Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting Information

A method for estimating the risk of drug-induced phototoxicity and its application to Smoothened inhibitors

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Contents include characterization for compounds **2**-**8**, information on the marketed oral drugs database and the classification into three phototoxicity categories, the method of calculating the HOMO-LUMO energy gap, information on the UV spectroscopic data obtained from the literature, the method of recording UV spectra and the experimental details for the 3T3 NRU phototoxicity assay.

Chemistry.

NMR spectra were recorded on a Bruker AV400 (Avance 400 MHz) instrument. Analytical LC-MS was conducted using an Agilent 1100 series with UV detection at 214 and 254 nm and an electrospray mode (ESI) coupled with a Waters ZQ single quadmass detector using acetonitrile/water gradients with various modifiers. Analytical HPLC UV purity was assessed at both 254 and 214 nm using an Agilent 1100 HPLC system and one of the following methods. For method A, an Inertsil 150 mm × 4.6 mm C18 column was used at a flow rate of 1.2 mL/min with a gradient of 10-95% acetonitrile/water with 0.1% TFA over 15 min. For method B, an Acquity UPLC BEH 1.7 μm 2.1 mm × 50 mm C18 column was used at a flow rate of 0.5 mL/min with a gradient of 5-95% acetonitrile/water with 5 mM ammonium formate as modifier additive in the aqueous phase over 4.5 min. LC/ESI-MS data were recorded using a Waters LCT Premier mass spectrometer with dual electrospray ionization source and Agilent 1100 liquid chromatograph. The resolution of the MS system was approximately 12 000 (fwhm definition). HPLC separation was performed at 1.0 mL/min flow rate with a gradient from 10% to 95% in 2.5 min. Ammonium formate (10 mM) was used as the modifier additive in the aqueous phase. Sulfadimethoxine (Sigma, protonated molecule *m*/*z* 311.0814) was used as a reference and acquired through the LockSpray channel every third scan.

The synthesis of the compounds **2**-**8** was conducted following the protocols in He, Feng; Peukert, Stefan; Miller-Moslin, Karen; Yusuff, Naeem; Chen, Zhuoliang; Lagu, Bharat, pyridazine derivatives as Smo inhibitors and their preparation, pharmaceutical compositions and use in the treatment of cancer and inflammation, PCT Int. Appl. (2010), WO 2010007120 and Dai, Miao; He, Feng; Jain, Rishi Kumar; Karki, Rajesh; Kelleher, Joseph, III; Lei, John; Llamas, Luis; Mcewan, Michael A.; Miller-Moslin, Karen; Perez, Lawrence Blas; Peukert, Stefan; Yusuff, Naeem, nitrogen-containing heterocyclic organic compounds as inhibitors of the hedgehog pathway and their preparation and use in the treatment of diseases, PCT Int. Appl. (2008), WO 2008110611. Full characterization of compounds is given below:

N-((6-(4-(4-(4-(trifluoromethyl)phenyl)phthalazin-1-yl)piperazin-1-yl)pyridin-3-yl)methyl)acetamide (2):
white powder: R_f = 3.06 min (method B), purity: 96.8% @ 254 nm; ^{1}H NMR (400 MHz, METHANOL-d_4): δ= 8.36 (d, *J* = 8.08 Hz, 1H), 8.09 (d, *J* = 2.02 Hz, 1H), 8.03 (td, *J* = 4.11, 8.46 Hz, 1H), 7.84 - 7.98 (m, 6H), 7.59 (dd, *J* = 2.27, 8.84 Hz, 1H), 6.94 (d, *J* = 8.59 Hz, 1H), 4.26 (s, 2H), 3.84 (dd, *J* = 3.79, 6.32 Hz, 4H), 3.69 (dd, *J* = 3.79, 6.32 Hz, 4H), 1.97 (s, 3H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{27}H_{25}F_3N_6O$: 507.2115, found: 507.2112.

