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In vitro baselining of new pyrrolopyrimidine EGFR-TK inhibitors with Erlotinib

Eirik Sundby [a], Jin Han [a,b], Svein Jacob Kaspersen [b,1], Bård Helge Hoff [b,*]

[a] Sør-Trøndelag University College, E. C. Dahls gt. 2, NO-7004 Trondheim, Norway
[b] Norwegian University of Science and Technology, Department of Chemistry, Høgskoleringen 5, NO-7491 Trondheim, Norway

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ABSTRACT

Epidermal growth factor receptor tyrosine kinase inhibitors are useful in treatment of non-small cell lung cancer, and show promise in combination therapy settings. Two novel chiral pyrrolopyrimidines have been baselined towards Erlotinib, Lapatinib and Dasatinib using in vitro cellular studies and ADME profiling. One of these, (*S*)-2-((6-(4-(hydroxymethyl)-2-methoxyphenyl)-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-2-phenylethan-1-ol, was more active than Erlotinib in lung and breast cancer cell models. The compound also had promising activity towards ovarian cancer cell lines, while low activity was seen towards cells of haematological origin. ADME profiling revealed good solubility, higher metabolic stability than Erlotinib and no inhibitory effect towards the hERG voltage-gated ion channel. Investigation of inhibitory potency towards 6 CYP isoforms generally revealed low inhibitory potency, but in the case of CYP3A4, a substrate dependent inhibition was noted using testosterone as substrate (IC_{50}: 12.5 μM). No cellular or gene toxicity was noted for the compounds or products of phase I metabolism. However, permeability studies using Caco-2 cells revealed a high efflux ratio. Further experiments using ABC transporter inhibitors revealed that the pyrrolopyrimidines are actively transported by the breast cancer resistant protein and P-glycoprotein transporters, which might prevent their further development into drugs.

1. Introduction

Epidermal growth factor receptor (EGFR) signalling is important in the progression of several tumour types (Yarden and Pines, 2012). Most importantly, in non-small-cell lung cancer (NSCLC) the activating EGFR mutations L858R and exon 19 deletions account for approximately 90% of the primary EGFR dysregulation (Brewer et al., 2013; Pao and Chmielecki, 2010). For patients harbouring these mutations, EGFR inhibitor therapy increases progression free survival (Koehler and Schuler, 2013; Sgambato et al., 2012). The human epidermal growth factor receptor 2 (HER2) is also often overexpressed, amplified or mutated in a significant fraction of lung adenocarcinomas. Given its role as a dimerization partner for EGFR, anti-HER2 therapies might therefore be envisioned (Landi and Cappuzzo, 2013). In breast cancer, HER2 overexpression is associated with low overall survival (Cobleigh et al., 1999), and in subsets of breast cancers, elevated levels of both HER2 and EGFR are observed (Lee et al., 2015).

EGFR amplification or mutation is also seen in pancreatic cancer (Cook et al., 2014), subgroups of ovarian (Siwak et al., 2010), prostate (Zellweger et al., 2005) and head and neck cancer. In a number of in vitro studies, EGFR inhibitors are shown to have anti-proliferative effects even though expression of HER receptors is low or non-detectable (Irwin et al., 2011; Trinks et al., 2010). This could be due to secondary target/off target effects, or the presence of non receptor bound HER like proteins. Therefore, new EGFR inhibitors are constantly being evaluated in vitro and in vivo as single drugs, or as combination with other anti-cancer agents or treatments (Tebbutt et al., 2013; Wu et al., 2013; Ratti and Tomasello, 2014; Pickhard et al., 2011; Qi et al., 2009; Tang et al., 2008; Weickhardt et al., 2012).

By exploring the thienopyrimidine and pyrrolopyrimidine scaffolds, we have discovered new inhibitors with high potency in enzymatic assays (Bugge et al., 2014; Kaspersen et al., 2014). Based on initial cell testing, kinase panel screen and estimated druglike properties, the pyrrolopyrimidines **1** and **2** were selected for further evaluation by baselining their cellular potency and in vitro ADME properties against Erlotinib (EGFR inhibitor), Lapatinib (HER2 inhibitor) and Dasatinib (multi-kinase inhibitor).

