Prevention of colorectal carcinogenesis by DNA binding small molecule curaxin CBL0137 involves suppression of Wnt signaling

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ABSTRACT
Chemoprevention is considered a valid approach to reduce the incidence of colorectal cancer (CRC), one of the most common malignancies worldwide. Here, we investigated the tumor preventive activity of curaxin CBL0137. This compound represents a new class of non-mutagenic DNA-binding small molecules that alter chromatin stability and inhibit the function of the histone chaperone FACT. Among downstream effects of CBL0137 treatment are activation of p53 and type I interferons and inhibition of NF-κB, HSF1 and MYC. In addition, our data show that in both human and mouse CRC cells in vitro CBL0137 inhibits the APC/WNT/β-catenin signaling pathway, which plays a key role in colon carcinogenesis. Using quantitative RT-PCR and microarray hybridization, we have demonstrated decreased expression of multiple components and downstream targets of the WNT pathway in colon cancer cells treated with CBL0137. At the same time, CBL0137 induced expression of WNT antagonists. Inhibition of WNT-signaling activity by CBL0137 was also confirmed by luciferase reporter assay. Tumor preventive activity of CBL0137 in vivo was tested in a murine model of colorectal carcinogenesis induced by 1,2-dimethylhydrazine (DMH), which is known to involve WNT pathway dysregulation. After DMH subcutaneous treatment, mice were administered CBL0137 in drinking water. Efficacy of CBL0137 in suppressing CRC development in this model was evidenced by reduced incidence of adenocarcinomas and adenomas in both males and females, decrease in tumor multiplicity. These data support the prospective use of CBL0137 in chemoprevention of CRC as well as of other malignances associated with activated WNT signaling.

INTRODUCTION
Colorectal cancer (CRC) is one of the most common types of malignancies, representing the third most frequently diagnosed cancer (1). While CRC treatment options and outcomes have improved, recent advances have been more modest than anticipated. High cost of CRC treatment is often not justified by the amount of benefit it provides, at the same time, it is out of reach for many patients (2). Globally, there remain 1.2 million cases of CRC diagnosed each year and CRC accounts for more than 600,000 deaths annually (8% of all cancer deaths). The global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030 (1).

CRC presents an example of the combined impacts of genetics, epigenetics and environment/lifestyle. Significant part of individuals diagnosed with CRC has a family history of cancer (3). Among genetic syndromes predisposed to CRC are familial adenomatous polyposis (FAP) caused by mutations in the APC gene and hereditary non-polyposis colorectal cancer (HNPPC or Lynch syndrome) caused by mutations in MMR genes encoding DNA mismatch repair proteins (4). CRC frequently develops on the background of inflammatory bowel disease, and it is the most common cancer among such patients (4). The mechanisms underlying development of sporadic CRC involve mutations in specific oncogenes (e.g., APC, KRAS) and tumor suppressor genes (e.g., P53) as well as epigenetic changes (5).

While much research and development effort has focused on CRC treatment, pharmacological strategies aimed at prevention of the disease are both appealing and gaining support. Chemopreventive activity against CRC has been reported for a number of drugs, including non-steroidal anti-inflammatory drugs (NSAIDs), hormone replacement therapy in postmenopausal women, statins, bisphosphonate, and angiotensin inhibitors (6). The most promising data on CRC chemoprevention were obtained for NSAIDs, which showed tumor preventive effects in many animal studies (7).

Epidemiological studies also indicate that anti-inflammatory drugs have a chemopreventive effect on colon carcinogenesis (8). These data are consistent with the well-established role of inflammatory factors in CRC development (9). Unfortunately, both aspirin and other NSAIDs are associated with significant adverse effects, including increased risk of upper gastrointestinal and of cardiovascular toxicity, haemorrhagic stroke and internal bleeding (10). Thus, while chemoprevention is a promising approach to reduce the incidence of CRC, new cancer preventive drugs that are both safe and effective are needed.