(R)-2-(5-(4-(4-benzylphthalazin-1-yl)-2-methylpiperazin-1-yl)pyrazin-2-yl)propan-2-ol (3):
white powder: R_f = 3.03 min (method B), purity: 99.1% @ 254 nm; ^{1}H NMR (400 MHz, METHANOL-d_4): δ= 8.39 (d, *J* = 1.52 Hz, 1H), 8.32 (d, *J* = 7.58 Hz, 1H), 8.13 - 8.21 (m, 2H), 7.94 (dt, *J* = 1.26, 7.71 Hz, 1H), 7.86 (dt, *J* = 1.26, 7.71 Hz, 1H), 7.19 - 7.33 (m, 4H), 7.13 - 7.19 (m, 1H), 4.73 - 4.81 (m, 1H), 4.63 (s, 2H), 4.26 (d, *J* = 12.63 Hz, 1H), 3.98 (dd, *J* = 2.53, 12.13 Hz, 1H), 3.79 - 3.90 (m, 1H), 3.62 (dt, *J* = 3.54, 12.63 Hz, 1H), 3.40 (dd, *J* = 3.54, 12.63 Hz, 1H), 3.16 - 3.24 (m, 1H), 1.54 (s, 6H), 1.48 (d, *J* = 6.57 Hz, 3H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{27}H_{30}N_6O$: 455.2554, found: 455.2550.

6-(4-(4-(4-(trifluoromethyl)phenyl)phthalazin-1-yl)piperazin-1-yl)nicotinonitrile (4):
pale yellow powder: R_f = 3.60 min (method B), purity: 96.3% @ 254 nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ= 8.55 (d, *J* = 2.02 Hz, 1H), 8.31 (d, *J* = 8.08 Hz, 1H), 8.04 (d, *J* = 1.01 Hz, 1H), 7.86 - 8.01 (m, 7H), 7.05 (d, *J* = 9.09 Hz, 1H), 3.94 - 4.11 (m, 4H), 3.50 - 3.70 (m, 4H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{25}H_{19}F_3N_6$: 461.1696, found: 461.1712.

(R)-2-(5-(4-(4,5-dimethyl-6-(4-(trifluoromethyl)phenyl)pyridazin-3-yl)-2-methylpiperazin-1-yl)pyrazin-2-yl)propan-2-ol (5):
pale yellow powder: R_f = 3.37 min (method B), purity: 97.9% @ 254 nm; ^{1}H NMR (400 MHz, METHANOL-d_4): δ= 8.38 (d, *J* = 1.52 Hz, 1H), 8.16 (d, *J* = 1.52 Hz, 1H), 7.80 - 7.86 (m, *J* = 8.08 Hz, 2H), 7.66 - 7.73 (m, *J* = 7.58 Hz, 2H), 4.68 - 4.80 (m, 1H), 4.22 (d, *J* = 13.14 Hz, 1H), 3.68 (dd, *J* = 2.53, 12.13 Hz, 1H), 3.53 - 3.62 (m, 1H), 3.48 (dt, *J* = 3.54, 12.63 Hz, 1H), 3.27 (d, *J* = 3.54 Hz, 1H), 3.13 (dt, *J* = 3.54, 12.38 Hz, 1H), 2.47 (s, 3H), 2.26 (s, 3H), 1.54 (s, 6H), 1.41 (d, *J* = 6.57 Hz, 3H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{25}H_{29}F_3N_6O$: 487.2428, found: 487.2436.

2-(6-(4-(6-(4-Fluorobenzyl)-4,5-dimethylpyridazin-3-yl)piperazin-1-yl)pyridin-3-yl)propan-2-ol (6):
white powder: R_f = 6.08 min (method A), purity: 100.0% @ 254 nm; ^{1}H NMR (400 HMz, CD_2Cl_2): δ = 1.54 (s, 6H), 2.11 (s, 3H), 2.23 (s, 3H), 3.28-3.31 (m, 4H), 3.65 – 3.68 (m, 4H), 4.24 (s, 2H), 6.70 (d, *J* = 8.5 Hz, 1H), 6.94-6.99 (m, 2H), 7.16-7.20 (m, 2H), 7.67 (dd, *J* =2.5 Hz, 9.0 Hz, 1H), 8.29 (s, 1H); ^{13}C NMR (100 HMz, CD_2Cl_2): δ = 14.45, 15.17, 31.93, 39.82, 46.04, 50.64, 71.20, 106.92, 115.51, 115.72, 130.63, 130.70, 134.70, 135.08, 137.30, 144.77, 157.44, 159.07, 163.22, 163.31; HRMS: *m/z* $[M+H]^+$ calcd for $C_{25}H_{30}FN_5O$: 436.2513, found: 436.2532.