* Corresponding author at: Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491, Norway.
E-mail addresses: Eirik.Sundby@hist.no (E. Sundby), Jin.han@chem.ntnu.no (J. Han), Svein.jacob.kaspersen@chem.ntnu.no (S.J. Kaspersen), bard.helge.hoff@chem.ntnu.no (B.H. Hoff).
[1] Current address: Jotun Marine and Protective Coatings, Rosenvoldsgt. 19, NO-3202 Sandefjord, Norway.

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2. Material and methods

2.1. Chemicals

Erlotinib was purchased from Apollo, Lapatinib and Dasatinib were acquired from Selleckchem, while (*S*)-2-((6-(4-(hydroxymethyl)-2-methoxyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino)-2-phenylethan-1-ol (**1**) and (*S*)-2-((6-(4-(hydroxymethyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-2-phenylethan-1-ol (**2**) were synthesised by us previously (Kaspersen et al., 2014). All in silico calculations were performed within the Schrodinger Maestro programme suite. ADME calculations were performed using QuickProp (Anon, 2015a), and "P450 site of metabolism" via Maestro's Physics-Based ADME/Tox suite.

2.2. Cell proliferation studies

Cell proliferation studies using CAL-27 was performed as previously reported (Bugge et al., 2014).

2.2.1. Cell assay: BT-474, Calu-3, HL-60, SkBr3 and U937 cells

Cell proliferation assays were performed by VivoPharma. The compounds were diluted in DMSO at a concentration of 10 mM and stored at room temperature. Vehicle control was DMSO, positive control: saline and negative control: 0.1% Triton X-100 in saline. A pre-study was performed in order to determine whether a final concentration of 1% DMSO was tolerated by the cell lines. For the pre-study 24 h after plating the cells was incubated in medium containing 1% DMSO or 1% PBS in duplicates for 48 h and viability of the cells was assessed. The cells (BT-474, Calu-3, HL-60, SkBr3 and U937) were seeded in 96-well plates in the appropriate cell culture medium (50 μl) and incubated for 24 h. Cell seeding density has been pre-optimized for these cell lines. Following the 24-h incubation, the test compounds and above-mentioned controls (DMSO, Triton X 100, and saline) were added in the appropriate cell culture medium (50 μl, 2× final concentration). Ten compound concentrations were evaluated in triplicate: 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μM for BT-474, HL-60, SkBr3 and U937 and 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 80 μM for Calu-3. DMSO was added to a final concentration of 1% and kept equal in all wells. Saline positive control and 0.1% Triton X-100 negative control will be evaluated in sextuplicate. The cells were incubated with the test compounds/controls for 72 h. Cell proliferation data was obtained using CellTiter-Blue® Cell Viability Assay with fluorescence read-out.

