Supplementary Material (Online Resource 1)

Article Title: A coumarin derivative (RKS262) inhibits cell-cycle progression, causes pro-apoptotic signaling and cytotoxicity in ovarian cancer cells

Journal Title: Investigational New Drugs

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Description of NCI $_{60}$ cell line in vitro Screening and Methodology

RKS262 was screened in the DTP-NCI_{60} cell line panel under the *in vitro* Cell Line screening Project (IVCLSP) and growth percent of treated cells estimated relative to control (100%) [DTP/NCI web site (http://dtp.nci.nih.gov)]. The output from a single dose screen (at 10 µM RKS262) was reported as a mean graph and is available for analysis by the COMPARE program. RKS262, which exhibited significant growth inhibition, was further evaluated against the 60 cell panel at five concentration levels.

Human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96 well microtiter plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37℃, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50µg/mL gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 µL of these different drug dilutions were added to microtiter wells containing 100 µL of medium, resulting in the required final drug concentrations. Following drug addition, the plates were incubated for an additional 48 h at 37℃, 5% CO_2, 95% air, and 100% relative humidity. The assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µL of cold 50 % (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4℃. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µL) at 0.4 % (w/v) in 1% acetic acid was added to each well,

and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain was solubilized with 10 mM trizma base, and the absorbance read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µL of 80 % TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as: [(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti>/=Tz and [(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz.

Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from [(Ti-Tz)/Tz] x 100 = -50. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested.

Protocol For the Synthesis of RKS262 [*3*] (Scheme 1)

Synthesis of diol [*1*]

10.31 mL of 2-mercapto ethanol (*1*) was added into the suspension of 10 g of sodium ethoxide in ethyl alcohol (160ml) and stirred at room temperature. After the

addition of propylene oxide (11.32 mL), the resulting solution was warmed to reflux for overnight. The reaction was then combined with ice water and stirred. After removal of the brownish-colored suspended particles by filtration, the excess water was evaporated in vacuo. The resultant solution was extracted with ethyl acetate. The combined organic layers were dried with Na2SO4, filtered and dried in vacuo to afford diol compound *ii*.

^{1}H NMR (CDCl3): δ 3.868 (1H), 3.734(2H), 2.725(4H), 2.465(2H), 1.222(3H)

MS (FAB): 159 [M+Na]+

Synthesis of sulfone diol [*iii*] from diol [*i*]

35% hydrogen peroxide (3.28 mL) was added dropwise to 2.6 g of the diol [*i*] in the presence of a catalytic amount of H_3PO_4 in a stirred ice water bath. After the completion of hydrogen peroxide addition, the solution was warmed to reflux in a silicon oil bath and heated to 130 ºC overnight. The resulting solution was monitored until all hydrogen peroxide had been consumed. The resultant solution was then dried in vacuo to afford the sulfone diol compound [*iii*].

^{1}H NMR (CDCl3): δ 4.48 (1H), 4.12 (2H), 3.31 (3H), 3.13 (1H), 1.31 (3H)

MS (FAB): 191 [M+Na] +.

Synthesis of Intermediate [*4*] from Sulphone diol [*iii*]:

Sulphone diol [*iii*] (5 g) was dissolved in the 30% NaOH solution (25 mL) and tert-butyl carbazate (10 g) was added to the reaction mixture which was stirred and refluxed for 24 hrs. Vigorous stirring was required. The reaction mixture was cooled to room temperature and the organic layer extracted with EtOAC. The organic layer was collected and dried over anhydrous sodium sulphate and concentrated under reduced pressure to afford [*4*] as a cream-colored viscous liquid, which upon drying and storage at 40℃ turned into mixed solid-liquid. Intermediate [*4*] was characterized by Mass spectrometry. MS [m/z] = 187 [M+Na+, 100%] and was used as such without further purification.

Synthesis and characterization of RKS262 [*5*]:

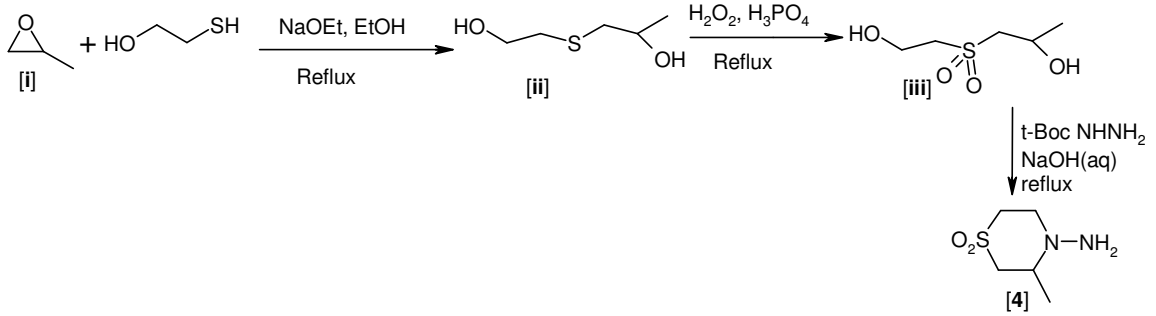
Intermediate *4* (1.5 eq) was dissolved in anhydrous EtOH and to this solution was added coumarin aldehyde *3* (1 eq.). The reaction mixture was refluxed and stirred vigorously for 1 hr. Progress of the reaction was monitored every 15 min. The reaction mixture was cooled to room temperature and concentrated to reduce the volume of EtOH by half and then the solution was filtered under suction. The solid collected was washed once with cold EtOH and then repeatedly washed with diethyl ether and dried under reduced pressure to afford a yellow powder (RKS262, compound *5*) in good yield (60-65%).

^{1}H NMR (DMSO-d6): δ 8.05(1H), 7.82-8.04(1H), 6.69(1H), 7.43-7.46(1H), 4.45(1H), 3.38-3.99(2H), 2.82-3.24(4H) and 1.05-1.07(d, 3H).

MS(FAB): 421 $[M+H]^+$, 443 $[M+Na]^+$.

Scheme 1: Synthesis of intermediates and RKS262

Part 1: Synthesis of N-amino-1,4-thiazane:



Part 2: Synthesis of RKS262: