Targeting FACT complex suppresses mammary tumorigenesis in *Her2/neu* transgenic mice

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Running title: Curaxin-137 prevents mammary tumors in cancer-prone mice

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List of abbreviations used

- CK2 – casein kinase 2
- ER – estrogen receptor
- H&E – hematoxylin and eosin
- HSF1 – heat shock factor 1
- FACT - facilitates chromatin transcription
- MMTV – mouse mammary tumor virus
- MTD – maximal tolerated dose
- SPT16 – suppressor of Ty
- SSRP1 – structure specific recognition protein 1

Competing Interests

A.V.G. is Board of Directors member and consultant of Incuron, a biotech company which develops Curaxins for cancer treatment. A.A.P., A.V.G. and K.V.G. are co-inventors on a patent application 61/102,913, filed on October 6, 2008. “Carbazole compounds and therapeutic uses of the compounds” that describes structure and anticancer properties of curaxins”. K.V.G. has research grant from Incuron and also consultant of Incuron.

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Abstract

Development of safe and effective tumor-preventive treatments for high-risk patient populations and therapies for early stage cancer remains a critical need in oncology. We have recently discovered compound with anti-cancer activity, Curaxin-137, which modulates several important signaling pathways involved in even the very early stages of cancer. In tumor cells, Curaxin-137 inhibits NF-κB- and HSF1 dependent transcription (pro-survival pathways) and activates p53 (a pro-apoptotic pathway) without inducing DNA damage. These effects result from chromatin trapping and inhibition of activity of the FACT (Facilitates Chromatin Transcription) complex by Curaxin-137. FACT has not been previously implicated in cancer, but we found that its subunits are overexpressed in breast cancer. Based on this background, we tested whether Curaxin-137 could suppress tumorigenesis in MMTV-neu transgenic mice, which spontaneously develop mammary carcinoma due to steroid receptor-regulated expression of the Her2 proto-oncogene. We found that chronic administration of Curaxin-137 in a preventive regimen to MMTV-neu mice did not cause any detectable changes in normal organs and tissues, yet inhibited tumor onset, delayed tumor progression, and prolonged survival of mice in a dose-dependent manner. Curaxin-137 induced changes in FACT, altered NF-κB localization, and activated p53 in tumor cells as expected from its defined mechanism of action. These results support further investigation of Curaxin-137 as a potential preventive and/or early stage therapeutic agent for breast cancer.
Introduction

With better understanding of cancer genetics and availability of advanced sequencing technologies, cancer risk prediction may become accurate and reliable in the near future. This creates a high demand for safe and effective cancer prevention agents. In addition new diagnostic tools are capable of detecting cancer at early stages when use of standard chemotherapy is questionable due to its harmful effects on normal tissues. We have recently discovered a new class of anti-cancer small molecules with novel mechanisms of activity, named Curaxins (1). Curaxins simultaneously modulate several signaling pathways that are commonly involved in transformation as well as other stages of cancer (e.g., the p53, NF-κB, and HSF1 pathways); this results in tumor-specific cytotoxicity (1, 2). These pathways are involved in transformation as well as other stages of cancer and their modulation may influence tumor appearance and progression in tumor prone individuals.

The anti-cancer effects of Curaxins on the p53, NF-κB and HSF1 pathways in tumor cells are due to suppression of activity of the Facilitates Chromatin Transcription (FACT) complex (1). FACT is a heterodimeric complex of two subunits, Structure Specific Recognition Protein 1 (SSRP1) and Suppressor of Ty 16 (SPT16), which is involved in transcription of genes with highly ordered chromatin structure through its bidirectional nucleosome remodeling activity (destabilizing nucleosomes to allow passage of RNA polymerases and stabilizing nucleosomes afterwards) (for review see (3)). FACT is also involved in replication and mitosis (4), (5). We found that FACT is overexpressed in many types of
tumor cells and in mammary tumors in mice and that survival of tumor cells depends on FACT levels (1). We also showed that FACT is required for NF-κB-dependent transcription (1). Curaxins impact FACT function by altering chromatin structure such that FACT becomes tightly bound (or “trapped”) in chromatin. This depletes the pool of soluble active FACT in the nucleoplasm and thereby inhibits NF-κB- (and presumably HSF1 (6)) dependent transcription (1).

Chromatin-trapping of FACT by Curaxins also leads to casein kinase 2 (CK2)-dependent phosphorylation and activation of p53 (1). Therefore, Curaxins alter the activity of FACT, suppress transcription of NF-κB- and HSF1-dependent genes, and activate p53 – all properties which compromise tumor cell survival.

