washed with ice-cold PBS, and lysed for 10 minutes in ice-cold Lysis Buffer containing protease and phosphatase inhibitors (Cell Signaling Technology). Homogenates were cleared by centrifugation (30 minutes, 14,000 rpm, 4°C). SDS-PAGE and Western blotting was performed as described previously (33). The antibodies used are described in the Supplementary Materials and Methods section. Lysates were analyzed using ProteomeProfiler Antibody Arrays (R&D Systems) according to the manufacturer’s instructions.

Surface plasmon resonance

Surface plasm resonance (SPR) experiments were performed using the Sprint platform (Beactica AB). GST-IGF1R and GST-INSR proteins were immobilized by anti-GST capture using Amine coupling buffer (10 mmol/L PBS, 0.05% Tween 20) and capture buffer (10 mmol/L TBS, 20 mmol/L MgCl2, 0.05% Tween 20). After capture, the surface was cross-linked with N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Interaction experiments were performed at 25°C in running buffer (10 mmol/L TBS, 20 mmol/L MgCl2, 0.05% Tween 20, 5% DMSO). BI 885578 was injected for 60 seconds over the immobilized proteins. Sensorgrams were analyzed with the BiacoreT200 evaluation software 1.0. A 1:1 interaction model was fitted globally to sensorgrams in multisensor experiments.

X-ray crystallography

The INSR kinase domain (V1005-K1310, with mutations C1008S, D1159N) was used for crystallization using the hanging-drop method. One microliter of INSR solution (13 mg/mL INSR in 50 mmol/L Tris HCl pH 7.5, 170 mmol/L NaCl) was incubated with BI 885578 for 1 hour at 4°C with 1 µL reservoir solution (100 mmol/L Tris pH 7.6, 22% PEG8000, 50 mmol/L NaCl, 5 mmol/L DTT). Crystals were flash-frozen in liquid nitrogen after immersion in 20% ethylene glycol. Data were collected at the SLS beam line X06SA (Swiss Light Source, Scherrer Institute) using a PILATUS 6M detector with a crystal that diffracted to 2.26 Å. Data processing and analysis was performed using the autoPROC toolbox (34). The structure was solved by molecular replacement using a published INSR structure (PDB ID 1IRK).
In vivo tumor models

The protocol for the GEO xenograft model was described previously (33). CL-14 xenograft tumors (80–110 mm³) were established from CL-14 cells (5 × 10⁶ in matrigel) by subcutaneous injection into the right flanks of female BomTac:NMRI-Foxn1 nu/nu mice. After six weeks, mice were randomized (7 per group) for treatment. Tumor volumes were determined three times per week with a digital caliper and body weight was measured daily. BI 885578 was formulated in 0.5% Natrosol (hydroxyethylcellulose natrosol 250HX, VWR International), 3% Tween 80, and 1 mol/L citric acid. For 10 mL of solution, a mixture consisting of 9.8 mL of 0.5% Natrosol and 20 µL of 3% Tween 80 was prepared and citric acid then used to adjust to pH 3. All doses were calculated relative to the mouse body weight on the treatment day. BI 885578 and the vehicle control (0.5% Natrosol) were administered orally (per os) with a volume of 10 mL/kg body weight.

One-sided decreasing Mann–Whitney tests were used to compare tumor volumes (efficacy). Two-sided decreasing Mann–Whitney tests were used to compare body weights (tolerability). The P values for the tumor volume assessment were adjusted for multiple comparisons according to Bonferroni–Holm (39), whereas P values for body weight remained unadjusted. For all analyses, P values under 0.05 represented a statistically significant effect.

