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Sorafenib (Nexavar®) Treatment Enhances Trail-Mediated Apoptosis in Human Pancreatic Cancer Cells

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Background. TRAIL (TNF-related apoptosis-inducing ligand) is a novel anti-cancer drug that activates the death receptor (DR)-mediated apoptotic pathway. However, most human cancer cells require a mitochondrial amplification step after DR activation that is inhibited by anti-apoptotic Bcl-2 proteins, including Mcl-1. Mcl-1 sequesters the pro-apoptotic BH3-only protein Bim and the multidomain Bak protein to disable them. Given that sorafenib, a multi-target kinase inhibitor, has been shown to downregulate Mcl-1, we determined whether sorafenib can potentiate TRAIL-mediated apoptosis. Materials and Methods. Human pancreatic cancer cell lines (PANC-1 and BxPC-3) were pre-incubated with sorafenib (Nexavar®, Bayer Pharma) followed by TRAIL and DNA fragmentation were measured (Cell Death Detection ELISA kit). Caspase activation and poly(ADP-ribose) polymerase (PARP) cleavage were analyzed by Western blotting. In sorafenib-treated cells, immunoprecipitation of Bak, Bim, or Mcl-1 proteins was performed and Bcl-xL, Mcl-1, or Bak was probed by immunoblotting. Lentiviral shRNA strategies were utilized to knockdown both Mcl-1 and Bim and cell viability was determined using the MTS assay. Results: TRAIL (0-10 ng/ml) or sorafenib (8, 16 µM) treatment was shown to induce apoptosis, as shown by DNA fragmentation in PANC-1 and BxPC-3 cells. Furthermore, pre-treatment with sorafenib (8, 16 µM) significantly potentiated TRAIL-mediated apoptosis that was accompanied by caspase-8,-9,-3 activation and Bid and PARP cleavage in both cells. Sorafenib (4, 8,16 µM) was shown to downregulate Mcl-1 protein levels with a resultant release of Bim and Bak from their sequestration by Mcl-1. Sorafenib also induced a conformational change in Bak, but not Bax. Furthermore, sorafenib enhanced TRAIL-mediated Bak conformational change. Mcl-1 shRNA was shown to markedly sensitize tumor cells to TRAIL-induced caspase-3 cleavage and cytotoxicity, whereas Bim knockdown attenuated TRAIL-mediated cytotoxicity, Conclusion. Sorafenib can potentiate TRAIL-mediated apoptosis by downregulating Mcl-1 expression that enables the release of sequestered Bim and Bak. Therefore, sorafenib represents a novel strategy to enhance the therapeutic efficacy of TRAIL against pancreatic cancer.

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Identification of Novel Small Molecule Compounds That Inhibit the Pro-Proliferative KrüPpel-Like Factor 5 By High-Throughput Screening

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BACKGROUND and AIM: Krüppel-like factor 5 (KLF5) is a highly biologically active molecule crucial for regulating cell proliferation. Our lab previously showed that KLF5 is a critical mediator of activated KRAS, frequently found in colorectal cancer. Inhibition of *KLF5* expression by either pharmacological or genetic means in intestinal epithelial cells and colorectal cancer cells containing activated KRAS leads to reduced rates of proliferation and anchorage-dependent growth. Thus, the identification of novel small molecule compounds that potently inhibit *KLF5* expression may aid in the development of therapeutic agents in treating colorectal cancer containing activated RAS. **METHODS**: We established a cell line, DLD-1/pGL4.18hKLF5p, that was stably transfected with a luciferase reporter under the control of 2 kb of the human *KLF5* promoter. The cells were used to screen the 1,280 compounds in LOPAC (Library of Pharmacologically Active Compounds; Sigma), for their ability to inhibit luciferase activity, using an automated process developed by the Emory Chemical Biology Discovery Center, one of the Molecular Libraries Screening Centers Network (MLSCN) funded by the NIH. DLD-1/pGL4.18hKLF5p cells were seeded at $3x10^4$ cells/well in 100 µl RPMI-1640 in 96-well plates and treated with individual compounds in LOPAC at a concentration of 10 µM in 1% DMSO. After 8 hr, luciferase activity was determined by Steady-Glo Luciferase Assay. Negative control contained DMSO alone and positive controls included PMA and LY29004, known to activate and repress *KLF5* expression, respectively. Data obtained from the screen were validated by two well-established parameters: signal-to-background ratio and Z' factor. To identify positive hits, we used the traditional criteria for hit threshold selection of μ-3σ, where μ is the mean value and σ is the standard deviation of the entire assay. The activity of the identified inhibitors was validated by luciferase assay, cell proliferation assay, Western and Northern analyses in established colorectal cancer cells. **RESULTS**: We identified 8 compounds that significantly inhibited *KLF5* promoter activity. Three were characterized further: Wortmannin, Tyrphostin 9 and AG879. All three inhibited *KLF5* expression at the levels of the promoter, mRNA and protein, in a time- and dose-dependent manner. Importantly, each inhibited proliferation of DLD-1, HCT116 and HT29 colorectal cancer cells, which contained high levels of endogenous KLF5. **CONCLUSION**: Our data demonstrate that high-throughput screening has the potential for identifying novel small molecule compounds that inhibit *KLF5* promoter activity, some of which may have therapeutic implication.