2-(6-(4-(4-(4-fluorobenzyl)-6,7-dihydro-5H-cyclopenta[d]pyridazin-1-yl)piperazin-1-yl)pyridin-3-yl)propan-2-ol (7):
white powder: R_f = 3.02 min (method B), purity: 100.0% @ 254 nm; ^{1}H NMR (400 MHz, METHANOL-d_4): δ= 8.25 (d, *J* = 2.02 Hz, 1H), 7.72 (dd, *J* = 2.53, 9.09 Hz, 1H), 7.16 - 7.24 (m, 2H), 6.95 - 7.04 (m, 2H), 6.87 (d, *J* = 8.59 Hz, 1H), 4.18 (s, 2H), 3.60 - 3.70 (m, 4H), 3.53 - 3.60 (m, 4H), 3.00 (t, *J* = 7.33 Hz, 2H), 2.75 (t, *J* = 7.58 Hz, 2H), 2.08 (t, *J* = 7.58 Hz, 2H), 1.52 (s, 6H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{26}H_{30}FN_5O$: 448.2507, found: 448.2503.

(R)-2-(5-(4-(4-benzyl-6,7-dihydro-5H-cyclopenta[d]pyridazin-1-yl)-2-methylpiperazin-1-yl)pyrazin-2-yl)propan-2-ol (8):
white powder: R_f = 3.09 min (method B), purity: 100.0% @ 254 nm; ^{1}H NMR (400 MHz, METHANOL-d_4): δ= 8.36 (d, *J* = 1.01 Hz, 1H), 8.13 (d, *J* = 1.01 Hz, 1H), 7.22 - 7.31 (m, 2H), 7.14 - 7.22 (m, 3H), 4.63 - 4.77 (m, 1H), 4.19 - 4.25 (m, 2H), 4.03 - 4.13 (m, 1H), 3.89 (d, *J* = 12.63 Hz, 1H), 3.38 (dt, *J* = 3.28, 12.51 Hz, 1H), 3.26 (dd, *J* = 3.54, 12.63 Hz, 1H), 3.13 (dt, *J* = 3.54, 12.38 Hz, 1H), 3.02 (t, *J* = 7.33 Hz, 2H), 2.75 (t, *J* = 7.58 Hz, 2H), 2.02 - 2.16 (m, 2H), 1.53 (s, 6H), 1.30 (d, *J* = 6.57 Hz, 3H); HRMS: *m/z* $[M+H]^+$ calcd for $C_{26}H_{32}N_6O$: 445.2710, found: 445.2708.

Oral drugs database and phototoxicity classification.
A list of 763 oral marketed drugs from the FDA Orange Book [25th Edition of FDA's *Approved Drug Products with Therapeutic Equivalence Evaluations*, or *Orange Book* 2005] was compiled. 18 large molecules failed conformer/AM1 calculations and were excluded for further analysis. The remaining 745 oral drugs (marketed oral drug database = MODD 2005) were binned into three categories according to their level of clinical signs of phototoxicity. For evaluation of phototoxicity the following process was employed: An initial search was conducted by accessing the entries for the oral drugs in the Thomson Micromedex Drugdex® (Drugdex® systems [internet database], Greenwood Village, CO, Thomson Micromedex, updated periodically) and curating information on phototoxicity/photosensitivity. SciFinder searches were used to further clarify the phototoxicity potential of some of these drugs. The criteria used for the three categories are as following:

Medium/high:	well known phototoxin, frequent and/or severe phototoxicity observed.
Low:	phototoxic effects were reported as rare or minimal; also, any mention of phototoxicity without information on incidence or severity.
Non-phototoxic:	no phototoxicity listed in the literature.

HOMO-LUMO energy gap calculation.
These calculations were performed using the Chemical Computing Group's MOE version 2005. Multiple conformers were generated using the High Throughput conformer generator and each was energy minimized using AM1. The HOMO-LUMO gaps were then calculated for each conformer. The lowest

HOMO-LUMO gap energy was used i.e. the gap energy corresponding to the most reactive conformer. (Note that MOE incorporates the publicly available versions of MOPAC to compute AM1 calculations including energy minimization and HOMO, LUMO energies).

Literature UV spectroscopic data.

Data on UV spectra were obtained from the CD-ROM database "UV and IR Spectra: Pharmaceutical Substances (UV and IR) and Pharmaceutical and Cosmetic Excipients (IR), H.-W. Dibbern, R. M. Müller, E. Wirbitzki (Eds.), 2002, Editio Cantor Verlag, Aulendorf. This database contains pdf files of 1,637 UV spectra. 409 oral drugs in our MODD 2005 list were found to have UV spectra taken in methanol from 220 nm to 360 nm in this database. Their maximum absorption at or above 290 nm and concentration were extracted from the pdf files and the molar absorptivities were calculated for the 409 oral drugs.

