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PRECLINICAL STUDIES

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

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Summary Coumarin derivative RKS262 belongs to a new class of potential anti-tumor agents. RKS262 was identified by structural optimization of Nifurtimox which is currently undergoing phase II clinical trials to treat high-risk neuroblastoma. In a NCI_{60} cell-line assay RKS262 exhibited significant cytotoxicity in ovarian cancer cells and a variety of other cell lines exceeding effects of commercial drugs such as cisplatin, 5-FU, cyclophosphamide or sapacitabine. Various leukemia cell-lines were most sensitive (GI_{50}:~ 10 nM) while several non-small cell lung cancer cell lines and few cell lines from other tissues were relatively resistant (GI_{50}>1 μM) to RKS262 treatment. The mechanism of cytotoxicity was examined using ovarian cancer cell-line OVCAR-3 as a model. RKS262 treatment resulted in a reduced mitochondria-transmembrane-depolarization potential. RKS262 effects included up-regulation of apoptotic markers and were not correlated with activation of pro-apoptotic MAP-Kinases (p38, SAP/JNK). RKS262 exerted strong inhibitory effects on oncogene ras, down-regulated DNA-pk KU-80 subunit expression and caused activation of Akt. A signature effect of RKS262 is the regulation of the mitochondrial Bcl2-family pathway. Pro-apoptotic factors Bid, Bad and Bok were up-regulated while expression of pro-survival factors Bcl-xl and Mcl-1 was inhibited. Moreover, at sub-cytotoxic doses RKS262 delayed OVCAR-3 cell-cycle progression through G2 phase and up-regulated p27 while cyclin-D1 and Cdk-6 were down-regulated, indicating that RKS262 is a specific cyclin/CDK inhibitor. In summary, RKS262 has been identified as a molecule belonging to a new class of potential chemotherapeutic agents affecting the viability of multiple cancer cell-lines and causing selective adverse effects on the viability of ovarian cancer cells.

Keywords Coumarin derivative RKS262 · Ovarian cancer · Cytotoxicity · Cell-cycle regulation · Bcl2 class proteins · MAPK · Akt signaling

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Abbreviations

DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
MAPK	mitogen activated protein kinases
PBS	phosphate-buffered saline
GI	growth inhibition
TGI	total growth inhibition
LC	lethal concentration

Introduction

Ovarian cancer is the eighth most common cause of death caused by cancer in women with worldwide more than 190,000 newly diagnosed cases each year. Incidence rates vary considerably, with the highest found in the United States and Northern Europe and the lowest in Africa and Asia [1]. The majority of patients with ovarian cancer present late with advanced disease (FIGO stage III/IV) and despite multimodality treatment with surgical debulking followed by platinum-taxane combination chemotherapy, median survival is only 3 years. While re-treatment with a platinum-based drug is possible for some women the response rate to current second line chemotherapy is 15–30% due to the rise of resistance to these drugs requiring the development of new drugs to treat this cancer.

Nifurtimox [Fig. 1A; compound ***1***], an agent originally employed to treat Chagas' disease, causes cytotoxic and antitumor effects in neuroblastoma cell lines [2, 3], has shown promise in a phase 1 trial [4] and is currently being evaluated in a phase 2 trial for the treatment of childhood neuroblastoma. Nifurtimox also exhibited cytotoxicity against cells lines derived from ovarian cancer even though with a lower efficacy [unpublished data]. In order to identify an analog of Nifurtimox with higher potency we carried out a structural optimization. A variety of analogs were synthesized and their cytotoxicity evaluated against a platinum-resistant ovarian cancer cell line (OVCAR-3) as a model system. RKS262 [Fig. 1A; compound ***5***], a coumarine derivative that includes the 1-aminotetrahydrothiazine ring of Nifurtimox, revealed the most potent activity among Nifurtimox analogs tested and was selected as 'lead' molecule for our study.

The antitumor potential of RKS262 was examined in multiple assays. To evaluate the tumor type selectivity of this compound a NCI_{60} cell line growth/viability assay featuring cell-lines derived from refractory tumors of 12 different tissues (http://dtp.nci.nih.gov/screening.html) was utilized. Next, the mechanism of cytotoxicity was analyzed in OVCAR-3 cells which, apart from platinum-resistance, feature various mutations present in a variety of ovarian and other solid tumors [5]. In addition, the antitumor potential of RKS262 to treat ovarian cancer cells was investigated by measuring anti-proliferative effects in conjunction with the expression profile of pro-apoptotic and pro-survival Bcl2 family proteins. Furthermore we studied the effects of sub-cytotoxic concentrations of RKS262 on cell-cycle progression and the expression of regulatory proteins such as p27, cyclins and CDKs in OVCAR-3 cells.

RKS262 revealed potent cytotoxicity in multiple chemoresistant cancer cell types including ovarian cancer. In