Pharmacodynamic biomarker analyses

For analysis of pharmacodynamic biomarkers, GEO tumor-bearing mice were treated with BI 885578 and blood, tumor, and muscle samples isolated (3 animals per time point). Blood was withdrawn retroorbitally under isoflurane anesthesia and the glucose concentration in each sample measured with the Accu-Check compact plus device (Roche). Blood was centrifuged for 5 minutes (10,000 rpm, 4°C) to isolate plasma for ELISA measurement of IGF1 (#MG100, R&D Systems) and insulin (#90080, Crystal Chem) according to the manufacturer’s instructions.

Mice were sacrificed prior to explanting tumors and muscle (right hind limb). Tumor and muscle samples were homogenized in ice-cold Cell Lysis Buffer using Eppendorf micropestles (Sigma). Homogenates were incubated on ice for 60 minutes and cleared as described above. Bio-Plex kits (Bio-Rad) were used according to the manufacturer’s instructions to measure phosphorylated IGF1R (Tyrosine 1131) and INSR (Tyrosine 1146). The statistical comparison of data was performed using unpaired parametric tests.

Histomorphologic and immunohistochemical analyses

GEO tumor-bearing mice were treated with BI 885578 as described above. Two hours prior to tumor excision, mice were administered with 10 mL/kg of a bromodeoxyuridine (BrdUrd) solution (Invitrogen). GEO tumors were explanted and cryopreserved hepatocytes (Celsis IVT) for 30 minutes followed by quantitation of BI 885578 by LC/MS/MS and using the well-stirred model.

Results

Chemical structure and target binding mode

BI 885578 is a chiral compound bearing a methyl-group at the stereogenic center located in the cycloaliphatic bridge linking the pyrimidine and pyrazole rings (Fig. 1A). The capped piperezine moiety exposed to the solvent serves as a solubilizing group with moderate basicity. The crystal structure of INSR with BI 885578 shows a typical bilobal architecture of protein kinases (Fig. 1B). BI 885578 binds noncovalently to the ATP-binding pocket in the INSR catalytic site and forms two hydrogen bonds to the backbone of Met1079 at the kinase hinge region (Fig. 1B and C).

Surface plasmon resonance (SPR) assays demonstrated that BI 885578 has a high affinity for the IGF1R and INSR kinase domains (KD = 9 and 12 nmol/L, respectively; Table 1). BI 885578 showed rapid association and dissociation kinetics for the IGF1R and INSR indicative of short residence times, in the range of minutes.

Molecular and cellular potency and selectivity

The potency and selectivity of BI 885578 were investigated using recombinant in vitro kinase assays. BI 885578 potently inhibited the kinase activity of the IGF1R and INSR with an IC₅₀ value of 1 nmol/L for both kinases (Table 1). When tested on 199 other kinases, BI 885578 was found to be highly selective, with only 25 enzymes displaying IC₅₀ values below 1,000 nmol/L (Supplementary Table S3). A selectivity window of
above 30-fold was demonstrated between IGF1R/INSR and the next most potently inhibited kinases (IC₅₀ = 33 nmol/L for CLK2 and FER).

The effect of BI 885578 on ligand-stimulated IGF1R and INSR autophosphorylation in cells was assessed. IGF1R null mouse MEFs stably expressing human IGF1R or INSRB (6) were stimulated with IGF1 or insulin, respectively, in the presence of BI 885578. IGF1R and INSRB autophosphorylation was potently inhibited by BI 885578 (IC₅₀ = 5 nmol/L for both targets; Table 1). BI 885578 also potently inhibited the proliferation of the human cancer cell lines TC71 (bone sarcoma; IC₅₀ = 4 nmol/L) and GEO (colon carcinoma; IC₅₀ = 9 nmol/L), which have previously been shown to be sensitive to the IGF pathway inhibitors BMS-754807, linsitinib, BI 836845, and/or figitumumab (28, 33, 41, 42). BI 885578 was comparatively inactive (IC₅₀ > 2000 nmol/L) in proliferation assays using the IGF pathway inhibitor insensitive cell lines HCT 116 (colon carcinoma) and Calu-6 (non–small cell lung carcinoma), indicative of a lack of off-target cytotoxicity.