Based upon these findings, we hypothesized that FACT might be involved in tumor transformation and that Curaxins, therefore, might suppress tumor formation by modulating FACT activity in precancerous cells. To test this hypothesis, we used the MMTV-neu transgenic mouse model of mammary carcinogenesis, in which mammary tumor formation is induced through ectopic expression of the Her2/neu proto-oncogene driven by the steroid receptor-responsive promoter from the long terminal repeats of Mouse Mammary Tumor Virus (MMTV) (7). Transgene expression in these mice is limited to tissues that express steroid receptors in female, such as mammary epithelial cells and ovary. Mammary tumors in these animals recapitulate histopathological features of human breast adenocarcinomas (8). A role for p53 suppression and NF-κB activation in promoting tumor formation in the MMTV-neu model was previously demonstrated (9-12). Inflammation, which is always associated with increased
NF-κB activity, is a marker of more aggressive breast cancer and a promoter of tumor formation in the MMTV-neu mouse model (for review see (9)). In addition, we observed gradual elevation of FACT subunit expression beginning at the very early stages of transformation in these mice. The lead Curaxin compound, Curaxin-137 (equivalent to CBLC137 from ref. (1)), had effects on FACT, p53 and NF-κB that are consistent with its described mechanism of action (1). These effects were translated into anti-cancer efficacy in MMTV-neu mice treated chronically with Curaxin-137 provided with drinking water: tumor onset and progression were suppressed, and the lifespan of MMTV-neu animals treated with Curaxin-137 was significantly prolonged as compared to control animals.

Material and Methods

Chemical and Reagents

Curaxin-137 (CBLC137, >97% pure by HPLC and LC/MS) was synthesized by Dalton Pharma (Toronto, Canada). For administration to animals, the drug was dissolved in water at 0.1 or 0.2 mg/ml and stored at room temperature (RT). Stability of the compound in water at RT was tested over 2 months in standard p53 activation reporter assay (1). No difference between fresh and stored solutions was found (data not shown).

Hoechst 33358, R1881, TNF, type III collagenase, hydrocortisone, insulin and EGF were purchased from Sigma (Sigma- Aldrich, Inc). 100X penicillin/streptomycin and fungizone solutions, DMEM, glutamine, N-(2-
hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), cholera toxin and Trizol were purchased from Invitrogen, Inc.

**Cells**

All human breast cancer cells were obtained from ATCC. Cells were frozen after 2 passages and used after thawing for no longer than 10-20 passages. Primary human mammary epithelial cells (HMEC) were purchased from StemCell Technologies. H1299-κB-Luc and RCC45-p53-Luc cells were described (13). κB-Luc and p53 reporter activity in these cells were confirmed by treatment with specific inducers (TNF and Curaxin-137 respectively). No additional authentication was done. Cells were grown in DMEM with 10% fetal bovine serum (Hyclone) and other standard supplements. HMEC cell medium was purchased from StemCell Technologies.

**Ex-vivo cell culture**

Tumor free mammary glands or necrosis-free mammary tumors were isolated from deeply anesthetized mice under sterile conditions, washed in PBS, minced with scissors and then incubated in 0.1% Type III Collagenase in complete culture medium (DMEM supplemented with 100 U/ml penicillin, 100 pg/ml streptomycin, 2 mM glutamine, 10 mM HEPES, 0.075% BSA, 10 ng/ml cholera toxin, 0.5 pg/ml hydrocortisone, 5 pg/ml insulin, and 5 ng/ml EGF) overnight at 37°C on a shaking platform. After incubation, the cell suspension was centrifuged at 40Xg for 1 min. The pellet was washed once with PBS using the same centrifugation conditions. The final pellet was resuspended in complete culture
medium and plated into plastic plates. The resulting “organoid” culture was used for an experiment within one week without additional replating.

**Western blotting and immunofluorescent staining** were performed using standard procedures. Antibodies used in the study are described in the Supplementary Materials.

**Immunohistochemistry**

Paraffin sections were cut at 5µm, placed on charged slides, and dried at 60°C for one hour. Slides were cooled to RT, deparaffinized in three changes of xylene, and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H₂O₂. For antigen retrieval, slides were heated in a microwave for 20 min in citrate buffer (pH 6.0), cooled for 15 min and washed in 0.1%PBS/Tween20 solution. Slides were then loaded onto a Dako Autostainer and blocked for 5 min with serum-free protein block (Dako). After blocking, the slides were incubated with 0.2 µg/ml goat anti-mouse SSRP1 polyclonal antibody (Santa Cruz, sc-5909) for 1 h. An isotype-matched control antibody (0.2 µg/ml goat IgG) was used on a duplicate slide in place of the primary antibody as a negative control. After washing, slides were incubated with biotinylated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.) , followed by staining with the Elite ABC Kit (Vectastain), and DAB chromagen (Dako). Finally, stained slides were counterstained with Hematoxylin, dehydrated, cleared and coverslipped. All slides were scanned using Aperio scanscope (Aperio...
Technologies, Inc). Images were made using Image scope software (Aperio Technologies, Inc).