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Effect of a Novel Telomerase Inhibitor, Grn163l, On Growth of Barrett's Esophageal Adenocarcinoma Cells *In Vitro* and *In Vivo*

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INTRODUCTION: Telomerase activity, although detected in germ-line and primitive hematopoietic cells, is either completely absent or very low in other somatic tissues. Telomerase is re-activated in most cancers and provides replicative advantage by preventing telomere shortening. The aim of this study was to evaluate the impact of telomerase inhibition in Barrett's Esophageal Adenocarcinoma (BEAC). METHODS: Impact of telomerase inhibition was demonstrated by treating BEAC cell lines (SEG-1, BIC-1, FLO-1) with a specific inhibitor of telomerase GRN163L, a lipid - conjugated oligonucleotide targeting the template region of RNA component of telomerase. The drug was used alone or in combination with other agents which may affect telomere length by other mechanisms. The cell lines were treated for various durations and evaluated for telomere length, telomerase activity, cell viability, and several markers of cell proliferation. Telomerase activity was assessed by an improved version of the original (Telomeric Repeat Amplification Protocol) assay whereas telomere length evaluated by quantitative real time polymerase chain reaction. RESULTS: GRN163L was efficiently taken by nuclei of BEAC cells without any need of a transfection procedure or reagent. Treatment with GRN163L led to loss of telomerase activity, reduction in telomere length, and inhibition of BEAC cell growth through induction of both the senescence and apoptosis. The inhibition of cell growth was associated with loss of several proliferation associated proteins including c-myc, whose elevated expression is associated with progression of Barrett's to adenocarcinoma and loss of expression is implicated in senescence. GRN163L induced cell death could also be significantly expedited by addition of chemotherapeutic agents, doxorubicin and ritonavir, to BEAC cultures pre-treated with GRN163L. Efficacy of GRN163L was also demonstrated in a murine model of human Barrett's esophageal adenocarcinoma. The SCID-mice were subcutaneously inoculated in the interscapular area with 2.5 to 10^6 SEG-1 cells and following appearance of palpable tumors, mice were injected intraperitoneally with PBS alone or GRN163L. A significant reduction in tumor size following treatment with daily intraperitoneal injections of 45 mg/kg GRN163L was observed, demonstrating the impact of telomerase inhibition *In Vivo*. CONCLUSIONS: These results demonstrate that suppression of telomerase by a specific inhibitor GRN163L inhibits BEAC cell growth both *In Vitro* and *In Vivo* and suggest that telomerase is an important therapeutic target for BEAC therapy and should be evaluated in human clinical trials.