Recent strategy for CRC chemopreventive drug development is based on improved understanding of the molecular events involved in colon carcinogenesis (11, 12). Aberrant activation of the
APC/WNT/β-catenin signaling pathway (referred to herein as the WNT pathway) plays a major role in both sporadic and hereditary CRC, and targeting of this pathway for CRC prevention was recently highlighted as a priority research gap (13, 14). Importantly, NSAIDs, in addition to their anti-inflammatory activity, were shown to suppress a number of COX-independent mechanisms and the WNT signaling pathway, in particular. Chemopreventive activity against CRC was also described for natural polyphenols (e.g., resveratrol, genistein and others), which was shown to inhibit WNT pathway in CRC cell lines and in chemically induced CRC in mice (15-17).

Paying attention to the fact that many natural polyphenols demonstrating CRC chemopreventive activity represent DNA-binding small molecules, we decided to study cancer preventive effects of a new anticancer drug, small molecule curaxin CBL0137. Recently it was shown that this compound binds DNA non-covalently through intercalation and minor groove binding, inhibits topoisomerases (18), influences epigenetic regulation (19) and DNA packaging (18). Chromatin alterations caused by CBL0137 lead to functional inhibition of the histone chaperone FACT and modulation of the activity of several transcription factors, including P53, NF-B and HSF-1 (20, 21). Moreover, our recent study revealed CBL0137 influence on type I interferon signaling (19). Recently using CRC cell line HCT116 we demonstrated CBL0137 inhibitory effect on COX2 transcription (22). As COX2 catalyzes biosynthesis of prostaglandin E2, which is a known modulator of the WNT pathway, we proposed that CBL0137 might influence WNT signaling pathway (23). Thus, the main goals of the present study were to analyze the effect of CBL0137 on the WNT signaling pathway in CRC cell lines in vitro and to evaluate its tumor preventive effect in vivo in an animal model. We used DMH-induced colon carcinogenesis in mice (24, 25) as a well-established CRC model in which tumor development is known to be associated with activation of the WNT signaling pathway (26-28).

MATERIALS AND METHODS

Chemicals and reagents
DMH (1,2-dimethylhydrazine 2-HCl) was purchased from Sigma-Aldrich Co. LLC, USA; CBL0137 and hydroxy-propylmethylcellulose were provided by Incuron, Inc., Russia. The following reagents were used: chloroform (Vekton, JSC, Russian Federation), histomix (BioVitrum LLC., RF), xylol (Merck & Co. Inc., USA), Haematoxylin (Ferak-Berlin GmbH, Germany), Eosin B (Sigma-Aldrich Co. LLC, USA) and Mayer’s Mucicarmine Stain Solution (Sigma-Aldrich) and dimethylsulfoxide (DMSO, Sigma-Aldrich Co. LLC, USA).

Cell lines
Cell lines HT29, HCT116, SW480, and Caco2 were obtained from ATCC. Cells were used no longer than 20 passages. Micoplasma cell culture contamination was routinely checked using DAPI-staining followed by fluorescent microscopy. Cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin (Paneco LTD, RF) in a humidified incubator at 37°C, 5% CO2. Mouse CRC cell line MC-38 (29) was obtained from the Istituto Scientifico San Raffaele, Milano.

Cell viability (MTT) assay
HT29, HCT116, SW480, and Caco2 cells were seeded in 96-well plates (4000 cells/well). After overnight incubation, CLB0137 was added to the medium to final concentrations of 0.075, 0.15, 0.3, 0.6 and 1.2 µM. After 72 hours 20 µl of freshly prepared 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Merck) (5 mg/ml) was added to wells and the plates were incubated for 4 hours. Then, medium was removed, 150 µL of DMSO was added to each well. Relative cell viability was determined respectively to the untreated cells (30).

Microarray analyses
HCT116 cells were treated with 1µM of CBL0137 for 5 or 16 hours. RNA was isolated from cells using TRIZOL reagent (Invitrogen) and processed for hybridization with GeneChip™ Human Genome U133A 2.0 Array (Applied Biosystems™, TermoFisher Scientific, USA) in Genomics Facility of Roswell Park Cancer Institute. Two biological replicates of each condition were used for microarray
hybridization. Raw data quantitation, background subtraction and quantile normalization was done according to manufacturer instruction. Comparison of gene expression between samples was done using GeneSpring GX software (Agilent).