The ability of BI 885578 to inhibit signal transduction downstream of the IGF1R/INSR in GEO cells was examined by Western blotting. Treatment with BI 885578 at concentrations above 20 nmol/L led to a marked reduction in the levels of phosphorylated IGF1R/INSR. The antibody used for this Western blot analysis does not distinguish between the phospho forms of the IGF1R, INSR A, and INSR B. BI 885578 also potently reduced the phosphorylation of the downstream proteins Akt, Erk1/2, and S6 (Fig. 2A). Levels of total IGF1R, Akt, Erk1/2, and S6 protein were not affected.

The specificity of BI 885578 for the IGF1R and INSR was next investigated in a cellular context. GEO cells were treated for 24 hours with 500 nmol/L BI 885578, which is 100-fold higher than the cellular IC₅₀ for reduction of IGF1R and INSR phosphorylation (5 nmol/L, Table 1). The levels of 49 phospho receptor tyrosine kinases (pRTK) were examined in GEO extracts using pRTK arrays (Fig. 2B). Phospho signals for the EGF R, ErbB2, c-Met, IGF1R, and INSR were detected in control GEO extracts, and BI 885578 treatment only reduced the intensity of the phospho-IGF1R and –INSR signals. This assay format cannot distinguish between the phospho-INSRA and –INSRB, owing to the complete identity of the intracellular domains of these proteins. The EGF R/ErbB2 inhibitor afatinib (43) and the c-Met inhibitor PHA665752 (44) reduced the phospho signals for their targets, without affecting the phospho-IGF1R or –INSR signal intensity.

Table 1. In vitro potency and target-binding properties of BI 885578

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC₅₀ (nmol/L)</th>
<th>Kₐ (10² M⁻¹s⁻¹)</th>
<th>Kₜ (10⁻² s⁻¹)</th>
<th>K_d (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1R kinase</td>
<td>1 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>INSR kinase</td>
<td>1 ± 0.7</td>
<td>5.0 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>IGF1R cellular autophosphorylation</td>
<td>5 ± 3</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>INSRB cellular autophosphorylation</td>
<td>5 ± 3</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>TC71 proliferation*</td>
<td>4 ± 3</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>GEO proliferation*</td>
<td>9 ± 3</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>HCT 116 proliferation#</td>
<td>&gt;2,000</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Calu-6 proliferation#</td>
<td>&gt;2,000</td>
<td>5.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: For each assay, mean values ± standard deviation are presented, which were determined from three or more experiments.

*IGF pathway–dependent cell line.
#IGF pathway–independent cell line.

The molecular weight of BI 885578 is 527.66 and the empirical formula is C₂₉H₃₇N₉O. The chemical structure of BI 885578 is shown in Figure 1.
Pharmacokinetic properties

As mentioned above, we hypothesized that an IGF1R/INSR inhibitor with a short systemic half-life may achieve an improved therapeutic window. We therefore aimed to generate a compound with a PK profile characterized by fast absorption, an early $t_{\text{max}}$ and subsequent rapid elimination. BI 885578 displays aqueous solubility, high permeability and low Caco-2 efflux ratio translating into rapid in vivo absorption following per os dosing in mice, rats, and dogs with $t_{\text{max}}$ values under 2 hours and terminal elimination half-lives in the range of 2 to 6 hours (Table 2). BI 885578 undergoes extensive metabolism in vitro when incubated with hepatocytes, which enables the desired short initial and terminal in vivo half-lives.