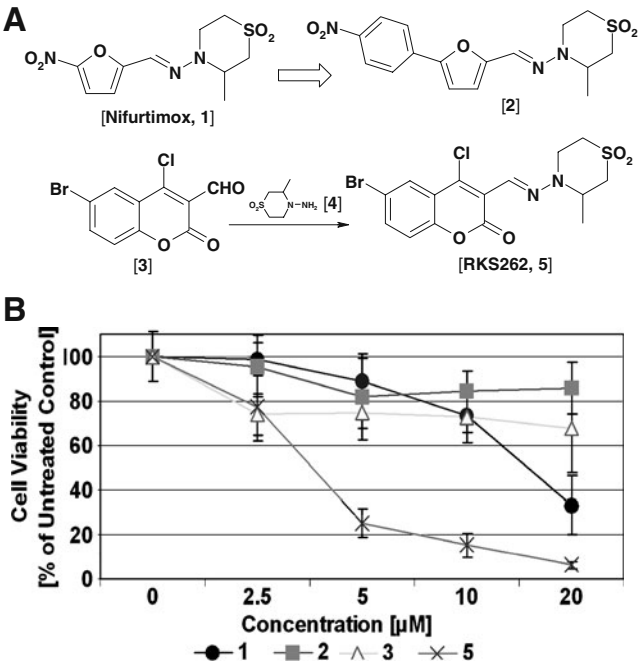

Fig. 1 Design of coumarin derivative RKS262 and cytotoxic effects in platinum resistant OVCAR-3 ovarian cancer cells. **A** RKS262, related to Nifurtimox [***1***] and Nifurtimox derivative [***2***] was synthesized in multiple steps; the synthesis of a key intermediate, the 1-aminotetrahydrothiazane ring [***4***] was carried out by developing a more efficient, less toxic and safer methodology (details to the experimental protocol are described in the Supplementary Materials, Online Resource 1). The condensation of commercially available coumarin aldehyde [***3***] with [***4***] in anhydrous EtOH at 60–70°C afforded RKS262 [***5***] as a yellow colored solid, which was characterized by NMR and Mass. Purity was ascertained by HPLC. **B** Comparative analysis of the cytotoxic effect of RKS262 [5], its precursor coumarin aldehyde [***3***], Nifurtimox [***1***] and its close structural analog [***2***] in OVCAR-3 cells (human ovarian epithelial adenocarcinoma cell line). Cells were treated with compounds in various concentrations (0–20 μM) for 48 h. An MTS viability assay was carried out as described (Materials and methods). Data are expressed as the mean of triplicate determinations (X±SD) of a representative experiment in % cell viability of samples with untreated cells [100%]

OVCAR-3 cells induction of apoptosis through RKS262 treatment is not directly correlated with activation of pro-apoptotic MAP-Kinases (p38, SAP/JNK) but with inhibitory effects on oncogene ras, down-regulation of DNA-pk KU-80 subunit expression and activation of AKT. RKS262 up-regulated pro-apoptotic Bcl2 family members such as Bad, Bid and Bok and down-regulated pro-survival Bcl2 family proteins Mcl-1 and Bcl-xl. Moreover, at sub-cytotoxic concentrations, RKS262 delayed progression of OVCAR-3 cells through G2 phase and upregulated p27 while cyclins-D1, cyclin-D3 and Cdk-6 were down-regulated.

Materials and methods

Synthesis of RKS262 and analogs

RKS262 was synthesized in multiple steps [Fig. 1] and details of the synthesis and experimental protocol are described in the supplementary material (Online Resource 1). The synthesis of a key intermediate, the tetrahydrothiazane ring, could not be achieved by the procedure described in the literature [6]. We developed a new route to synthesize this intermediate in multi-gram quantities with high yield. The treatment of sulfone diol with tertiary butyl carbazate in refluxing sodium hydroxide solution overnight afforded the tetrahydrothiazane ring. Removal of the tert-Boc protecting group on the primary nitrogen achieved the cyclization of the sulfone diol to its corresponding amine. This improved methodology provides a strategy to avoid the use of carcinogenic and highly explosive hydrazine reagents used previously [6]. Finally, the condensation of commercially available coumarin aldehyde [Fig. 1; compound ***3***] with the newly synthesized amine afforded RKS262 [compound *5*] as a yellow colored solid, which was characterized by NMR and Mass Spectroscopy. Purity was ascertained by HPLC. Stock solutions of RKS262 in 10 mM DMSO were stored at −20°C.

Cell lines (human)

OVCAR-3 (ovarian epithelial adenocarcinoma) cells were purchased from ATCC (Manassas, VA) and grown in T75/T150 cell culture flasks (Corning, New York, NY) in DMEM medium (Gibco, Rockville, MD) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2, in a humidified incubator. For all assays cells were allowed to attach overnight after seeding and treated in complete medium.

Cell viability assay

Viability of cells was determined by the 96® Aqueous-One-Solution Assay (Promega, Madison, WI) following the manufacturer's recommendations. This colorimetric assay is based on the ability of mitochondria to reduce a substrate (MTS) into a soluble formazan product with an absorbance at 490 nm (ELISA plate reader; (Thermo Labsystems, Waltham, MA) directly proportional to the number of living cells (Promega, Madison, WI). Cells (5×10^3) were plated into 96 well flat bottom plates (Corning, Inc., Corning, NY) and treated with RKS262 as indicated (Results section). Following incubation for 46 h the MTS reagent was added for an additional 2 h and absorbance measured at 490 nm. Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations (X±SD) of a representative experiment in % of absorbance of samples with untreated cells [100%]. The description of NCI_{60} cell lines assay can be found at http://dtp.nci.nih.gov and a protocol is provided in the Supplementary Material (Online Resource 1).

Mitochondrial transmembrane-potential

OVCAR-3 cells (1×10^6) were seeded in a 100 mm^2 petri-dish and treated with 5 μM of RKS262 for 3 h or 24 h. Following treatment cells were washed with PBS, re-suspended in fresh medium (5×10^5 cells/ml) and incubated with 15 nM $DiOC_{18}(3)$ (Invitrogen Corporation) for 30 min at 37°C. The cells were washed twice with DPBS, re-suspended in an equal volume of DPBS analyzed by flow cytometry (excitation at 488 nm, emission at 520 nm). Data was acquired on a BD FACSort flow cytometer using CellQuest software (BD Immunocytometry-Systems, San Jose, CA) and analyzed (ModFit LT software, Verity Software House, Inc., Topsham, ME). Ten thousand cells were analyzed for each sample.