Method for recording UV spectra.

UV spectra were recorded in methanol as solvent at a concentration of ~ 0.01 mg/ml in absorbance mode on a Shimadzu UV2101-PC UV/Visible scanning spectrophotometer between 200 and 700 nm. Molar absorptivity ε for the maximum in the range 290-400 nm was calculated at that wavelength using Beer's law: $A = \varepsilon lc$, where A ist the absorptivity, l is the pathlength in cm, and c ist he sample concentration in mol/L.

Method for measuring phototoxicity using the 3T3 NRU test system.

The "*In vitro* 3T3 NRU Phototoxicity Profiling Assay" was performed according to OECD TG 432 with the mouse Balb/c fibroblast cell line 3T3 clone A31. The cells were stored as frozen stocks in liquid nitrogen in the cell bank of the test facility. The original cultures were obtained from the European Collection of Cell Cultures (ECACC) / Health Protection Agency (Salisbury/UK). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with 10 % newborn calf serum (NCS) and used for the study in a passage between 3 and 17 after thawing (corresponds to total number of 85-100 passages). During creation of the master cell bank typical morphology and UV-A sensitivity as well as the absence of mycoplasms were verified. Exposure to UV-A light was done using the solar simulator SOL500 (Dr. Hoenle/Germany). The light source was filtered by the filter H1 (320-400 nm) in order to attenuate the cytotoxic UV-B range (280-320 nm).

Cell culture and treatment

Approximately 10'000 cells/well were seeded in 100 µl DMEM (10 % NCS) in all but the peripheral wells (blanks) of 96 well microtiter plates. Afterwards these plates were incubated for 20 - 24 hrs in a CO_2 incubator (37°C, 7.5 % (v/v) CO_2, and approx. 90 % humidity). Then test items or the positive control were applied (usually prepared from 100x stock solutions diluted into Hanks' Balanced Salt Solution (HBSS) serving as a medium replacement). In all cases standardized dilution series (8 values per plate) up to 1000 µM were applied. On each plate 2x 6 wells served as negative control (e.g. 1 % DMSO in HBSS). For the positive control (CPZ) as well as for the test item two plates (one for irradiation, one for respective dark period) per experiment were prepared using at least 3 replicates of each concentration.
After application of the HBSS solutions these plates were incubated for approx. 1 hr in the incubator and subsequently transferred to the light source. Then, 2 plates (CPZ and test item) were irradiated for ca. 50 min (1.7 ± 0.1 mW/cm^2 UV-A resulting in a UV-A dose of 5.0 ± 0.3 J/cm^2 UV-A), the remaining 2 plates were kept accordingly in the dark at similar conditions. After removal of the HBSS solutions all plates were further incubated with DMEM (10 %NCS) for additional 22 to 20 hrs.

Neutral red uptake measurement and cytotoxicity assessment

The DMEM (with NCS) was removed, the plate was washed with 150 µL HBSS and further incubated with 100 µL Neutral Red medium (DMEM, without NCS and with 50 µg/ml Neutral Red dye, CAS No.: 553-24-2) for three hours in a CO_2-incubator (37.0 ± 0.5°C,approx. 7.5 % (v/v) CO_2, and approx. 90 % humidity). The Neutral Red medium was totally removed by slight centrifugation of the plate against a filter paper. The remaining dye was then extracted for at least 5 min with 150 µL/well of Neutral Red desorb (acetic acid / ethanol/ H_2O demin, 1:50:49). The optical density (OD) of the resulting solution was measured at 540 nm in a microtiter plate spectrophotometer (Bio-Tek Synergy HT using the proprietary KC4 software). The blank corrected OD values were used in order to obtain concentration-response curves. All values were normalized to the mean of the negative control values of the same plate. The EC_{50} and PIF values were calculated using the "Phototox 2.0" software or alternatively, using "SigmaPlot 8.0" based on a four-parameters logistic curve fit. Photo Irritation Factors (PIF) were determined using the ratio of the EC_{50} values obtained without and with irradiation: PIF = EC_{50} (-irr)/EC_{50} (+irr). In some cases the PIF calculation was based on the highest soluble concentration tested without irradiation.

The positive control (reference item) used was chlorpromazine-HCl (CPZ, MW: 355.3 g/mol, CAS No.: 69-09-0) dissolved in DMSO. The solvent used for dilution of test item (usually DMSO) served as negative control.