### Table 2. Key physicochemical and DMPK properties of BI 885578

<table>
<thead>
<tr>
<th>Property</th>
<th>Solubility (mg/mL)</th>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5</td>
<td>&gt;0.98</td>
<td>65</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>0.052</td>
<td>ND</td>
<td>121</td>
<td>66</td>
<td>132</td>
</tr>
</tbody>
</table>

#### In vitro ADME and in vivo plasma PK properties across species

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{\text{app}}$ (a-b) Caco-2 cells at 10 $\mu$mol/L (10 $^{-6}$ cm/s)</td>
<td>65</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Efflux ratio Caco-2 cells at 10 $\mu$mol/L</td>
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<td>ND</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$V_{\text{m}}$ (% Q&lt;sub&gt;L&lt;/sub&gt;)</td>
<td>7.6</td>
<td>ND</td>
<td>6.0</td>
<td>7.0</td>
<td>21</td>
</tr>
<tr>
<td>$V_{\text{m}}$ (L/kg)</td>
<td>70</td>
<td>ND</td>
<td>47</td>
<td>47</td>
<td>88</td>
</tr>
<tr>
<td>$C_{\text{m}}$ (mmol/L)</td>
<td>809</td>
<td>ND</td>
<td>500</td>
<td>500</td>
<td>541</td>
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<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>6.9</td>
<td>ND</td>
<td>22</td>
<td>22</td>
<td>7.5</td>
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<tr>
<td>$C_{\text{max}}$ (mmol/L)</td>
<td>679</td>
<td>ND</td>
<td>98</td>
<td>98</td>
<td>134</td>
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<tr>
<td>$C_{\text{bioavailability}}$ (%)</td>
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<td>ND</td>
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<td>1.1</td>
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</tr>
<tr>
<td>$C_{\text{bioavailability}}$ (%)</td>
<td>40</td>
<td>ND</td>
<td>5</td>
<td>5</td>
<td>9</td>
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<tr>
<td>$C_{\text{bioavailability}}$ (%)</td>
<td>809</td>
<td>ND</td>
<td>500</td>
<td>500</td>
<td>541</td>
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<td>$C_{\text{bioavailability}}$ (%)</td>
<td>6.9</td>
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<td>679</td>
<td>ND</td>
<td>98</td>
<td>98</td>
<td>134</td>
</tr>
</tbody>
</table>

**NOTE:** Solubility data were derived from single determinations. Caco-2, plasma protein binding and hepatocytes CL data represent mean values from two separate experiments. In vivo data are mean values from 3 animals. Abbreviations: NA, not applicable; ND, not determined.
In vivo tolerability and efficacy

The in vivo efficacy and tolerability of BI 885578 was examined in mice bearing xenografted GEO tumors. The GEO cell line is derived from a human colon carcinoma and carries a mutation in KRAS (G12A) in addition to displaying high expression of IGF2 (45). Oral doses of 20 and 40 mg/kg applied daily (q.d.) and 80 mg/kg applied every other day (q2d) were tested over 22 days. A significant reduction in tumor growth compared with controls was observed in all treatment groups with median tumor growth inhibition (TGI) values of 64% (P = 0.0009), 85% (P = 0.0009), and 79% (P = 0.0012), respectively (Fig. 3A).

The 20 and 40 mg/kg q.d. treatments did not reduce mouse body weight gain (Fig. 3B), suggesting that these treatments were tolerated. In contrast, the 80 mg/kg q2d group displayed body weight reductions on the treatment days. Treatment-free days in this group were associated with a recovery of body weight. Following the final BI 885578 treatment in each group, blood glucose levels were monitored over 24 hours. The 20 and 40 mg/kg q.d. treatments induced a transient elevation of blood glucose, with normalization observable between 2 and 6 hours after dosing (Fig. 3C). The 80 mg/kg q2d treatment induced slightly more prolonged, yet not statistically significant, elevation of blood glucose compared with the other dose groups.

The efficacy and tolerability of the 40 mg/kg (q.d., per os) dose of BI 885578 was further examined in the CL-14 colon carcinoma xenograft model (Supplementary Fig. S1A–S1C). BI 885578 significantly inhibited the growth of CL-14 tumors with a median TGI of 89% (P = 0.0003). As in the GEO xenograft, this BI 885578 dose had no significant impact on body weight gain (P = 0.093) and was associated with only a transient (<6 hours) elevation of blood glucose.

In vivo pathway modulation in plasma, tumor, and muscle

To investigate mechanisms by which the 40 mg/kg dose of BI 885578 delivers efficacy and acceptable tolerability, we examined pharmacodynamic markers of the IGF pathway in plasma and tissues. GEO tumor-bearing mice were treated once with this dose of BI 885578 and plasma, tumor, and muscle samples were taken at multiple time points up to 24 hours after application.

Rapid absorption of BI 885578 with an early $t_{\text{max}}$ and subsequent rapid elimination was observed in plasma with concentrations at 24 hours below the LLOQ of 2.8 nmol/L (Fig. 4A). Similar to data shown above (Fig. 3C), a transient elevation of plasma glucose levels was observed 15 minutes after 40 mg/kg BI 885578 treatment, with normalization of glucose levels occurring by 1 hour (Fig. 4A). Plasma insulin levels were significantly increased with an $E_{\text{max}}$ of 34-fold above baseline observed 1 hours after dosing but normalized between 12 and 24 hours. A comparatively low variation (±2-fold of baseline) was observed for IGF1 levels and at no time point was the variation found to be statistically significant compared with untreated controls (Fig. 4A).

We next monitored the phosphorylation of the IGF1R and INSR in GEO tumors and muscle. The Bio-Plex assays used do not discriminate between the phospho-INSRA and -INSRB. BI 885578 treatment led to a significant and equivalent reduction of IGF1R and INSR phosphorylation in GEO tumors, with an $E_{\text{max}}$ of approximately 10% control at 1 hour after dosing (Fig. 4B). The phosphorylation of both IGF1R and INSR remained suppressed to 10% to 25% of control until 6 hours. In this experiment, phospho-INSR levels normalized after 24 hours. Partial recovery of insulin levels occurring by 1 hour (Fig. 4A). Plasma insulin levels were significantly increased with an $E_{\text{max}}$ of 34-fold above baseline observed 1 hours after dosing but normalized between 12 and 24 hours. A comparatively low variation (±2-fold of baseline) was observed for IGF1 levels and at no time point was the variation found to be statistically significant compared with untreated controls (Fig. 4A).

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of IGF1R phosphorylation in GEO tumors was observed between 6 and 24 hours, but did not reach levels observed in untreated control tumors. In a separate experiment analyzing later time points, IGF1R and INSR phosphorylation in GEO tumors normalized to control levels 48 hours after dosing (Supplementary Fig. S2).

In mouse muscle, the kinetics of IGF1R phosphorylation showed a similar profile to that of the IGF1R in GEO tumors, whereas the kinetics of INSR phosphorylation contrasted starkly to that of IGF1R and INSR phosphorylation in GEO tumors and the IGF1R in muscle. INSR phosphorylation in muscle was significantly reduced to 23% of control 15 minutes after BI 885578 dosing, but then rebounded to between 200% and 400% of control between 6 and 24 hours (Fig. 4B). This rebound of phospho-INSR levels in muscle coincided with the induction of plasma insulin and the normalization of blood glucose levels described above.

**In vivo modulation of cell proliferation and survival markers in tumors**

We next examined the effect of BI 885578 on markers of tumor proliferation, growth, and survival. GEO tumor-bearing mice were treated once with BI 885578 (40 mg/kg) and tumors excised for histologic and immunohistochemical analysis at multiple