Cell-cycle analysis (by FACS)

Cell-cycle analysis was carried out by flow cytometry. OVCAR-3 cells were seeded into 100 mm^2 tissue culture dishes (1×10^6 cells/dish) (Corning Inc., Corning, NY), treated with RKS262 (1 μM or 3 μM) for 24 h or 48 h and the assay carried out as described previously [7]. Appropriate gating was used to select the single cell population. The same gate was used on all samples, ensuring that the measurements were made on a standardized cell population. Experiments were performed in duplicate.

Western blot analysis

OVCAR-3 cells were seeded into 100 mm^2 tissue culture dishes (1×10^6 cells/dish) and treated with RKS262 (5 μM) for various time intervals as indicated (Results section). Preparation of cell lysates, PAGE and immunoblotting was carried out as described elsewhere [7]. Primary antibodies were all purchased from (Cell Signaling Technology, Beverly, MA). The bands were visualized using horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia Biotech, Piscataway, NJ), followed by enhanced chemiluminescence (Upstate, Waltham, MA) and documented autoradiography (F-Bx810 Film, Phoenix, Hayward, CA). As a size standard pre-stained Precision Plus Protein Kaleidoscope (Biorad, Hercules, CA) marker was used. Experiments were performed in duplicate.

Data analysis

Mean and standard deviation (SD) were calculated. Mean differences were determined by Student's t-test or determined by one-way ANOVA. Software used for these analyses was STATA 9.0 (StataCorp, College Station, TX).

Results

Development of a new route to synthesize RKS262 and analogs

We modified and improved the synthesis of 1-aminotetrahydrothiazane [4], the key intermediate in the synthesis of Nifurtimox, because the method previously described [6] could not be reproduced in our hands despite repeated attempts. In addition the previously described method [6] required the use of hydrazine, a known carcinogen and a highly explosive agent. We instead used tert-butyl carbazate to accomplish the ring cyclization and achieved tert-butyl Boc deprotection of the amine group *in situ* in one step to generate a 1-amino tetrahydrothiazane ring [coumpound ***4***] in multi-gram quantities in good yield (60–70%, un-optimized). Coupling of the ring with 6-bromo,4-choloro coumarin-3-aldehyde [compound ***3***] generated RKS262 in good yield (60%, un-optimized). Other analogs such as Nifurtimox [compound ***1***] and nitrophenyl analog [coumpound ***2***] were prepared under similar conditions (Fig. 1A).

Viability of various cancer cell types upon treatment with RKS262

In an initial approach to analyze the effects of RKS262 on ovarian cancer cells, we performed a cytotoxicity assay (Fig. 1B) comparing Nifurtimox [compound ***1***] and analog [compound ***2***] to RK262 [compound ***5***] and precursors i.e. coumarin aldehyde [compound ***3***]. RKS262 proved to be highly and dose-dependently cytotoxic to OVCAR-3 (75% cytotoxicity at 5 μM) cells. For Nifurtimox, similar toxicity required a concentration of 20 μM while the other derivatives displayed minimal toxicity even at 20 μM. To evaluate potential tumor-type selectivity, RKS262 was screened in a NCI_{60} cell-line growth/viability assay (Fig. 2A). RKS262 treatment resulted in cytotoxicity to most of NCI_{60} cell-lines tested. Specifically, leukemia cell lines (e.g. HL-60/MOLT-4/SR) were most sensitive (GI_{50}:10 nM) whereas several non-small cell lung cancer (incl. NCI322M/NCI-H226) as well as some cell lines from other tissues were relatively resistant (breast/BT549, CNS/SNB19, melanoma/UACC257, renal/786-0, ovarian/SKOV-3) to RKS262 treatment (GI_{50} >1 μM). The comparison of key parameters such as GI_{50}, TGI and LC_{50} (Fig. 2A) which define growth inhibition (50%), total growth inhibition and lethal concentration (50%), respectively, indicate that RKS262 is superior to various clinically used anti-cancer drugs (e.g. cisplatin, 5-FU, cyclophosphamide, sapacitabine) (http://dtp.nci.nih.gov/screening.html). In the same assay the dose-dependent effect of various concentrations of RKS262 (100 nM–100 μM) on a panel of ovarian cancer cell lines was also compared (Fig. 2B). The growth of IGROV-1 and OVCAR-3 were highly affected, OVCAR-8, OVCAR-4 and OVCAR-5 responded strongly to treatment, whereas SKOV-3 revealed less reduction in growth upon RKS262 treatment. The NCI_{60} cell-line screen and our cytotoxicity assays clearly suggest RKS262 to be highly damaging to cell lines derived from a variety of tumor types, including ovarian cancer. Similarly cell-line selectivity, yet not tumor-type specificity, has been observed in other classes of drugs screened in NCI_{60} cell-lines [7, 8].

Cell-cycle analysis of OVCAR-3 cells after RKS262 treatment

Cytotoxic agents at subcytotoxic concentrations often display cell-cycle regulatory effects. For example, isothiocyanate 7Me-IEITC arrested progression of SKOV-3 ovarian cancer cells in G2/M phase and its close structural analog NB7M in SKOV-3 caused G1 phase arrest [7]. Using FACS we analyzed the effect of subcytotoxic (1 μM) and IC_{50} (~3 μM) concentration of RKS262 (24 h treatment) in OVCAR-3 cells. Our analysis revealed a significant increase in the G2/M-phase cell population followed by reduction of cells in G1-phase as compared to untreated control (Fig. 3A). This indicated that proliferation of OVCAR-3 cells was adversely affected, while a block of cell-cycle progression in G2/M-phase in this non-synchronized culture appeared within 24 h of treatment. We performed western blot analysis of the lysates of OVCAR-3 cells to investigate the expression of cell-cycle regulatory factors upon drug treatment (Fig. 3B). Western blotting revealed that RKS262 caused cyclin D1 down-regulation and a p21-independent inhibition of CDK-6. In contrast p27 which previously has been suggested as a target for cancer therapeutics [9] was up-regulated by RKS262 treatment. Immunoblotting further revealed that RKS262 treatment of OVCAR-3 did not alter the expression of members of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors, p21 and p16 (Fig. 3B).