2.2.2. Cell assay for BV-173, H-9, Hs578T, SW480, MCF-7, MDA-MB-231, OVCAR-3, SKOV-3, MRC-5, FaDu, PC3 and Du145

Cell proliferation assays were performed by Reaction Biology Corp. The cell lines were obtained from American Type Culture Collection (Manassas, VA). Staurosporine was obtained from Sigma-Aldrich (Saint Louis, MI). Cell Titer-Glo® Luminescent cell viability assay reagent was obtained from Promega (Madison, WI). The cells were grown in the following media: FaDu cells were grown in MEM medium with 10% heat-inactivated foetal bovine serum (FBS); MCF-7, MDA-MB-231 and MRC-5 cells in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% FBS; Hs578T cells were grown in DMEM medium supplemented with 10% FBS and 0.01 mg/mL bovine insulin; PC3 and Du145 cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FBS; SKOV-3 cells were grown in McCoy's 5A medium supplemented with 10% FBS; BV-173 cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 20% FBS; H9 cells were grown in RPMI medium supplemented with 10% FBS; OVCAR-3 cells were grown in RPMI medium supplemented with 20% FBS and 0.01 mg/mL bovine insulin and SW480 cells were grown in L15 medium supplemented with 10% FBS. All cell culture mediums were supplemented with 100 μg/mL penicillin, and 100 μg/mL of Streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Compounds **1** and **2**, Erlotinib and for collected cell lines Lapatinib and Dasatinib, and Staurosporine (positive control) were all dissolved in DMSO in 10 mM stock. Culture medium (10 μL) was added to each well of 384 well cell culture plates. The compounds were diluted in a source plate in DMSO at 3 fold serial dilutions starting at 10 mM, total 10 doses. The compounds (0.25 μL) were delivered from source plate to each well of the cell culture plates by Echo 550. Then, 15 μL of culture medium containing 5000 cells was added to the wells of the cell culture plates. The cells were incubated with the compounds at 37 °C, 5% CO_2 for 72 h. 25 μL of Cell Titer Glo reagent (25 μL) was added to each well according to the instruction of the kit. The contents were mixed on an orbital shaker for 2 min and incubated at room temperature for 10 min to stabilise luminescent signal. Luminescence was recorded by Envision 2104 Multilabel Reader (PerkinElmer, Santa Clara, CA). The maximum luminescence for each cell line in the absence of test compound, but in the presence of 0.4% DMSO, was similarly recorded after incubation for 72 h. The number of viable cells in the culture was determined based on quantitation of the ATP present in each culture well. The percentage growth after 72 h (*%-growth*) was calculated as follows: 100% × (*luminescence* t = 72 h / *luminescence* untreated, t = 72 h).

2.2.3. Cell assay for PC9, ASPC-1 and BXPC-3

Cell proliferation assays were performed by Fluofarma. The PC9 cell line was cultured in RPMI (Invitrogen) containing 10% SVF, Glutamax (Gibco) and HEPES (Sigma-Aldrich), while AsPC-1 and BXPC-3 were cultured with RPMI (Invitrogen) containing 10% SVF, Glutamax (Gibco), sodium pyruvate (Sigma-Aldrich), and HEPES (Sigma-Aldrich), and seeded in a 96 well plate. All cells were grown at 37 °C at pH 7.4 in the presence of 5% CO_2. From 30 mM stock solutions of the compound dilutions of 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM and 100 μM were made and added 24 h after cell seeding. Cell proliferation was measured by time-lapse imaging and by cytolysis. By the time-lapse imaging method the cell proliferation was measured as the surface of the well occupied by the cells. A confluence index was then calculated by making a ratio with the surface occupied at the time-point preceding the treatment. The cell count was also assessed at the last time-point of the kinetics (96 h). The number of lysed cells was quantified with the nuclear marker Sytox Green, able to selectively enter cells with compromised membranes. The IC_{50} measurement was based on cytolysis.

2.3. Turbidimetric aqueous solubility

The compound (10 mM in DMSO) was serially diluted to give solutions of 0.1, 0.3, 1 and 3 mM in DMSO. Each test compound concentration was then further diluted in 100 in buffer (typically 0.01 M phosphate buffered saline pH 7.4) so that the final DMSO concentration was 1% and the final test compound concentrations were 1, 3, 10, 30 and 100 μM. The experiment was performed at 37 °C and each concentration was incubated in 7 replicate wells. The plates were incubated for 2 h at 37 °C before the absorbance was measured at 620 nm. Nicardipine and pyrene were included as control compounds. The solubility of Nicardipine is pH dependent whereas the solubility of pyrene is pH independent. Data analysis: The solubility was estimated from the concentration of test compound that produces an increase in absorbance above vehicle control (i.e., 1% DMSO in buffer). This gave an estimated of precipitation range (lower and upper bound) and a calculated mid-range value.

2.4. hERG ion channel inhibition

The experiments were performed on an IonWorks™ HT instrument (Molecular Devices Corporation), which automatically performs electrophysiology measurements in 48 single cells simultaneously in a specialised 384-well plate (PatchPlateTM). All cell suspensions, buffers