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**Figure 4.**
Effects of BI 885578 on IGF pathway pharmacodynamic biomarkers. GEO tumor-bearing mice were treated once orally with BI 885578 (40 mg/kg) and blood, tumors, and muscle sampled at 0, 0.25, 1, 2, 6, 12, and 24 hours after administration for analysis of PD biomarkers (3 animals per time point). A, levels of circulating BI 885578, glucose, insulin, and mouse IGF1 were examined. B, the phosphorylation of the IGF1R and INSR was measured in tumor and muscle protein extracts. *P < 0.05 for treated versus control group.
time points. Mice were also injected with a BrdUrd solution 2 hours prior to tumor sampling to enable monitoring of nucleoside uptake by GEO tumor cells as an indicator of proliferation. Erk1/2 and S6 are both downstream of the IGF1R and INSR and their phosphorylation is indicative of tumor cell proliferation and survival (46, 47). A reduction in the phosphorylation of Erk1/2 and S6 was observed 12 to 48 hours after BI 885578 dosing (Fig. 5A; Table 3). A reduction of BrdUrd uptake at 24 hours indicated that GEO cell proliferation was inhibited (Fig. 5B; Table 3). Normalization of Erk1/2 and S6 phosphorylation and BrdUrd uptake to levels equivalent to that in untreated tumors was observed at 72 hours. An increase of cleaved caspase-3 levels was also observed in GEO tumors at 12 and 24 hours, indicative of apoptosis (Fig. 5B; Table 3).

Table 3. IHC scoring of the effect of BI 885578 on GEO tumor cell proliferation and survival markers.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tbody>
<tr>
<td>BrdUrd</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cleaved casp-3</td>
<td>–</td>
<td>–</td>
<td>–/+</td>
<td>+</td>
<td>+</td>
<td>–/+</td>
<td>–</td>
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<tr>
<td>pErk1/2</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>–/+</td>
<td>–/+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pS6</td>
<td>+++</td>
<td>++/+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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NOTE: IHC samples from experiments described in Fig. 5A and B were scored for staining intensities based on standard histology criteria for each marker analyzed.
Given the indispensable role of the INSRB in glucose homeostasis, the development of IGF1R/INSR TKIs that balance efficacy and tolerability represents a significant challenge. In preclinical studies conducted earlier, we noted that published compounds with relatively long half-lives in mice only achieved significant antitumor efficacy at the cost of poor tolerability, in particular if treatment was continued for more than one or two weeks. We hypothesized that an improved therapeutic window can be achieved with a potent and selective IGF1R/INSR TKI that undergoes rapid in vivo elimination and thus only transiently inhibits its targets. In our compound optimization campaign (to be described in a separate publication) we therefore included rapid elimination, based on susceptibility to hepatocyte-mediated metabolism, as a prioritized optimization parameter. These efforts led to the discovery of BI 885578.

BI 885578 potently and specifically inhibited the kinase activities of the IGF1R and INSR as well as downstream signal transduction and this translated into potent inhibition of the proliferation of IGF pathway–dependent, but not IGF pathway–independent, cell lines. The activity of BI 885578 in diverse tumor models and the genetic determinants of BI 885578 sensitivity are currently under investigation with particular focus on IGF2 expression (48, 49; M. Sanderson, manuscript in preparation). The physicochemical and metabolic properties of BI 885578 enabled the desired in vivo pharmacokinetic profile upon oral dosing, characterized by rapid intestinal absorption and fast elimination. In addition, the short residence times of BI 885578 for the IGF1R and INSR ensured that prolonged target engagement, subsequent to plasma elimination, could be avoided.

BI 885578 induced an elevation of blood glucose in mice, indicative of inhibition of INSRB-mediated glucose homeostasis.