RKS262 disrupted the mitochondrial membrane potential of OVCAR-3 and caused morphological hallmarks of apoptosis

To understand the mechanism of cytotoxic response of OVCAR-3 cell to RKS262, we examined the mitochondrial

transmembrane depolarization potential (ΔYm) cells by flow cytometry. RKS262 at a concentration of 5 μM caused a loss of ΔYm within 12 h of treatment (Fig. 4A). RKS262 treatment also led to similar reductions in the ΔYm of SMS-KCNR, a chemotherapy-resistant neuroblastoma cancer cell line (unpublished data). Loss of ΔYm due to chemical agents has been reported to be an indicator of onset of early and irreversible apoptotic events [10].

Induction of apoptosis in OVCAR-3 ovarian cancer cells by RKS262

Cell death via apoptosis is executed by initiator caspases (such as caspases-2, -8, -9 and -10) which leads to activation of downstream effector caspases (such as caspases-3, -6 and -7) and, subsequently, of many intracellular proteins and morphological and biochemical changes associated with apoptosis [11, 12]. RKS262 treatment of OVCAR-3 cells (5 μM) resulted in the activation/cleavage of executioner caspase-3 and caspase-7 within 1 h (reaching maximal activation at 18 h) as shown by immunoblotting (Fig. 4B). The activation of proteolytic caspases-3 and -7 following RKS262 exposure to OVCAR-3 cells resulted in the cleavage of PARP-1 within 1 h of treatment (Fig. 4B). PARP, a 116 kD nuclear poly (ADP-ribose) polymerase, is involved in DNA repair, and cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [13].

RKS262 treatment suppression of pro-survival markers in OVCAR-3 ovarian cancer cells

To define key signaling responses of OVCAR-3 cells upon treatment with RKS262, we analyzed the expression and activation/phosphorylation of cellular markers involved in pro-apoptotic signaling. Immunoblotting of cell lysates revealed that RKS262 (at 5 μM) caused only a minor up-regulation/activation of SAP/JNK and p38 MAPK (Fig. 3C). These two MAPKs are crucial factors in signaling cascades responding to inflammatory cytokines, stress, UV light, osmotic shock, cytotoxic drugs and diverse pro-apoptotic stimuli [14]. It appeared from our experiments that RKS262 induced cytotoxicity of OVCAR-3 cells was mainly independent of the MAPK studied.

In contrast, RKS262 (5 μM) exerted strong inhibitory effects on oncogene ras (Fig. 4D) within 1 h after treatment. Another protein of current interest in ovarian cancer treatment is DNA-pk (see Discussion section) essential for DNA repair. RKS262 treatment (5 μM) initially up-regulated the KU-80 subunit expression and after 6 h down-regulated KU-80 to background levels within 18 h of treatment (Fig. 4D) suggesting that RKS262 has a significant effect on DNA-pk holozyme expression and function. Moreover, RKS262 treatment (5 μM) initially and within 1 h activated Akt but over time down-regulated phosphorylation of Akt to reach background levels at 36 h, which was counteracted by the cells through increased expression of Akt (Fig. 4D). Among various other signal transduction pathways, Akt is emerging as a major therapeutic oncogenic target in several cancers including ovarian cancer (see Discussion section).

RKS262 selectively regulated expression of pro-apoptotic Bcl2 family member proteins

The expression of Bcl2 family members are a major determinant of cancer cell-survival. Relevant pro-apoptotic markers include Bid, Bad, Bax, Bak and Bok. Immunoblotting of OVCAR-3 cell lysates revealed that RKS262 (at 5 μM) caused a strong, rapid (within 1 h), and sustained (36 h) activation of Bid and Bad as well as an up-regulation of Bok after 36 h whereas levels of Bax and Bak remained unchanged (Fig. 5). Further analysis of the lysates proved that expression of anti-apoptotic Bcl2 family members Mcl-1 and Bcl-xL was strongly down-regulated by RKS262 treatment (Fig. 5).

Discussion

Nifurtimox, an agent originally employed to treat Chagas' disease displays antitumor effects in neuroblastoma *in vitro* and *in vivo* [2–4] and is currently being tested in a phase 2 treatment trial for childhood neuroblastoma. Nifurtimox forms a nitro-anion radical metabolite and may produce superoxide anions/hydrogen peroxide in cells [3]. These metabolites react with nucleic acids causing a significant breakage in deoxyribonucleic acid (DNA) [15]. As suggested for other chemotherapeutic drugs, Nifurtimox may be used in oxidation therapy by elevating H_2O_2 and superoxide radical in tumor cells above the survival/death threshold (see introduction) [16]. Production of reactive oxygen species results in apoptosis and/or necrosis and can be used for selective targeting of tumor cells which possess higher oxidative stress level and display alteration of antioxidant enzymes (catalase, SOD) as compared to normal cells. However, cytotoxicity screening in our lab revealed that cell-lines derived from a variety of tissues including ovarian cancer are relatively resistant to Nifurtimox treatment (unpublished data). The objective of the present study was to design a potent synthetic analog of Nifurtimox to potentially treat various human solid tumors including platinum-resistant ovarian cancers.