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Sulforaphane, An Antioxidant Derived from Broccoli, Induces Apoptotic Cell Death in Barrett's Esophageal Adenocarcinoma Cells

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INTRODUCTION: Sulforaphane, an anti-oxidant derived from Broccoli, has been shown to possess chemopreventive and chemotherapeutic actions. In this study, we evaluated the efficacy of this agent in Barrett's esophageal adenocarcinoma. METHODS: P53-positive and p53-negative BEAC cell lines (SEG-1 and BIC-1) and normal diploid fibroblasts (GM07675) were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and maintained in a state of logarithmic growth. Constant number of cells were plated in 100 mm dishes and treated with sulforaphane 1.0, 3.0, 5.0, and 7.0 µM concentrations and live cell number was determined by trypan blue exclusion and MTT assays, on alternate days. Apoptotic BEAC cells were detected using the Annexin V-Biotin Apoptosis Detection Kit (Oncogene Research Products, San Diego, CA). Impact of sulforaphane on the expression of various proliferation and apoptosis related proteins was determined by western blotting. RESULTS: Sulforaphane induced both time- and dose-dependent decline in survival of BEAC cells; at 3 µM concentration it induced 85% and over 60% cell death in SEG-1 and BIC-1 cells respectively at day 3 and >90% cell death in both the cell lines at day 7. In both the p53-positive (SEG-1) and p53-negative (BIC-1) BEAC cell lines tested, exposure to 3 µM EGCG led to a significant inhibition of cell proliferation as assessed by MTT assay, within 72 hrs treatment. Importantly, the same or even higher concentrations of sulforaphane (1, 3, or 5 µM) had no effect on survival of normal diploid fibroblasts. The mechanism of cell death following treatment of BEAC cells with sulforaphane seemed to be apoptotic. Following three day exposure to sulforaphane, 80±8% SEG-1 cells and 70±6% of BIC-1 cells were annexin V positive, whereas only 7±3% and 32% of untreated SEG-1 and BIC-1 cells respectively were annexin V positive, indicating that the antioxidant induces apoptosis in BEAC cells. Western blot analysis indicated that the treatment of BEAC cells with sulforaphane was associated with a significant reduction in the expression of hsp90, a molecular chaperon required for activity of telomerase and several other proliferation associated proteins. CONCLUSIONS: Sulforaphane, an antioxidant derived from Broccoli, induces apoptotic death in Barrett's esophageal adenocarcinoma cells at concentrations which do not affect proliferation of normal diploid fibroblasts. The inhibition of BEAC cell growth by this agent involves inhibition of an important molecular chaperon hsp90, required for activity of telomerase and several other proliferation associated proteins.

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Resveratrol Analogues with Potent Anticarcinogenic Properties

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Background: Recently we could show that the naturally occurring polyphenol resveratrol (Res) mediates chemopreventive effects by triggering the synthesis of endogenous ceramide [Biochem Pharmacol 2007]. Therapeutical relevance of resveratrol is often challenged due to unphysiological high concentrations used in *In Vitro* experiments. Thus, the aim of this project was to design and synthesize analogues which are more effective in effectuating anticarcinogenic effects in a colorectal cancer cell model. Methods: Res analogues have been synthesized by classical condensation reactions. Structures and purities have been confirmed by 1H-NMR, MS, IR, and elemental analysis. Caco-2 cells were treated with increasing concentrations of different resveratrol analogues (100 nM to 200 µM) for 24-72 h. Cytotoxicity was excluded using a commercially available kit. Cell growth was determined by crystal violet staining. Intracellular ceramide concentrations were determined by HPLC-MS. Results: Except for ST915 (IC50 ~50 µM) which showed similar effects to that of Res (IC50 ~46 µM) and ST916 which was inactive in Caco-2 cells, the other analogues were more effective in reducing cell growth after 48 h of incubation (IC50 values ~250 nM (ST911, p<0.001); ~5.7 µM (ST912, p<0.001); ~4.5 µM (ST913, p<0.001), ~37.3 µM (ST914, p<0.01)). The Z-isomers (cis) generally showed higher efficiencies than the related E-configured (trans) isomers. Furthermore, incubation with ST911 (10-50 µM, *p<0.05 vs. Res) or ST912 (10-50 µM, *p<0.05 vs. Res) featured significantly higher efficiencies in elevating intracellular ceramide levels after 24h. Conclusions: These preliminary data impressively show that targeting ceramide signaling by new resveratrol analogues potentially provide a novel promising strategy to control cancer cell growth.