**Quantitative RT-PCR**
HT29, HCT116, SW480, and Caco2 cells were seeded in 6-well plates (250000 cells/well) and after overnight incubation CLB0137 was added to final concentrations of 0.25µM, 0.5 µM, or 0.75 µM. After 48 hours of incubation, total RNA was extracted using TRI reagent (Sigma-Aldrich) and purified using RNase-free DNase (Promega). Quantitative RT-PCR was performed using the Bio-Rad CFX Real-Time PCR System. Each sample was tested in triplicate, and results were normalized to the expression of RPL27. PCR primers were designed using BLAST (Supplemental Table S1).

**Lentiviral TCF/LEF luciferase reporter construct and luciferase assay**
Lentiviral stocks were generated containing the construct Cignal Lenti TCF/LEF Reporter (Qiagen, USA). HCT116 cells were infected with the lentivirus following the manufacturer's protocol. The infected cells were then treated with CBL0137. Luciferase activity was measured using a commercially available Luciferase Assay (Promega, USA) and Luminometer TD 20/20 (TDI, USA) (30).

**Animals**
Tumor preventive effects of CBL0137 were studied in CBA mice obtained from the Stolbovaya Farm of Federal Medical and Biological Agency (http://www.scbmt.ru). Two-months old 20-22 g mice were used. The protocols for the experiments were approved by the Animal Care and Use Committee of the N.N. Blokhin NRMCO and corresponded to the guidelines for the welfare and use of animals in cancer research adopted by The United Kingdom Coordinating Committee on Cancer Prevention Research (31). All animals were housed 7-8 mice per cage with free access to drinking water and a pelleted basal diet.

**Testing CBL0137 against DMH-induced CRC carcinogenesis in mice**
Chemically induced colon carcinogenesis was reproduced on CBA mice by s.c. injections of 1,2-dimethylhydrazine according to the protocol described in the IARC monograph 71 (24, 32, 33). CBL0137 was administering in drinking water as 0.13 mg/ml solution that corresponds to the maximal tolerated dose 20 mg/kg/day that was detected and calculating according to the previously described protocol (34). 150 male and 153 female CBA mice were randomized into the groups of 35-44 animals as follows (see Figure 1): Groups 1.1 and 1.2 – once weekly s.c. injection of DMH (dissolved in sterile water) at a dose of 8 mg/kg for 15 weeks (males) or 20 weeks (females) (32, 33), followed by treatment with CBL0137 in drinking water at a dose of 20 mg/kg/day during the weeks 18-40 (males) or 23-40 (females); Groups 2.1/2.2 – DMH treatment only (as described for Group 1.1/1.2); Groups 3.1/3.2 – CBL0137 treatment only (as described for Group 1.1/1.2); Groups 4.1/4.2 – no treatment. Animals were euthanized after the 40th week. All organs were examined for gross pathology and were also collected for histological examination.

**Histological analysis**
Gross lesions and normal-appearing tissues were fixed in 10% buffered formalin for 3 days. The samples were then dehydrated with alcohol followed by chloroform and embedded in Histomix. After deparaffinization by m-Xylol and rehydration sections were stained with hematoxylin-eosin (H&E) according to the standard procedure (35). Mayer’s Mucicarmine Staining (according to the protocol of Sigma-Aldrich Co. LLC., USA) was used to reveal mucin-depleted foci (MDF).

**Statistical analysis**
Data processing was carried out using Statistics software (StatSoft Inc., 2001, version 6.0) and Origin 8.0 (OriginLab Corporation, USA). The statistical significance of the differences between two samples in RT-PCR analysis and luciferase reporter assay were evaluated by paired samples Student’s t-test. The statistical significance of the difference between animal groups was calculated with Pearson’s chi-squared test ($\chi^2$).

**RESULTS**

**CBL0137 shows cytotoxicity to CRC cell lines**

As an initial step towards investigation of the potential utility of CBL0137 as a CRC preventive agent, we tested the effect of the drug on viability of four human CRC cell lines (HCT116, HT29, SW480, Caco2) and murine adenocarcinoma cell line MC-38 growing in culture. HCT116 harbor three base deletion in the *CTNNB1* (β-catenin) gene and in the rest three lines mutations of *APC* gene were described (36, 37). Cells were treated for 72 hours with doses of CBL0137 ranging from 0.075 µM to 1.2 µM. Dose-dependent toxicity of CBL0137 was observed for all tested CRC cell lines, with IC50 values of 0.70 µM, 0.67 µM, 0.63 µM and 0.86 µM in HT29, HCT116, SW480, and Caco2 cells, respectively (Supplemental Figure S1).