The cytotoxic potential of RKS262, a Nifurtimox derivative with specific modifications in the chemical structure including a coumarin scaffold was identified by

A

Panel/Cell Line	Percent Growth Log10 Concentration -8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia								
HL-60(TB)	48	45	33	-43	-50	< 1.00E-8	2.74E-6	8.98E-5
K-562	105	76	67	6	2	1.87E-6	> 1.00E-4	> 1.00E-4
MOLT-4	55	56	48	-31	-31	4.97E-7	4.05E-6	> 1.00E-4
SR	30	38	22	-47	-31	< 1.00E-8	2.07E-6	> 1.00E-4
Non-Small Cell Lung Cancer								
A549/ATCC	94	95	87	38	-81	5.73E-6	2.09E-5	5.47E-5
EKVX	99	94	96	47	-96	8.88E-6	2.15E-5	4.80E-5
HOP-62	109	105	102	17	-96	4.07E-6	1.41E-5	3.91E-5
NCI-H226	106	116	101	64	-45	1.33E-5	3.84E-5	> 1.00E-4
NCI-H23	93	98	93	-38	-61	2.12E-6	5.11E-6	3.33E-5
NCI-H322M	108	104	107	66	-92	1.26E-5	2.62E-5	5.44E-5
NCI-H460	103	102	104	7	-71	3.63E-6	1.24E-5	5.42E-5
NCI-H522	97	105	57	-89	-88	1.12E-6	2.47E-6	5.43E-6
Colon Cancer								
COLO 205	105	107	97	-62	-85	1.97E-6	4.07E-6	8.43E-6
HCT-116	139	102	74	-88	-99	1.41E-6	2.86E-6	5.84E-6
HCT-15	92	104	82	-31	-94	1.92E-6	5.32E-6	2.02E-5
HT29	100	99	86	-54	-67	1.80E-6	4.09E-6	9.29E-6
KM12	115	116	104	.	-94	3.32E-6	1.01E-5	3.44E-5
SW-620	108	110	94	-64	-100	1.89E-6	3.93E-6	8.16E-6
CNS Cancer								
SF-268	104	108	100	4	-80	3.33E-6	1.12E-5	4.42E-5
SF-295	102	103	103	53	-67	1.06E-5	2.76E-5	7.17E-5
SF-539	97	99	92	50	-97	1.01E-5	2.20E-5	4.81E-5
SNB-19	100	108	109	45	-96	8.40E-6	2.09E-5	4.74E-5
SNB-75	90	89	82	33	-94	4.45E-6	1.81E-5	4.47E-5
U251	102	97	92	-1	-95	2.83E-6	9.71E-6	3.32E-5
Melanoma								
LOX IMVI	103	93	88	-76	-93	1.70E-6	3.44E-6	6.96E-6
MALME-3M	97	106	96	-16	-66	2.57E-6	7.17E-6	4.75E-5
M14	97	110	94	-50	-93	2.03E-6	4.52E-6	1.02E-5
MDA-MB-435	94	91	89	-22	-92	2.25E-6	6.37E-6	2.53E-5
SK-MEL-2	101	112	110	19	-96	4.53E-6	1.46E-5	3.99E-5
SK-MEL-28	118	125	121	-35	-100	2.85E-6	5.94E-6	1.68E-5
SK-MEL-5	97	81	90	-53	-98	1.90E-6	4.27E-6	9.58E-6
UACC-257	106	111	105	43	-92	7.59E-6	2.07E-5	4.88E-5
UACC-62	101	105	104	-53	-91	2.20E-6	4.58E-6	9.55E-6
Ovarian Cancer								
IGROV1	111	81	111	-69	-96	2.18E-6	4.12E-6	7.80E-6
OVCAR-3	115	112	104	-100	-100	1.84E-6	3.24E-6	5.69E-6
OVCAR-4	99	98	92	6	-97	3.08E-6	1.15E-5	3.51E-5
OVCAR-5	108	102	104	36	-84	6.20E-6	1.99E-5	5.22E-5
OVCAR-8	106	105	96	18	-83	3.89E-6	1.51E-5	4.72E-5
NCI/ADR-RES	104	109	96	-5	-25	2.85E-6	8.87E-6	> 1.00E-4
SK-OV-3	103	106	102	79	-99	1.45E-5	2.78E-5	5.32E-5
Renal Cancer								
786-0	95	98	88	-75	-100	1.70E-6	3.45E-6	6.99E-6
A498	111	107	103	-39	-100	2.35E-6	5.30E-6	1.52E-5
ACHN	104	98	92	-23	-100	2.30E-6	6.26E-6	2.22E-5
CAKI-1	97	97	101	16	-94	3.97E-6	1.39E-5	3.97E-5
RXF 393	106	104	78	-82	-79	1.50E-6	3.08E-6	6.34E-6
SN12C	111	108	101	-49	-94	2.18E-6	4.71E-6	1.06E-5
TK-10	74	93	78	-24	-97	1.87E-6	5.78E-6	2.25E-5
UO-31	96	94	68	-67	-95	1.36E-6	3.20E-6	7.52E-6
Prostate Cancer								
DU-145	104	105	106	-100	-100	1.87E-6	3.27E-6	5.72E-6
Breast Cancer								
MCF7	100	91	85	-25	-46	2.07E-6	5.88E-6	> 1.00E-4
MDA-MB-231/ATCC	107	106	96	-60	-99	1.97E-6	4.13E-6	8.66E-6
HS 578T	111	114	103	-21	-48	2.67E-6	6.78E-6	> 1.00E-4
BT-549	102	90	90	35	-93	5.27E-6	1.87E-5	4.59E-5
T-47D	104	103	99	-17	-25	2.65E-6	7.13E-6	> 1.00E-4
MDA-MB-468	90	87	61	-96	-93	1.18E-6	2.46E-6	5.12E-6