**Microarray-based gene expression profiling**

Total RNA was isolated from frozen tissue samples using TRIZOL reagent (Invitrogen). mRNA labeling and hybridization to MouseWG-6 v2.0 Expression BeadChips, image scanning, and intensity processing were performed according to the manufacturer’s instructions (Illumina, San Diego, CA). BeadChip data files were analyzed with Illumina’s GenomeStudio software and R-based Bioconductor package to determine gene expression signal levels. A hierarchical clustering algorithm based on the average linkage of Pearson correlations was employed. Data are deposited at NCBI GEO database, accession number GSE33285.

**Measurement of Curaxin-137 concentrations in mouse tissues and plasma**

Drug compound extraction was done in the Department of Chemistry at Cleveland BioLabs. From an original tissue specimen, a 70-100 mg sample was weighed out. Chilled Methanol (9x sample weight) was added to the sample and the sample was mechanically homogenized (Fisher Scientific PowerGen 125). The resulting sample/methanol suspension was then rocked overnight in a 4º C refrigerator. The samples were then centrifuged and the supernatant was taken for analysis by LC/MS/MS. The plasma samples were extracted using an extraction solution of 0.1% Trifluoroacetic acid in Acetonitrile (4x sample volume). The diluted samples were vortexed thoroughly then centrifuged and the resulting
supernatant was taken for analysis by LC/MS/MS. All prepared samples were stored at 4°C until analyzed. The samples were analyzed for Curaxin-137 using an Applied Biosystems API 3000 LC/MS/MS system. A gradient HPLC method was employed with mobile phases: (A) 2 mM Ammonium acetate, 0.1% Trifluoroacetic acid in Water and (B) 2 mM Ammonium acetate, 0.1% Trifluoroacetic acid in Methanol. The injection volume was 20 µL and the flow rate 0.20 mL/min. A Phenomenex Luna column C18(2), 50 x 2.00 mm, 5 µm particle size was coupled with a Phenomenex C18, 4 x 2.00 mm guard cartridge/column. The mass spectrometer used multiple reaction monitoring (MRM) with the singly charged Curaxin-137 selected at m/z 337.20 giving a fragment ion at m/z 86.00.

**Animal Experiments**

All animal experiments were performed according to the Roswell Park Cancer Institute IACUC-approved protocol and with guidance from the “Guide for the Care and Use of Laboratory Animals” by the National Research Council [ISBN 0-309-05377-3].

FVB/N-Tg(MMTVneu)202Mul/J mice (referred to herein as “MMTV-neu mice”) were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred as necessary in the department of Laboratory Resources (LAR) of RPCI. The transgene carried by these mice directs expression of the Her2/neu proto-oncogene from the steroid receptor. 100% of female MMTV-neu mice develop spontaneous mammary carcinomas between 6 and 12 months of age (8).
Detection of the maximal tolerated dose (MTD) of Curaxin-137 administered to mice in their drinking water

Groups of 5 MMTV-neu mice (males and females, 4 weeks old) were placed in a cage and provided with a solution of Curaxin-137 (various concentrations as described below) in water in dark bottles (at least 150ml per bottle) as the only source of water. Bottles were weighed before the start of the experiment and once a week thereafter. Bottles were refilled once a week. Mice were given a standard diet ad libitum. Mice were observed daily for changes in appearance and behavior and were weighed daily during the first week and then once weekly. The experiment was run for one month or until any of the following conditions were observed: >10% loss of weight (all 5 mice in a group); >15% loss of weight by 2 and more animals in a group; consistent changes in mouse visual appearance or behavior; death of more than one animal in a cage.

Starting doses tested in this experiment were calculated based on average mouse daily liquid consumption of 150ml/kg/day taken from several reference sources and then adjusted based in actual liquid consumption in LAR facility of RPCI.

Safety of chronic administration of Curaxin-137 at MTD and ½ MTD in drinking water

Groups of 5 male MMTV-neu mice (4 weeks of age) were given either plain water or solutions of Curaxin-137 (at MTD or ½ MTD) in water ad libitum for 10 weeks. Mice were weighed once a week. After 10 weeks, mice were deeply anesthetized and blood was collected by cardiac puncture for plasma isolation.
Major organs were collected for histopathological analysis, RNA isolation and measurement of Curaxin-137 concentration.

**Cancer prevention study**

Virgin female MMTV-neu mice (19-27 per group) were given either plain water or Curaxin-137 in water *ad libitum*. Curaxin-treated groups were given either 0.1 mg/ml Curaxin-137 starting at 4 weeks of age or 0.2 mg/ml Curaxin-137 starting at 10 weeks of age. Animals were monitored daily for signs of abnormalities and tumor appearance. They were weighed once a week for the first 24 weeks and then once a month. Animals that developed at least one visible tumor were transferred to drug-free water and kept until their cumulative tumor volume reached 2000 mm$^3$. At that time, mice were sacrificed and all mammary glands with and without tumors were excised, fixed in buffered formalin and paraffin embedded for sectioning.

Histopathology examination was done blindly by qualified pathologists at RADIL facility, University of Missouri, Columbia, MO. In addition, H&E-stained slides of mouse parenchymal organs, bone marrow and tumors were analyzed by a qualified animal pathologist in house (I.T.) and representative photographs were taken using a Zeiss Axio Observer A1 inverted microscope with N-achroplan 100x/1.25 oil lens, Zeiss MRC5 camera and AxioVision Rel.4.8 software.

**Quantitation and statistical evaluation of data**

All in vitro experiments were run in triplicates and repeated 2-5 times. Data within each experiment were averaged and compared using Student t-test (SigmaStat 4, Aspire Software). If necessary intensities of bands of immunoblotting
experiments were assessed using Image J software from NCI. Overall and tumor free survival of mice were compared using Log-rank test (MedCalc v.11.3.3) and Friedman Repeated Measures Analysis of Variance on Ranks (www-users.cs.umn.edu). Distribution of different histological types of tumors between groups was compared using Chi-square test (SigmaStat 4, Aspire Software).

Results and Discussion

Curaxin-137 does not directly interfere with steroid induced activity of MMTV-promoter

In MMTV-neu transgenic mice, mammary tumorigenesis in females is driven by Her2/neu proto-oncogene expression from the steroid receptor regulated MMTV promoter. To test the potential anti-tumor effect of Curaxin-137 in this model, we first checked that Curaxin-137 does not have any direct effect on the activity of the MMTV promoter that would alter ectopic expression of Her2/neu. Therefore, we evaluated the effect of Curaxin-137 on luciferase reporter gene expression from the MMTV promoter in a human breast cancer cell line (MDA-KB2). As a positive control, we used cells carrying an NF-κB-dependent luciferase reporter, expression of which is known to be inhibited by Curaxin-137 (1). Transcriptional activity of steroid receptor (androgen receptor in MDA-KB2 cells) and NF-κB were induced in cells with their corresponding ligands, synthetic androgen, R1881, or TNF respectively, in the presence or absence of different concentrations of Curaxin-137 (Fig.1A and B). Curaxin-137 clearly inhibited NF-κB-dependent reporter activity as early as 3 h post-treatment, but there was no
effect of the drug on luciferase expression from the MMTV promoter at 3 or 6 h post-treatment, even with high Curaxin-137 doses. 50% inhibition of MMTV-Luc expression was only seen following 24 h treatment with drug which most probably was due to toxic effect of Curaxin-137 seen at this time point (>50% reduction in number of cells from concentrations of 0.6uM and higher, data not shown).

Curaxin-137-targeted factors, FACT, p53 and NF-κB, are frequently deregulated in mammary tumors of MMTV-neu mice

We next examined whether major factor known to be affected by Curaxin-137 in tumor cells, chromatin remodeling complex FACT, is involved in mammary carcinogenesis. We observed elevated expression of FACT in several human breast cancer cell lines comparing with normal primary and immortalized mammary epithelial cells (Fig.2A). We also noticed that while in tumor free mammary gland tissue of MMTV-neu animals SSRP1 and SPT16 were very low, in tumors isolated from the same animals levels of both subunits were elevated (Fig.2B). Both SSRP1 and SPT16 protein levels were variable but in all cases higher than in tumor free tissue. To identify the stage of tumor formation at which FACT becomes overexpressed in this model, we stained sections of tumor free mammary glands and lesions of different degrees of malignancy with anti-SSRP1antibodies. As shown in Figure 2C, SSRP1-positive mammary epithelial cells were detected in sections of tumor-free mammary glands from MMTV-neu mice, but not in similar sections from age-matched wild type animals of the same FVB background. A much higher degree of SSRP1 staining was seen in
abnormal mammary lesions of all stages from MMTV-neu mice (Fig.2C). These data suggest that FACT levels are elevated early in the process of mammary epithelia transformation in MMTV-neu animals.

According to its established mechanism of action, Curaxin-137 causes chromatin trapping of FACT what results in reduction or disappearance of FACT subunits from nucleoplasm (1). To check that treatment of mice with Curaxin-137 leads to FACT chromatin trapping in vivo we administered one oral dose of the drug to several tumor-bearing mice and collected tumor tissue 1 hour later at the peak of plasma concentration of Curaxin-137 after oral delivery (data not shown). We observed different degree of reduction of soluble SSRP1 and SPT16 in protein extracts of tumors from Curaxin-137 treated animals comparing with vehicle treated mice (Fig. 3A).

Variability in soluble FACT reduction in tumors of different mice may suggest difference in sensitivity of tumors to Curaxin-137 or just difference in intra-tumor drug concentration due to the structures of individual tumors. To get better insight into the effect of Curaxin-137 on FACT we established short term ex-vivo cultures from freshly isolated and disaggregated normal and tumorous mammary tissues of untreated control animals and treated them with Curaxin-137 in vitro. As shown in Figure 3B, Curaxin-137 treatment resulted in disappearance of both FACT subunits, SSRP1 and SPT16, from soluble protein extracts of mammary tumor cells, while little or no SSRP1 or SPT16 protein was detected in tumor free mammary gland extracts (Fig.2B). The degree of FACT subunits reduction was
proportional to the basal level of FACT in individual tumors and effect of Curaxin-137 was similar in all tumors tested (Fig.3B).

Chromatin trapping of FACT leads to inhibition of NF-κB dependent transcription due to depletion of soluble FACT from the nucleoplasm (1). The lack of soluble FACT in Curaxin-treated cells blocks elongation of NF-κB dependent transcripts, but does not prevent nuclear translocation of NF-κB per se. Moreover, blocked NF-κB-dependent transcription leads to the depletion of IκBα normally keeping NF-κB complex in cytoplasm. Therefore, Curaxin treatment leads to accumulation of NF-κB in the nuclei of cells (1, 13). It should be noted that while nuclear translocation of NF-κB is typically interpreted as a sign of NF-κB activation, in this case, it is actually an indicator of NF-κB inhibition.

To determine the NF-κB status of mammary tumors from MMTV-neu mice, samples of several tumors were used for immunofluorescent staining with an antibody against the p65 subunit of NF-κB. Weak background staining was observed in tumor-free mammary gland, while all 5 tumor samples demonstrated visible p65 positivity with cytoplasmic localization in most of the cells and nuclear staining in some cells (Fig. 3C). Importantly, Curaxin-137 treatment led to significant nuclear relocalization of p65 without any additional stimulus, which is consistent with the previously defined specific mechanism of NF-κB inhibition by Curaxin-137 (Fig. 3D). Inflammation and activation of the NF-κB pathway through overexpression of p65 subunit was also shown to promote tumorigenesis in this model (11), (9). Therefore Curaxin-137 induced inhibition of NF-κB may have protective effect at early stages of tumor transformation.
Additional consequence of FACT chromatin trapping is activation of p53 via phosphorylation by FACT-associated Casein Kinase 2 (CK2) (1). Evaluation of p53 protein levels in the same ex vivo cultures (from treatment naïve animals), showed that p53 levels were increased in a dose-dependent manner 6 hours after Curaxin-137 treatment of cells from a tumor-free mammary gland and mammary tumor with low basal level of p53 (Fig. 3E). Several other tumors assessed showed high basal p53 levels and no induction in response to Curaxin-137 treatment, likely due to p53-stabilizing mutations (Fig. 3E). We observed the similar result in tumors of mice treated with Curaxin-137 (Fig.3A). These data confirmed the known fact that a high proportion of mammary tumors in this model have p53 pathway inactivated by p53 mutations (10). The roles of p53 in mammary carcinogenesis in general and in this particular model have been well-studied (10, 12). It was shown that Her2 overexpression induces senescence in normal mammary cells and that this phenotype is overcome by inactivation of p53 (14). Reported high incidence (37%) of p53 mutations in tumors developed in MMTV-neu mice (12) suggests that p53 activity interferes with tumor development and p53-inducing Curaxins treatment at pre-cancerous stage may enforce anti-cancer protective activity of p53.

These data, together with those described in the previous section, demonstrate that Curaxin-137 has desirable effects on multiple pathways proposed (FACT) and established (ER, Her2, p53, NF-κB) to be involved in mammary carcinogenesis in the MMTV-neu mouse model as well as human breast cancer.
Curaxin-137 can be administered to mice chronically with drinking water

Curaxin-137 is orally available and soluble in water (1). To establish a regimen of chronic administration for a cancer prevention study, we tested whether mice could be given the compound in their drinking water. MMTV-neu mice did not refuse drinking solutions of 0.1 or 0.2 mg/ml Curaxin-137, consuming the same volume of liquid in a given period of time as mice exposed to plain water (Fig. 4A). However, less liquid was consumed for more concentrated solutions of Curaxin-137 (data not shown). Conversion of the amount of solution consumed by mice into the actual dose of the drug indicated an average daily dose of Curaxin-137 of 13.8+/−2.2 mg/kg for the 0.1 mg/ml solution group as and 28.5+/−2.5 mg/kg for the 0.2 mg/ml group (Fig. 4B). MMTV-neu mice given solutions of Curaxin-137 at these dose levels in place of regular drinking water between 4 and 14 weeks of age showed no difference in weight gain as compared to control animals given plain water (Fig. 4C). In addition, there were no observed differences between the mice in the Curaxin-137 treated and control groups during this period, except that one mouse had a hunched appearance for 2 days without any other changes in behavior or weight. No morphological differences were found between the study groups upon histopathological examination of internal parenchymal organs at the end of the observation period (10 weeks) (Supplementary Figure S1). Moreover, hierarchical clustering of global gene expression profiles of genes in liver and spleen (these organs were selected as organs with the highest level of Curaxin-137 accumulated, see below) did not separate samples based on the treatment.
what demonstrates that there is very little difference between Curaxin-treated and control animals (Fig. 4E). These data indicate that chronic administration of Curaxin-137 does not cause any apparent systemic toxicity.