**CBL0137 modulates expression of WNT-signaling pathway components, target genes, and regulators in CRC cell lines**

Since CRC is known to be associated with over-activity of the WNT-signaling pathway, we next tested whether the observed influence of CBL0137 on human CRC cells might be associated with altered expression of genes involved in the WNT pathway. We compared RNA prepared from HCT116 cells left untreated or treated with 0.5 µM CBL0137 for either 5 or 16 hours using microarray-based global gene expression profiling. We revealed CBL0137-dependent reductions in the expression of WNT-signaling pathway receptors *FZD1*, *FZD2*, and *FZD5* and 18 target genes (Figure 2A, Supplemental Table S2). In contrast, CBL0137 activated expression of two known negative regulators of the WNT signaling pathway, *APC2* and *WiF1*, by up to 2.83- and 37-folds, respectively.

To extend our findings from the HCT116 microarray hybridization experiment, we used quantitative RT-PCR to analyze the effect of CBL0137 on expression of several target genes of the WNT signaling pathway in all four CRC cell lines (HT29, HCT116, SW480, Caco2). RNA was analyzed from cells treated with 0.25µM, 0.5 µM or 0.75 µM CBL0137 for 48 hours. Three tested β-catenin transcriptional targets, pro-proliferative *Cyclin D1*, pro-proliferative and anti-apoptotic *cMYC*, and anti-apoptotic *Survivin (BIRC5)* all showed significant CBL0137 dose-dependent inhibition of expression (Figure 2B). On the other hand, significant dose-dependent increases in expression were observed for three negative regulators of WNT signaling: *DKK3*, *SFRP1*, and *WIF-1* (Figure 2C).

It is well known that T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors are the major end point mediators of Wnt pathway throughout metazoans. To determine whether the observed CBL0137-dependent changes in gene expression indeed resulted in altered WNT/β-catenin signaling in treated cell lines, we tested the effect of the compound on expression of a TCF/LEF luciferase reporter construct stably integrated into the genome of HCT116 cells. As shown in Figure 2D, CBL0137 inhibited expression of the reporter in a dose-dependent manner (up to 3-fold with 0.5 µM CBL0137) after 24-hour treatment. CBL0137 dose-dependent inhibition of reporter expression was also observed when WNT signaling was activated by LiCl (up to 5-fold with 0.5 µM CBL0137), as LiCl enhances WNT pathway via GSK3β inhibition. These data were in accordance with the results obtained by quantitative PCR: the treatment of cells with CBL0137 (0.50 µM) decreased luciferase expression up to 5 folds.

Together, these results show that CBL0137 treatment of human CRC cell lines cause reduced expression of WNT pathway components and target genes yet increased expression of antagonists of the pathway, resulting in suppression of WNT/β-catenin signaling.

Before the analysis of CBL0137 preventive effect in vivo we checked whether CBL0137 influences Wnt pathway in mouse CRC cells in vitro using MC-38 adenocarcinoma cells, which was obtained by Corbett et al. using DMH-induced chemical carcinogenesis (35a). We revealed strong inhibiting effect
of CBL0137 on the expression of the main Wnt targets: cMyc, Ccnd1, Birc5, CD44, and Cox2 in MC-38 cells (Figure 3)

**Effect of CBL0137 on DMH-induced colon carcinogenesis in mice**

To determine whether the effects of CBL0137 on Wnt signaling both in human and mouse CRC cells observed in vitro would contribute to the tumor preventive effect in vivo, we used a well-established mouse model of colon carcinogenesis induced by DMH. Mice were given subcutaneous injections of DMH once weekly for 15 weeks (males) or 20 weeks (females) after which CBL0137 was provided via drinking water ad libitum. Four treatment groups were established both for males and females as shown in Figure 1. Group 1 received DMH and CBL0137, Group 2 received DMH alone, Group 3 received CBL0137 alone, and Group 4 did not receive either compound (untreated control group).

Over the course of the experiment general animal health, as determined by cage-side observations, was similar between all four study groups. As expected, a steady increase in mean animal body weight was observed in all groups during the experiment. However, consistent with the previously published results of Turusov et al (33), groups of animals injected with DMH gained weight more slowly than groups not given DMH. CBL0137 (20 mg/kg in drinking water) did not affect weight gain in either DMH-treated or DMH-untreated mice. For both males and females, there was no significant difference in mean body weight at the end of the experiment between groups that received DMH and CBL0137 versus DMH alone or between groups that received CBL0137 alone versus no treatment (Supplemental Table S3). Thus, CBL0137 did not in itself have any effect on animal body weight by itself and it did not alter the effect of DMH.

DMH-induced colon carcinogenesis was evaluated in all study animals through gross and histopathological observation following euthanasia at 40th week, with particular focus on the colon as described in the Methods. In collected colons, we analyzed the frequency of adenomas and adenocarcinomas, tumor multiplicity, and the frequency of mucin-depleted/mucin-producing tumors and mucin-depleted foci (MDF). The last ones are crypt foci with absent or scant mucous production. They are considered to be pre-neoplastic lesions, as mucus layer play an important role in the intestine homeostasis: it provides a physical separation between the epithelial cell monolayer and the luminal contents, a chemical and biochemical barrier that supports the structure of the mucus gel, as well as the ability of the gel to concentrate biological factors secreted by mucosal epithelial cells (38). Recently it was shown that Muc2 deficiency generates a chronic, low-level inflammatory response, and eventual tumor development. Representative images of stained histological colon sections are shown in Figure 4. There were no signs of colon carcinogenesis in any study animal that did not receive DMH. Thus, no colon adenomas or adenocarcinomas were detected in the untreated group or in the group that received only CBL0137. These animals also lacked MDF in normal-appearing tissue. These findings are presented in Supplements, Table S4.

Histologically, the morphology of the colon epithelium of males and females treated with CBL0137 showed no differences compared to corresponding samples from the untreated control group. Normal colon tissue is shown in the Figure 4A, B, E.

In contrast, adenomas and adenocarcinomas were observed in the colons of mice treated with DMH (Figure 4C, 4D). In addition, some DMH-treated mice had foci within normal-appearing epithelium and/or adenomas characterized by absent or scarce mucous production (Figure 4F).

Comparisons of overall tumor incidence (adenoma and adenocarcinoma) and tumor multiplicity (number of tumors per animal) clearly demonstrated a tumor-preventive effect of CBL0137 in this study (Figure 5). Among the 36 males treated with DMH, 28% of animals did not show any colon tumors, 42% developed a single tumor, and 30% had more than 2 tumors (Figure 5A, Table S4). In contrast, among 44 males treated with DMH and CBL0137, tumors did not appear in 77% of animals, 14% of mice had 1 tumor, and only 4 animals (9%) had more than 2 tumors. A similar tumor-preventive effect of CBL0137 was observed in females. In the group of 39 mice given DMH alone, 28% of mice did not develop any colon tumors, 33% developed a single tumor and 36% developed more than 2 tumors. Of 44 females treated with DMH and CBL0137, 69% of animals did not bear any tumors, 21% animals developed 1 tumor and only 11% had 2 or more tumors. Thus, CBL0137
administering after DMH injections both greatly increased the proportion of animals without tumors and decreased tumor multiplicity per animal. While adenomas were detected much more frequently than adenocarcinomas (Figure 5B, Table S4), incidence of both tumor types was reduced by CBL0137 administering, resulting in increased proportions of tumor-free animals. The difference in the proportion of animals with adenomas (irrespective of tumor multiplicity) between the group of DMH-treated mice and the group of mice treated with DMH and CBL0137 was highly statistically significant (P<0.01) for both males (54% and 23%, respectively) and females (64% and 25%, respectively) (Figure 5B). CBL0137 similarly reduced the proportions of male and female animals with adenocarcinomas, but the difference was statistically significant only in males (Figure 5B). Notably, in males, CBL0137 treatment following DMH injection completely eliminated the appearance of adenocarcinomas, as compared to their development in 19% of mice that received DMH alone. In females, the incidence of adenocarcinomas was reduced from 21% to 12%.

The incidence of mucin-depleted adenomas was also significantly lower in both males and females treated with CBL0137 after DMH injection compared to those that received DMH alone (24% vs 55% for males; 19% vs 68% for females; Figure 5C). It should be noted that CBL0137 treatment in males led to a substantial reduction in the proportion of mucin-depleted adenomas: from 17/19 in the group of DMH-treated mice to 8/15 in the group of males treated with DMH and then CBL0137 (Figure 5D). In the group of females treated with DMH alone, the multiplicity of adenomas was 6.12 (adenomas per mouse) while in the group of females treated with DMH followed by CBL0137, this parameter was 1.64. Therefore, CBL0137 decreased the multiplicity of adenomas in females by 3.73 fold.

For accurate presentation of the in vivo experiment results we should point out that besides carcinogenesis in the small and large intestine we observed appearance of the following malignant tumors: anal tumors, renal angiosarcomas in male mice and uterine sarcomas and ovarian hemangiomas. All the results are presented in the Supplemental Figure S2. We observed statistically significant tumor-preventive influence of CBL0137 on the frequency of DMH-induced renal capsule angiosarcomas in males and uterine sarcomas in females.

Overall, the results of this in vivo experiment demonstrate high chemopreventive efficacy of CBL0137 against CRC in both male and female mice.
DISCUSSION

WNT- and NFκB- signaling pathways have been identified as playing key roles in colon carcinogenesis, and thus they are well recognized as promising drug targets for CRC prevention and/or treatment (4, 12). Inhibition of NFκB by a new class of DNA binding small molecules (curaxins) represented by CBL0137 has been demonstrated in various CRC cell lines (20, 22). Here we report for the first time that CBL0137 also inhibits WNT signaling.

This was demonstrated in CRC cell lines using three independent approaches: microarray-based gene expression analysis, quantitative RT-PCR and luciferase reporter assay. Microarray analysis in HCT116 cells revealed a significant inhibitory effect of CBL0137 on expression of many components and downstream targets of the WNT signaling pathway, while expression of WNT-antagonists WIF1 and APC2 was enhanced. Using quantitative RT-PCR, we demonstrated a dose-dependent inhibitory effect of CBL0137 on expression of several WNT signaling target genes (cMYC, CCND1 and BIRC5) in four different human CRC cell lines. We also observed dose-dependent activation of WNT signaling antagonists SFRP1, WIF1 and DKK3. It should be noted that naturally occurring inactivation of these WNT signaling antagonists in human CRC (39), and in DMH/AOM-induced mouse colon tumors occurs mainly via epigenetic silencing (40). CBL0137 is a DNA-binding small molecule, and is known to alter chromatin structure through its inhibition of the histone chaperone FACT (18, 21). Our recent demonstration of CBL0137 epigenetic activity using HeLa-TI cells harboring an epigenetically silenced GFP reporter gene (18) suggests that such activity may underlie the observed upregulation of WNT signaling antagonists in CRC cell lines. Reduced expression of a luciferase reporter gene controlled by the TCF/LEF promoter in CRC cells treated with CBL0137 provided additional support for our conclusion that CBL0137 is an efficient inhibitor of WNT signaling.

This conclusion is consistent with and further supported by our previously published works showing that CBL0137 has anti-inflammatory effects including COX2 inhibition (20, 22), since one of the bioactive products of COX-2 function is prostaglandin E2 which regulates WNT signaling (23). Moreover, CBL0137 was previously shown to activate NOTCH1 expression in SCLC and an interplay between b-catenin signaling and NOTCH1 effectors has been demonstrated in intestinal tumorigenesis (41). Finally, it is possible that the effect of CBL0137 on cMyc expression that was previously thought to be mediated by CBL0137’s modulation of FACT function (18), could be also due to the impact of the drug on WNT signaling.

The significance of our finding that CBL0137 inhibits WNT signaling is highlighted by the well-appreciated fact that aberrant activation of WNT signaling plays a pivotal role in colorectal carcinogenesis in mammals. Germ-line mutations in the adenomatous polyposis coli (APC) gene result in familial adenomatous polyposis, the major hereditary predisposition event leading to CRC development (42).

Having demonstrated that CBL0137 is cytotoxic towards human and mice CRC cell lines and inhibits the Wnt signaling pathway both in human and murine adenocarcinoma cells, we next tested the drug for chemopreventive activity in vivo using a murine model of CRC induced by DMH. Rodent models of DMH-induced CRC were developed after it was recognized in a Guamanian population study that hydrazines from cycad flour induce colon cancer (43). The hydrazine and, in particular, unsymmetric dimethylhydrazine (UDMH), represent rather widespread human chemical carcinogens (24). UDMH is an industrial chemical that enters the environment primarily by emissions from its use in aerospace fuels and from industrial facilities that manufacture, process, or use it (44). DMH is used in experimental study of colon tumors with morphological and histological features and pathogenesis similar to those seen in human sporadic CRC (24). In DMH-induced colon carcinogenesis, similarly to human CRC, Wnt signaling activation is regarded as critical early events (45, 46). In spite of the fact that the patterns of somatic mutations between mouse and human CRC are in some way different (47), both of them affect Wnt signaling (28, 44). Notably, aberrant activation of Wnt signaling both in human CRC and in DMH-induced colon tumors is due to a lack of β-catenin phosphorylation (14, 27, 28). In mammals, Wnt signaling is mainly regulated via proteasomal degradation of β-catenin after its phosphorylation by the β-catenin destruction complex and subsequent ubiquitination. In human CRC, this process is often disrupted by APC mutations that inactivate the β-catenin destruction complex. In
mouse tumors, phosphorylation of β-catenin by the destruction complex is obstructed by point mutations in the GSK-3β phosphorylation consensus motif of β-catenin. Thus, the consequences of β-catenin mutations in mouse DMH-induced tumors and APC and β-catenin mutations in human CRC are similar. COX2 overexpression is another molecular alteration that is observed both in mouse DMH-induced tumors and in human CRC (28). Together, these facts indicate that the murine DMH-induced CRC model is an appropriate animal model for testing the effect of CBL0137 colon carcinogenesis in vivo.

The results of our in vivo study demonstrated a clear, statistically significant chemopreventive effect of CBL0137 against CRC in both male and female mice. At the same time, chronic administering of CBL0137 via drinking water did not cause any detectable changes in normal organs and tissues of mice of either gender. The latter finding is consistent with the results of previous studies demonstrating that CBL0137 is generally safe and well-tolerated in mammals (48). It is well known that colon adenomas and carcinomas appear in men at an earlier age and at a higher rate than in women. This was also observed in rodents, particularly in mice treated with azoxymethane (48). These gender-based difference in colon carcinogenesis are likely due to the tumor-promoting effects of testosterone. Taking this into consideration, different protocols of carcinogen treatment have been developed for males and females (32, 33). Applying this to our study, we administered DMH to males for 15 weeks and to females for 20 weeks. Among DMH-treated males, CBL0137 administering reduced adenoma incidence by approximately 2.5 fold and completely prevented appearance of adenocarcinomas. The proportion of males that remained tumor-free till the end of the study increased from 24% to 73% with CBL0137 treatment. Moreover, while the proportion of mucin-producing adenomas in the group of DMH-treated males was 2/17, in the group of males given CBL0137 after DMH the corresponding proportion changed to 5/8. As Blache et al. (49) showed that MUC-2 gene, encoding the main intestinal mucin, is suppressed by SOX9, which is one of the main targets of Wnt-signaling pathway, statistically significant difference (p value = 0.06) in the proportions of mucin-producing and mucin-depleted adenomas between the analyzed groups may be considered as an indirect confirmation of CBL0137 influence on Wnt signaling pathway in DMH-transformed cells in vivo (Fig. 4C; Table S4). In DMH-treated females, CBL0137 inhibited adenoma and adenocarcinoma incidence up to 2.6 and 1.8 fold, respectively. The proportion of DMH-females without colon tumors increased from 28% to 68% with CBL0137 treatment and the proportion of females. Consistent with our findings in the mouse DMH-induced CRC model, chemopreventive activity of CBL0137 was previously demonstrated in two other preclinical cancer models. First, chronic administering of CBL0137 was shown to inhibit tumor onset in MMTV-neu mice without causing any detectable changes in normal organs and tissues (34). The preventive effect of CBL0137 was evidenced by reduced incidence of spontaneously developing mammary carcinomas, delayed tumor progression, and prolonged animal survival. Second, in a murine model of MYCN-driven neuroblastoma, low dose CBL0137 administering on days 6-28 after birth significantly delayed tumor growth in TH-MYCN+/+ mice (50). Thus, there is a growing body of evidence supporting the potential clinical usefulness of CBL0137 as a chemopreventive agent.

In summary, our results indicate that CBL0137 is an efficient inhibitor of WNT signaling in CRC cell lines. This reveals an additional mechanism of action for multi-targeted anti-cancer agents, the curaxins. Together with the previously demonstrated NFκB-inhibitory and anti-inflammatory activities of CBL0137, suppression of WNT signaling provides a mechanistic explanation for the significant chemopreventive influence of CBL0137 on DMH-induced murine colon carcinogenesis. These data highlight the strong potential of CBL0137 as an agent with broad applicability against many types of tumors associated with aberrant activation of WNT signaling.

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REFERENCES


FIGURE LEGENDS

Figure 1. Scheme of the experimental design.

Figure 2. Effect of CBL0137 treatment on WNT signaling in CRC cell lines. A. Changes in gene expression in HCT116 cells upon 5 or 16h CBL0137 treatment (color scale shows log2 fold-change relative to untreated cells). Data is shown for genes coding for WNT pathway component (n=26), ligand (n=1), receptors (n=3), negative regulators (n=4) and targets (n=18). B. Relative expression level (fold-change in cells treated with CBL0137 during 48h compared to untreated cells) of WNT target genes determined by quantitative RT-PCR. C. Relative expression level (fold-change in CBL0137-treated cells compared to untreated cells) of WNT signaling antagonists determined by quantitative RT-PCR. D. Luciferase TCF/LEF reporter analysis. B,C,D Influences of CBL0137 on the gene expression levels were significant with p<0.05.

Figure 3. Effect of CBL0137 treatment on WNT signaling in MC-38 adenocarcinoma cells: relative expression level (fold-change in cells treated with CBL0137 during 48h compared to untreated cells) of WNT target genes determined by quantitative RT-PCR.

Figure 4. Histologic analysis of colon tissue observed in control and DMH–treated CBA mice. A-D. Microphotographs of H&E-stained sections of mouse colon tissue. A,B. Normal colon epithelium (x40; x200). C. Tubular adenoma induced by DMH (x100). D. A representative adenocarcinoma induced by DMH (x100). E,F. Mouse colon tissue stained with mucicarmine, which reveals mucus (pink), and Haematoxylin staining basophilic cell structures (blue). E. Mucin-producing normal epithelial tissue (x500). F. Mucin-depleted epithelium of early stage adenoma (marked area) (x400).

Figure 5. Prevention of DMH-induced colorectal carcinogenesis in mice by CBL0137. A. Colon tumor (adenoma + adenocarcinoma) multiplicity in male and female mice given DMH injections alone or followed by CBL0137 treatment. B. Adenoma and adenocarcinoma incidence in male and female mice left untreated (control) or treated with DMH alone, DMH+CBL0137, or CBL0137 alone. C. Incidence of MDF in normal-appearing colon tissue, adenomas with mucin-depleted epithelium and adenomas with mucin-producing epithelium among male and female mice treated with DMH alone or DMH+CBL0137. B,C. Stars indicate statistically significant differences between DMH-treated and DMH+CBL0137-treated groups (Pearson's chi-squared test, p<0.01). More detailed information could be found in the Table S4. D. The number of mucin-depleted and mucin-producing adenomas in the DMH-treated and DMH+CBL0137-treated male and female groups. The difference in the proportions of mucin-depleted/mucin-producing adenomas in males is significant, p-value = 0.06.
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Figure 3

Relative mRNA level

- Cmyc: Control 1, CBL0137 - 0.7 μM 0.1, CBL0137 - 0.9 μM 0.4
- Ccnd1: Control 1, CBL0137 - 0.7 μM 0.65, CBL0137 - 0.9 μM 0.7
- Birc5: Control 1, CBL0137 - 0.7 μM 0.5, CBL0137 - 0.9 μM 0.6
- Cd44: Control 1, CBL0137 - 0.7 μM 0.15, CBL0137 - 0.9 μM 0.35
- Cox2: Control 1, CBL0137 - 0.7 μM 0.5, CBL0137 - 0.9 μM 0.7
Prevention of colorectal carcinogenesis by DNA binding small molecule curaxin CBL0137 involves suppression of Wnt signaling

Kirill Igorevich Kirsanov, Timur Fetisov, Ekaterina A Lesovaya, et al.

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