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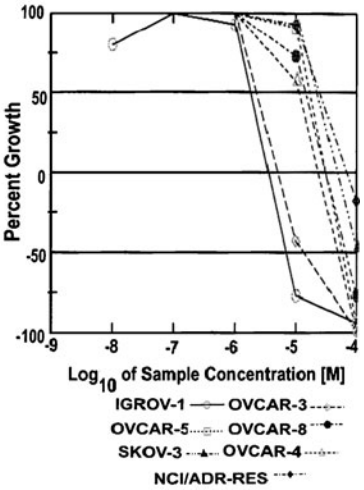

Fig. 2 Effect of RKS262 [NSC-D-746733-1] on cell growth in a NCI60 cancer screen. **A** Cells were treated in 96-well plates with RKS262 (10 nM–10 μM) or vehicle and cell growth of the TCA fixed treated and untreated cells was assessed after 48 h with sulphorhodamine-B (SRB) solution and absorbance read at 515 nM. (For details see Supplementary Material, Online Resource 1). **B** Dose-dependent effect of RKS262 on ovarian cancer cell lines in the NCI60 screen

using an ovarian cancer cell line as a model system. In RKS262 [Fig. 1A; compound ***5***] the tetrahydrothiazane ring was retained and linked to a 6-bromo, 4-chloro coumarin ring which replaced the nitrofuran ring present in Nifurtimox [compound ***1***]. Another Nifurtimox analog [compound ***2***] was synthesized by inserting a phenyl ring spacer between the nitro-functionality and the furan ring while the tetrahydrothiazane ring of Nifurtimox and the imine spacer were maintained. RKS262, now possessing the tetrahydrothiazane ring and a coumarin/benzopyranone scaffold with enhanced lipophilicity (bromide-functionality) revealed potent cytotoxicity in a viability assay and was chosen as the 'lead' structure for our studies. Viability experiments with OVCAR-3 cells settled some intriguing structure-activity relationship questions: (i) RKS262, multiple-fold more cytotoxic than Nifurtimox or analog [compound ***2***] (inactive at concentrations ≤ 20 μM), is structurally devoid of a nitro group which appears to not be essential for cytotoxicity; (ii) the phenyl spacer present only in inactive compound ***2*** appears to be detrimental to cytotoxicity; (iii) the tetrahydrothiazane ring present in Nifurtimox and RKS262 likely contributes to cytotoxicity; (iv) the imine spacer common to Nifurtimox, inactive compound ***2*** and RKS262 is not important for the activity

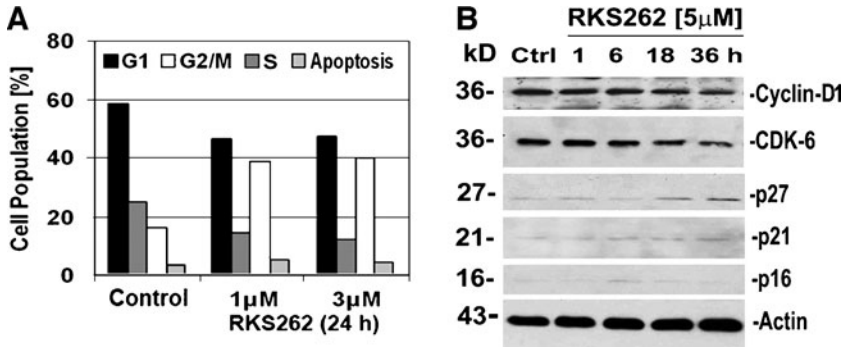

Fig. 3 Effect of RKS262 on cell-cycle progression and regulators in OVCAR-3 cells. **A** OVCAR-3 cells were treated with RKS262 (1or 3 μM) or vehicle for 24 h. Cell-cycle analysis of treated and untreated cells by FACS was carried out as described (Materials and Methods). Data are presented as the relative fluorescence intensity of cell subpopulations in a bar chart. **B** Expression of Cyclin-D1, CDK-6, p16, p21 and p27 in RKS262 and vehicle-treated OVCAR-3 cells was analyzed by Western blotting (Materials and Methods) of cell lysates using appropriate primary and secondary antibodies. As an internal standard for equal loading the blots were probed with an anti-β-actin antibody. Experiments were performed independently in duplicates showing identical outcomes

of these compounds. In summary, we postulate that the superior activity of RKS262 is based on an additive cytotoxic effect of the synthetic lipophilic coumarin scaffold in conjunction with the 1-aminotetrahydrothiazane ring originally derived from Nifurtimox.

Screening the effects of RKS262 in a NCI_{60} cell-line growth/viability assay [17] revealed remarkable responses in 48 of 60 cell lines. This panel represents cell lines derived from tumors of 12 different tissues types for which current chemotherapy regimens are inadequate to provide