Since concentration of Curaxin-137 in drinking water higher than 0.2 mg/kg impeded liquid consumption by mice, this dose (0.2 mg/ml Curaxin-137 in drinking water; equivalent to 28.5 mg/kg/day) was defined as the MTD for this administration regimen. This dose was very close to the previously established repetitive MTD for administration to mice by oral gavage (30 mg/kg).

Following administration of 0.1 or 0.2 mg/ml Curaxin-137 in drinking water for 10 weeks, the compound was detected in the plasma of mice at a median concentration of 56.2 and 111.2 ng/ml, respectively (equivalent of 0.17 and 0.33 μM, Supplementary Table 1). Substantially higher concentrations of Curaxin-137 were found in several mouse organs, with spleen showing the highest levels (1286 and 2414 ng/ml in low and high dose groups respectively (equivalent to 3.8 and 7.2 μM) (Supplementary Table 1).

Importantly of Curaxin-137 at concentrations exceeding the LC50% for most tumor cells in vitro (0.2-0.6 μM) did not cause any pathological changes in spleen or other organs analyzed (Supplementary Fig. S1). Therefore, we concluded that chronic administration of Curaxin-137 was safe enough to be tested as a tumor preventive regimen.

**Curaxin-137 delays tumor onset and increases survival in MMTV-neu transgenic mice**
Three groups of female MMTV-neu transgenic mice were given (i) regular water throughout their lives ("untreated" control), (ii) 0.1 mg/ml solution of Curaxin-137 in water from 4 weeks of age, or (iii) 0.2 mg/ml Curaxin-137 in water from 10 weeks of age. Several mice in each group (including the untreated control group) died without tumors during the course of the study. The reasons underlying these deaths were not established upon necropsy and histopathological examination. There was no statistically significant difference in the number of tumor free deaths between the three groups (Fig. 4D).

In the control group as well as the 0.1 mg/ml Curaxin-137-treated group, mammary tumors started being detected at 23-25 weeks of age (Fig. 5B). In contrast, tumor appearance was delayed until 40 weeks of age in the 0.2 mg/ml Curaxin-137-treated group. Despite the fact that the kinetics of initial tumor appearance in the low dose Curaxin-treated group was similar to that in the control group, both the low and high dose Curaxin-treated groups were statistically different from the control group based on the comparison of Kaplan-Meyers curves of tumor-free survival using Log-rank test (Fig.5B).

The median duration of tumor-free survival was 44 weeks in the control group and 57 and 78 weeks in the low and high dose Curaxin-treated groups, respectively (Fig. 5B). There were minimal difference in the size of tumors between groups (Fig.5C) and no difference in the life-span of tumor-bearing animals (Fig.5D) what most probably resulted from the regimen of administration used when Curaxin-137 administration was stopped after tumor appearance.
Overall survival of animals treated with Curaxin-137 was longer than control animals in both treatment groups (Fig. 5A). Some animals in both Curaxin treated groups were still tumor-free at >18 months of age, while no control animals lived longer than 13 months (Fig. 5B and Supplementary Table 2). Besides in house pathological evaluation (Supplementary Fig. S1), the oldest surviving animals from each group were subjected to blind histopathological evaluation by phenotyping facility of University of Missouri (Supplementary Table 2). The oldest Curaxin-137-treated animals did not have any abnormalities that were different from those seen in the oldest surviving control mice (Supplementary Table 2). This provides additional support for the general non-toxicity and safety of systemic Curaxin administration.