Figure 6. Model for the differential kinetic effects of BI 885578 on GEO tumors and muscle. Top, under basal conditions, GEO tumors express a dominant proportion of IGF1R homodimers and a smaller proportion of IGF1R:INSRA hybrids, which both bind circulating mouse IGF1 and autocrine human IGF2 to promote cell proliferation and survival. Muscle cells dominantly express INSRB homodimers that bind insulin to promote glucose uptake. INSRB:IGF1R hybrids, of low abundance in muscle, bind circulating IGF1. Middle, BI 885578 achieves peak concentrations soon after oral dosing and inhibits the IGF1R and INSR isoforms in all tissues, leading to an elevation of blood glucose. Glucose in turn stimulates β cells of the pancreas to immediately release their depot of insulin into the circulation. In contrast, blockade of the IGF1R does not result in increased IGF levels. Blockade of IGF1R and IGF1R:INSRA hybrids in GEO cells results in shutdown of signal transduction, inhibiting cell proliferation and inducing apoptosis. Bottom, BI 885578 is rapidly eliminated, enabling the high circulating insulin levels to promote hyperphosphorylation of inhibitor-free INSRB homodimers in muscle. This leads to rapid glucose disposal by muscle and normalization of blood glucose levels. With IGF1/2 levels remaining constant, re-equilibration of IGF1R homodimer and IGF1R:INSRA hybrid phosphorylation is slow and GEO cell proliferation and survival signaling never fully recover before the next BI 885578 dose is administered.
We noted that doses associated with only a transient (<6 hours postadministration) elevation of blood glucose levels in mice were acceptably tolerated, even during prolonged treatment. Intriguingly, these doses demonstrated efficacy in GEO and CL-14 tumor xenografts, indicating that BI 885578 was able to deliver a therapeutic window.

Pharmacokinetic/pharmacodynamic analyses indicated that BI 885578 differentially modulated IGF pathway components responsible for glucose homeostasis and tumorigenic signaling. INSR phosphorylation in muscle, a pharmacodynamic marker of glucose homeostasis and tumorigenic signaling, was only brieﬂy suppressed and subsequently increased even beyond baseline levels, potentially as a result of the sharp increase in circulating insulin levels. Meanwhile, the phosphorylation of the IGF1R in GEO tumors and muscle recovered only at a much slower rate. This could be potentially explained by the finding that unlike insulin, levels of IGF1, the predominant ligand for the IGF1R, did not undergo compensatory induction. Unlike in muscle, the INSR in GEO tumors did not appear to be sensitive to increased circulating insulin levels. This could potentially be explained by two separate mechanisms. First, the expression of the IGF1R by GEO cells is approximately 15-fold higher than that of the INSR, while the INSR isoform predominates over INSRB (33). This likely results in the dominant representation at the GEO cell surface by IGF1R homodimers and IGF1R:INSRB hybrids, which both display low afﬁnity for insulin (3, 4). A consequently low proportion of INSR or INSRB homodimers and INSRB:INSRB hybrids, capable of high-afﬁnity insulin binding, could explain the inability of GEO tumor INSRs to respond to elevated plasma insulin levels. In contrast, muscle dominantly expresses the INSRB (6), thus providing ample high-afﬁnity receptor sites for insulin binding. Second, the autocrine expression of IGF2 by GEO cells (45) could result in a high level of INSRB occupancy, thus reducing the availability of ligand-free receptors for insulin engagement. It would be of interest for future studies to examine the kinetic effects of BI 885578 on INSR phosphorylation in tumor models that either lack autocrine IGF1/2 expression, or dominantly express the INSRB relative to the IGF1R.

In conclusion, we propose a model based on our observations to explain the mechanism by which the properties of BI 885578 exploit the rapid adaptability of INSRB signaling in metabolic tissues and slower recovery of IGF1R/INSRB signaling in GEO tumors to achieve a therapeutic window (Fig. 6). Translation of our encouraging ﬁndings from mouse models to cancer patients requires that the favorable pharmacokinetic characteristics of BI 885578 are maintained in humans. In initial experiments using human hepatocytes, we observed rapid in vitro BI 885578 metabolism indicating that the desired short initial and terminal in vivo half-life may be achieved in humans. Evaluation of the efﬁcacy and tolerability of BI 885578 in cancer patients thus seems warranted.

Disclosure of Potential Conﬂicts of Interest
No potential conﬂicts of interest were disclosed.

Authors’ Contributions
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