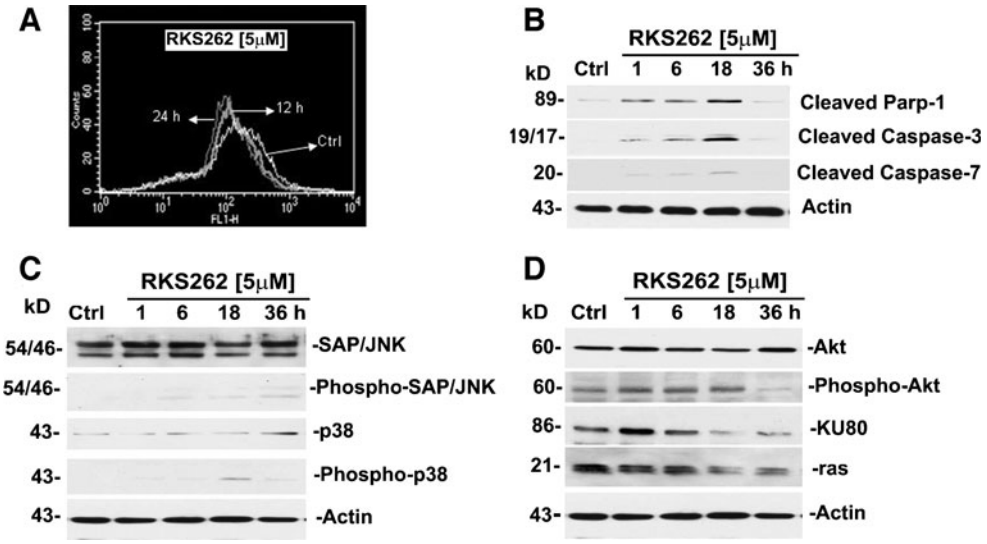

Fig. 4 RKS262 causes apoptosis in OVCAR-3 platinum-resistant ovarian cancer cells. **A** Mitochondrial membrane depolarization analysis: OVCAR-3 cells were treated for 12 or 24 h with vehicle or 5 μM RKS262 and stained with DiOC18(3) as described (Materials and Methods). Fluorescence of the single-cell population was measured by flow cytometry and the transmembrane depolarization potential of the single-cell populations plotted. Ten thousand cells were analyzed in each sample. **B** Caspase activation following RKS262 treatment: OVCAR-3 cells were treated with vehicle or 5 μM RKS262 for 1, 6, 18 or 36 h. Analysis of the expression of proteins in the lysates of treated and untreated cells was carried out by Western blot analysis as described (Material and Methods). Primary antibodies against activated caspases-3, -7 and inactivated/cleaved PARP-1 were used. **C** Expression of pro-apoptotic MAPK in OVCAR-3: Cells were treated with vehicle or 5 μM RKS262 for 1, 6, 18 or 36 h. Analysis of the expression of proteins in the lysates of treated and untreated cells by Western blot analysis was carried out as described (Material and Methods) using primary antibodies against pro- and activated/phosphorylated (P-) SAP/JNK and p38. **D** Inactivation of survival signaling proteins in OVCAR-3: Cells were treated with vehicle or 5 μM of RKS262 for 1 6, 18 or 36 h. Analysis of the expression of proteins in the lysates of treated and untreated cells by PAGE/western blot analysis was carried out using primary antibodies against ras, KU80, pro- and activated/phosphorylated Akt proteins

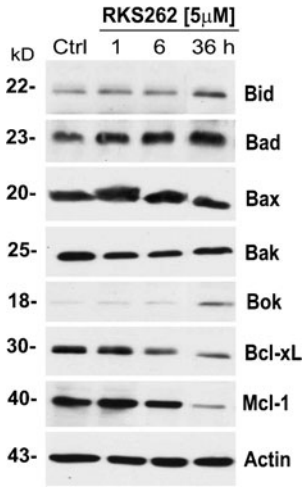

Fig. 5 Effect of RKS262 treatment on the expression profile of extended Bcl2 class proteins in OVCAR-3 cells. Expression of pro-apoptotic Bax, Bid, Bad, Bax, Bak and Boc and anti-apoptotic Bcl-xl and Mcl-1 in RKS262 (5 μM; for 1, 6, 18 and 36 h) and vehicle-treated OVCAR-3 cells were analyzed by Western blotting of lysates (Materials and Methods). As an internal standard for equal loading the blots were probed with an anti-β-actin antibody. Experiments were performed independently in duplicates showing identical outcomes

cure or extended disease free survival periods. While most of the cell lines responded to a standard single dose of 10 μM RKS262, we are intrigued by the differential activity with exceptional sensitivity of leukemia cells types such as HL-60 TB and SR (GI_{50}<10 nM) but minimal activity against several non-small cell lung cancer cells (NCI-H322H, NCI-226, HOP-62, EKVX and A549). A comparison analysis (COMPARE program, http://www.dtp.nci.nih.gov) of known cytotoxic drugs indicated that RKS262 is more potent than a variety of clinically used drugs, such as cisplatin, oxaloplatin, seliciclib, 5-FU and cyclophosphamide in most of the NCI_{60} cell lines including various ovarian cancer cells and compares well with paclitaxel and doxorubicin [18]. The high degree of cytotoxic action by RKS262 could be explained by its unique/native stereo-electronic properties. It combines lipophilic properties (e.g. modified coumarin scaffold) with an aminotetrahydrothiazane moiety of primarily hydrophilic character which likely endows this drug with enhanced solubility, improved uptake and cellular retention [19].

This present study reveals that RKS262, in addition to its selective cytotoxic properties, acts as a potent inhibitor of OVCAR-3 ovarian cancer cell proliferation at sub-cytotoxic concentrations. RKS262 treatment inhibited cell-cycle progression through G2/M phase and this arrest was correlated with the cumulative effects of up-regulation of p27 and down-regulation of CDK-6 and cyclin-D1. In contrast, the expression of p21 and p16 proteins which are primarily linked to G1-phase regulation [20] remained unaffected by RKS262 treatment. It is noteworthy that tumor suppressor gene p27 has been suggested as a target for cancer therapeutics [9]. Similarly, cyclin-D1 and CDKs, specifically CDK-6 and CDK-7, are key regulators during G2/M cell-cycle progression in cancer cells [21, 22] and targeting such checkpoints has been suggested as an alternative approach to anti-cancer therapies [23, 24]. Regulators of the cell-cycle machinery are frequently altered in human cancer and apparently transformed cells can be more sensitive to the cyclin-dependent kinases (CDK) inhibition [25, 26]. Based on the specific effect of RKS262 on G2/M phase, p27-, cyclin-D1- and CDK-6 expression in OVCAR-3 cells we suggest future studies on subcytotoxic concentrations of RKS262 and related compounds to include a full examination of the expression profile of cell-cycle regulators in synchronized ovarian cancer cell lines as well as in *in vivo* models.