**Curaxin-137 slows the progression of mammary tumors**

We performed histological analyses on mammary glands with tumors isolated from mice in the three study group described in the preceding section when the cumulative tumor size per mouse was close to \(2000\text{mm}^3\). Importantly this approach allowed analysis of tumors which were grown approximately the same time, therefore potential differences in their histology cannot be explained by the differences in tumor appearance. Tumors were excised and the morphology of each tumor was assessed on H&E stained sections. Tumors were graded according to the recommendations of ref. (15) as “undifferentiated” when the tumor mass was >90% solid cell mass with no traces of cells forming glandular or tubular structures (Fig. 6A), “high grade” when >50% of the tumor mass was comprised of poorly organized, but recognizable glandular-like structures (Fig.
6B) or thick tubular structures (Fig. 6C); “glandular” when 100% of the tumor mass resembled glandular or tubular structures (Fig. 6D) and “carcinoma in situ” when the tumor mass consists primarily of hyperplasic mammary epithelial structures (Fig. 6E and F). Analysis of the proportion of tumors of each type in each study group (control, 0.1 mg/ml or 0.2 mg/ml Curaxin-137-treated) demonstrated that more than half of the tumors in the control group had undifferentiated phenotypes and only minor proportions of the tumors were classified as more differentiated subtypes. In contrast, in both Curaxin-treated groups, most of tumors were more differentiated subtypes (Fig. 6G). Since loss of differentiation is usually associated with tumor progression, the prevalence of more differentiated tumors in Curaxin-137 treated groups of mice suggests that chronic administration of the drug slowed tumor progression in the MMTV-neu breast cancer model.

Conclusions

The results of this study show that Curaxin-137 has anti-tumor efficacy when administered to tumor-prone MMTV-neu female mice in a preventive regimen. The protective effect of Curaxin-137 was evidenced by reduced tumor incidence, delayed tumor progression and prolonged animal survival. Chronic administration of Curaxin-137 was safe; no drug-related mortality or changes in organ morphology or function were observed. At the same time, Curaxin-137 induced molecular changes in tumor cells that were consistent with its established mechanism of action: depletion of soluble FACT from the nucleoplasm, activation of p53 (in those cells with a functional p53 pathway), and accumulation of...
inactive NF-κB in nuclei. Importantly, Curaxin-137 did not directly affect activity of the MMTV promoter in MMTV-neu mice, which suggests that our demonstration of tumor preventive efficacy for Curaxin-137 in this model is likely relevant to inhibition of mammary carcinogenesis in general.

Author's contribution

IEK run animal experiments and drafted manuscript, MC and SP run most of in vitro assays, GP continued animal experiments after IEK left the lab, BH analyzed FACT and p53 levels in mouse tissues in vivo and AS did the same in vitro, IT assessed histology of mouse tissues, AP analyzed Curaxin-137 concentration in plasma and tissues of mice, DW and SL performed bioinformatics analysis of gene expression, CM oversaw IHC stainings and data interpretation, AG critically discussed and edited manuscript, KG oversaw the whole study and wrote the manuscript.

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Figure Legends

**Figure 1. Curaxin-137 does not affect MMTV promoter activity.** A-B. Curaxin-137 inhibits activity of the NF-κB promoter, but not the MMTV promoter at 3 and 6 hrs. Luciferase activity was measured in lysates of MDA-MB-453-MMTV-Luc (A) and H1299-κB-Luc (B) cells treated for 3, 6 or 24 h with different concentrations of Curaxin-137 in the presence of 10nM of R1881 (A) or 10ng/ml TNF (B). Luciferase activity is shown as the fold-change relative to that in untreated, uninduced cells (set at 1.0). Error bars indicate standard error of three replicates.

**Figure 2. FACT subunits levels are elevated in the process of tumor formation.** A. Protein levels of SSRP1 and SPT16 in human epithelial cells of mammary glands. Western blotting of total protein extracts of primary mammary epithelial cells (HMEC), immortalized mammary epithelial cells (MCF10A) and cells of breast cancer cell lines (all others). B. Increased FACT subunit expression in mammary tumors. Western blotting of total protein extracts from tumor free mammary glands (n) or mammary tumors (t) of two untreated female MMTV-neu mice with visible tumors. C. FACT expression is elevated in mammary glands of MMTV-neu animals at different stages of tumor formation. Immunohistochemical staining with anti-SSRP1 antibody of sections of mammary gland tissue from wild type (wt) and MMTV-neu animals and lesions of different stages from MMTV-new animals. TLDU – terminal lobular-ductal unit. DCIS - ductal carcinoma *in situ*.