Recent approaches to understand the mechanism of anti-cancer drugs in general, and in ovarian cancer cells in particular, have focused regularly on the involvement of MAP-Kinases [27, 28]. Based on immunoblot experiments, we assessed that contrary to many cytotoxic agents, RKS262-induced cytotoxicity is not significantly correlated with the activation of key pro-apoptotic MAPK P38 and SAP/JNK. These, as well as other MAPKs, mediate signaling pathways in cancer and control cells responding to inflammatory cytokines, UV light, diverse pro-apoptotic stimuli as well as cytotoxic drugs [14, 29–31] such as with cisplatin in ovarian cancer cells [27]. To confirm that RKS262 effects do not include major changes in the expression, activation and function of such MAPK, future studies could include detailed kinetic and dose-dependent cellular responses to RKS262 during co-treatment with MAPK inhibitors or growth factors (e.g. EGF) which can modulate drug induced MAPK activities in ovarian cancer cells [32, 33].

In addition to a variety of undefined factors, proto-oncogenes, repair enzymes and signaling factors such as ras, DNA-pk, and Akt are over-expressed in ovarian and other cancers. These factors play multiple roles in resistance to therapies and inactivation of a broad panel of critical pro-apoptotic events [34–36].

Constitutive expression of ras enhances ovarian cancer cell migration via the ras-MEK-1 kinase pathway [35] and may be a target for cancer treatment. Down-regulation of ras by RKS262 in OVCAR-3 cells indicates that this compound possesses the capability to interfere with tumor progression. Similarly, it has been suggested that agents that can down-regulate peptide components Ku-70, Ku-80 or p350 of the DNA-pk holozyme may have potential in the treatment of ovarian or other cancers [36]. DNA-pk essential for DNA repair is composed of a large catalytic subunit (M_r 460,000) and heterodimeric autoantigen Ku 70 and Ku 80, is a key

enzyme in determining the response to DNA-damaging agents [37, 38]. RKS262 treatment time dependently down-regulated KU-80 indicating that RKS262 has a significant effect on DNA-pk holozyme constitution and function. In addition, RKS262 treatment of OVCAR-3 cells resulted in a time-dependent down-regulation of Akt phosphorylation. Akt (a serine-threonine kinase) is emerging as a major therapeutic oncogenic target in several cancers including ovarian cancer where elevated signaling by the Akt pathway has been associated with poor prognosis and a more aggressive phenotype [36]. Therefore, RKS262 and other agents that directly target this pathway and its downstream molecules might be developed to mitigate the oncogenic effects of Akt.

A major determinant of cancer cell-survival is the expression of Bcl2 family members with wide ranging effects in ovarian and other cancer cells both *in vitro* and *in vivo*. It is known that the effective treatment of ovarian cancer is significantly hampered by drug resistance, which is directly correlated to the expression and function of Bcl-2 regulators [39].

We show here that RKS262 down-regulates the expression of pro-survival Bcl-xl and Mcl-1 proteins in platinum-resistant OVCAR-3 ovarian cancer cells within 6 h of treatment. These pro-survival proteins are crucial components of gynecological cancer cells where Bcl-xL over-expression is associated with a shorter disease-free interval in breast cancer patients [40] or over-expression of Mcl-1 correlates with an advanced clinical stage and poor survival of ovarian cancer patients [41]. RKS262 also up-regulates the expression of several pro-apoptotic Bcl2 class proteins such as Bid, Bad and Bok in OVCAR-3 cells. Expression of Bid is an adverse prognostic factor for radiotherapy outcome in carcinoma [42]. This protein plays a central role in apoptosis by connecting the intrinsic and extrinsic pathway, converging signals in mitochondria and acting agonistically in concert with other pro-apoptotic Bcl2 proteins [43]. The significance of the RKS262 effect on Bid, however, remains to be further investigated because the expression of Bax and Bak remained unaltered by RKS262 even though the pro-apoptotic activities of these two proteins are generally coupled to expression of Bid [44].

RKS262 also induces the expression of Bad in OVCAR-3 cells. Bad has been described as a crucial factor in chemotherapy-induced apoptosis of epithelial ovarian cancer cells and apparently Bad is capable of partly reversing paclitaxel chemoresistance in ovarian cancer through inhibition of Bcl-xL [45]. Bad like Bid possesses a BH3 domain which allows binding to other Bcl2 family members, thus triggering mitochondrial events associated with apoptosis. It can translocate between the cytosol and membrane-bound BCL-xL or Bcl2. Similarly Bok can induce apoptosis by promoting the release of pro-apoptotic mitochondrial factors to the cell cytosol. Bok expression is down-regulated through RKS262 and its activity has been shown to be inhibited by Mcl-1 but not Bcl-xl [46] (both of which are down-regulated by RKS262). These observations suggest a multilayer effect of RKS262 in the induction of Bcl2 protein class mediated apoptosis. While numerous antagonists of pro-survival Bcl2 class proteins are under investigation in clinical trials [47], similarly, the selective effect of drugs such as RKS262 on specific pro-apoptotic and pro-survival Bcl2 proteins deserves further investigation.

In conclusion, novel compound RKS262 displays characteristics of a therapeutic anti-cancer drug. In platinum-resistant OVCAR-3 ovarian cancer cells RKS262 regulates multiple aspects of cell viability such as cell-cycle regulation, mitochondrial integrity, induction of apoptosis and various oncogenic signaling events. The present report suggests that RKS262 is a potent growth-suppressing agent *in vitro* for cell lines derived from ovarian cancer. Future studies on RKS262 will include animal models to determine the compounds toxicity and potential to treat such tumors *in vivo*.

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