**Figure 3. FACT, NF-κB and p53 are involved in mammary carcinogenesis in MMTV-neu mice and are responsive to Curaxin-137 treatment.** A. Protein levels of FACT subunits and p53 in tumors from several MMTV-neu mice treated
with vehicle (#1 and 2) and 30mg/kg of Curaxin-137 (#3-5). Left panel - western blotting of soluble protein extracts of tumors isolated 1 hour after oral drug administration. Right panel quantitation of data presented on the left using Image J software (NCI). Bars – average intensities of SSRP1 and SPT16 bands normalized using intensities of corresponding β-actin bands. Error bars – range of data from different mice. ** - p-value (t-test) <0.01, * - <0.05. B. Curaxin-137 treatment depletes the soluble pool of FACT in mammary tumor cells of MMTV-neu mice. Western blotting of soluble extracts of cells isolated from several different tumors of two mice. Disaggregated tumor cells were left untreated (-) or treated ex vivo with increasing concentrations of Curaxin-137 (1-10μM) for 1 h. C. Increased level of NF-κB in mammary tumors of MMTV-neu mice as compared to normal mammary cells. Immunofluorescent staining of cells with antibodies to p65 (green). Blue – staining of DNA with Hoechst 33358. D. Curaxin-137 induces nuclear accumulation of NF-κB in mammary tumor cells from MMTV-neu mice. Immunofluorescent staining with anti-p65 antibody of disaggregated tumor cells treated ex vivo with 2μM Curaxin-137 for 6 h or 10ng/ml TNF for 2 h as a positive control. E. Effect of Curaxin-137 treatment on p53 protein levels in normal mammary cells and mammary tumor cells from MMTV-neu mice. Western blotting of protein extracts of cells isolated from tumor free mammary gland and several different tumors of two mice. Cells were left untreated (-) or treated ex vivo with increasing concentrations of Curaxin-137 (1-10μM) for 6 h. Based on the difference between basal and induced level of p53 protein p53 pathway in cells
was proposed: (+) – active (p53 is most probably wild type), (-) – non active (p53 is most probably mutated.

**Figure 4. Safety of chronic administration of Curaxin-137 with drinking water.** A-E. Groups of MMTV-neu mice (n=5/group) were given regular water (control) or solutions of Curaxin-137 (0.1 or 0.2 mg/ml) *ad libitum* from 4 to 14 weeks of age. A. The rate of liquid consumption by MMTV-mice was similar for groups given regular water or solutions of Curaxin-137 (0.1 or 0.2 mg/ml). Y-axis calculated amount consumed by every mouse in a cage per day based on weighting bottles once a week. B. Actual dose of Curaxin-137 delivered with drinking water. The average daily dose of Curaxin-137 was calculated using measurements from A and C. C. Curaxin-treated and control mice gained weight at similar rates. The average body weight of mice in the three study groups is shown normalized to the average weight for each group at 4 weeks of age. Error bars indicate standard deviation between 5 mice per group. D. Kaplan-Meier survival curves illustrating non-tumor-related mortality of MMTV-neu animals in the three study groups. 0.1mg/ml group started treatment at 4 weeks of age and 0.2mg/ml at 10 weeks of age. No tumors were visible or palpable in mice that died before 44 weeks of age. Data were analyzed using MedCalc v.11.3.3 and survival curves were compared using Log-rank test, p >0.1 for each Curaxin-treated group versus the control group. E. Hierarchical clustering analysis of gene expression profiles of samples isolated from 2 mice in each treatment group, control (plain
water), and treated 0.1 and 0.2 mg/ml of Curaxin-137 from 4 to 14 weeks of age. Liver and spleen RNAs were analyzed.

**Figure 5. Effect of chronic administration of Curaxin-137 on overall and tumor-free survival of female MMTV-neu transgenic mice.** A. Curaxin-137 (here, CBL 137) prolonged overall survival of mice in a dose-dependent manner. Kaplan-Meier survival curves are shown for each group of animals (control---regular drinking water, or 0.1 or 0.2 mg/ml Curaxin-137 (CBLC137) in drinking water; n=19-25/group). Data were analyzed using MedCalc v.11.3.3; survival curves were compared using the Log-rank test; p<0.0001 for both treatment groups. B. Tumor onset (appearance of visible tumors) was delayed in animals treated with Curaxin-137 (CBL 137) in a dose-dependent manner. Kaplan-Meier curves of tumor-free survival were generated for the groups described in A. and were analyzed as in A.; p<0.001 for both treatment groups. C. Tumor multiplicity in three groups of mice shown as average number of tumors of different size category (big, medium, small) in mice determined upon necropsy. Error bars – standard deviation between 19-25 animals analyzed per each group. P-values (t-test) are shown above bars different from the controls with p<0.05. D. Lifespan of mice with tumors was not different between treatment groups. Bars are average life span of mice in each group from the moment of tumor appearance and till the cumulative tumor size reached 2cm³. Error bars – standard deviation between (19-25 mice in each group), p values show insignificant difference of treated groups from control (t-test)
Figure 6. Analysis of histological types of mammary tumors observed in control and Curaxin-137-treated female MMTV-neu mice. A-F. Microphotographs of H&E-stained sections of mouse mammary tumors of different histological subtypes: “undifferentiated” (A), “low grade” with poorly differentiated, glandular-like structures (B) or thick tubular structures (C), “glandular” (D), carcinoma in situ (E), hyperplastic mammary epithelia (F). G. Proportion of tumors of different types (A-F) among experimental groups. P values demonstrates significance of difference in the number of tumors of different histological types between treated and control animals according to Chi-square test.
Figure 3
Figure 5
Targeting FACT complex suppresses mammary tumorigenesis in Her2/neu transgenic mice

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