Catechin complexed with lysine has potent antitumor activities in human breast cancer xenograft model

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Abstract

Aims: Mitochondrially-produced superoxide anion has been recently recognized as a factor responsible for metastasis induction and development of secondary tumor lesions. Certain polyphenols, such as catechins, have strong antioxidant properties that may be exerted specifically at the intracellular site of superoxide production and have been proved to be effective against the development and progression of various cancer types. The purpose of this work was the evaluation of the protective activities of a novel antioxidant conjugate of (+)-catechin:lysine 1:2 against the metastatic phenotype of breast cancer cells.

Methods: We utilized standard toxicity and viability assays to determine the anticancer activity of the complex in vitro in various cancerous cell lines, with emphasis given on the human breast cancer cell line MDA-MB231. The implicated protective mechanisms of (+)-catechin:lysine 1:2 against MDA-MB231 cell migration and invasion were studied using expression analysis of certain metastatic (epithelial-mesenchymal transition; EMT) and proliferation markers. The possibility of the complex to protect against breast cancer progression in vivo was investigated using a xenograft model of immunocompromised mice generated by orthotopic implantation of GFP-tagged MDA-MB231 cells. Results: We determined that the action of (+)-catechin:lysine 1:2 in vitro was time- and dose-dependent, and identified a sublethal concentration which was able to potently inhibit breast cancer migration and invasion by over 50%. These effects were notably accompanied by differences in expression of proliferation (p38), DNA-repair (p53), EMT (pyk2/SNAIL) and endoplasmic reticulum stress (GRP78) markers. In vivo, the complex was found to similarly affect primary tumors but not to inhibit metastasis incidence to lungs. Conclusion: Our results suggest that the antioxidant action of (+)-catechin:lysine 1:2 complex may exert protective effects against breast cancer progression in xenograft models and that further experiments are required to assess its efficacy in combination with other widely used treatments in clinic. Of note, manipulation of p53-dependent pathways may account for its anticancer potency.
**Comments:** This work was performed in Universite Catholique de Louvain Medical School, where the authors were occupied as postdocs in the lab of Pierre Sonveaux. The project was supervised by P. Sonveaux, but due to conflicts of interests, he is not placed as an author. However, the official publication and distribution of the current work is on P. Sonveaux’ discretion and judgement. We do acknowledge P. Niebes and H. May for the provision of the Catechin:lysine 1:2 complex.

1. **Introduction**

Breast cancer is among the most common gynecological cancers worldwide, which, despite treatment advances, breast cancer-associated mortality is still the second leading cause of cancer-related death among women in the United States [1]. In addition to hormonal-based therapy and chemo- and radio-therapy, therapeutic strategies are focusing on dietary-derived compounds which are showing promising potential. Such dietary factors are the polyphenols, members of a large family of phytochemical compounds which are present in almost all plant foods, particularly in fruit, vegetables, coffee and tea. The most extensively studied polyphenols in cancer research are the tea catechins, which among others, include (−)-epigallocatechin 3-gallate (EGCG) and (+)-catechin [2]. For instance, epidemiological studies have indicated an association between tea consumption and decreased risk of breast cancer [3, 4].

The anticancer activity of tea catechins has been attributed to alterations of various intracellular pathways of the cancer cell, almost all being primarily triggered by the antioxidant potency of the compounds. Reduction of highly required -by the cancer cell- intracellular reactive oxygen species (ROS) suppresses carcinogenesis [5]. This reduction is the result of the direct scavenging action of catechins on free radicals [6], as well as to the indirect increases of endogenous antioxidant enzymes, such as catalase and superoxide dismutase (SOD) [7]. These events gradually lead to stimulation of DNA repair and breast cancer cell apoptosis [8], and to inhibition of DNA methylation [9], angiogenesis [10] and metastasis [11].

Although among all catechins EGCG has been most extensively studied in cancer research and is currently being tested in 37 clinical trials, it has limitations as it can undergo self-oxidation [12] and trigger toxicity to normal tissues [13], in doses higher than 800mg/day [14]. On the other hand, (+)-catechin (cyanidanol-3) is more stable than EGCG and has the advantage that it can form complexes with one or two lysines, a property that can amplify (+)-catechin antioxidant potency over 400 times [15], and allow it to directly inhibit disease-associated enzymes such as xanthine oxidase, which is a superoxide-producing enzyme [16]. Complexed to lysine, (+)-catechin water solubility increases with an overall positive charge.
that allows the complex to enter mitochondria and exert its antioxidant effects predominantly at this site [17]. This is of particular importance, as our group has previously demonstrated that mitochondrial superoxide is a key driver of metastasis [18]. In support of this, (+)-catechin:lysine complexes have been demonstrated to prevent melanoma cell metastasis to lungs of mice [19] and also exert anti-migratory and pro-apoptotic effects on pancreatic, colorectal and breast cancer cells, without affecting normal cells [20]. In the light of these results, we sought to investigate and determine the anticancer and antimitastatic effects of (+)-catechin:lysine to experimental breast cancer, using in vitro MDA-MB231 cells and in vivo xenograft model of breast cancer, following orthotopic injections of MDA-MB231 cells to the mammary glands of immunocompromised mice.

2. Methods and Materials

2.1 Cells and treatments

Cell lines were purchased from ECACC, ATCC or Caliper. Super-invasive mouse melanoma B16M4 and human cervical cancer SiHa-F3 cells were obtained by serial in vivo and in vitro selection, respectively, as previously described [18]. The standard culture medium for the human breast cancer cell line MDA-MB-231 and its luciferase-expressing variant (MDA-MB-231-LUC-D3H2LN), as well as for mouse breast cancer 4T1 cells, mouse melanoma B16F10 and B16M4 cells and human cervical (SiHa and SiHaF3) and laryngeal cancer cells, SQD9, was DMEM containing 4.5 g/L glucose and GlutaMax (Gibco), 10% (v/v) FBS. The standard culture medium for the human super-metastatic breast cancer MCF10CA1a was DMEM/Ham’s F12 (1:1) supplemented with 5% (v/v) horse serum, 1.05 mmol/L CaCl2 and 10 mmol/L HEPES, while for MCF10 (normal breast epithelial) and MCF10AT1 (highly invasive) cell lines was the same, but further containing 10 µg/mL insulin, 20 ng/mL epidermal growth factor (EGF) and 0.5 µg/mL hydrocortisone. The standard culture medium for the U373 and T98G cell lines was RPMI containing 4.5 g/L glucose and GlutaMax (Gibco), 10% (v/v) FBS. All cells were routinely maintained at a sub-confluent level for no more than 15 passages in standard culture media, in a humidified atmosphere with 95% O2 and 5% CO2 at 37°C. All treatments took place in standard tissue culture media under low serum (1%) conditions. (+)-Catechin:lysine 1:2 was dissolved in PBS to yield a 5 mmol/L stock solution, which was serially diluted in treatment medium to yield the final treatment concentrations.

2.2 Chemicals

(+)-Catechin [(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(H)-benzopyran-3,5,7-triol], also known as cyanidanol-3, was extracted and purified from Uncaria gambir according to Niebes P et al. 1981.
For the production of the catechin/lysine complex, (+)-catechin was dissolved with D,L-lysine monohydrochloride at a 1:2 (mol/mol) ratio in culture medium buffered at pH 7.4. Unless specified otherwise, all other chemicals were from Sigma-Aldrich.

2.3 MTT assay

Cellular metabolism was assessed via measurement of enzymatic conversion of the yellow tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Calbiochem) into purple formazan, as previously described [21]. For the MTT assay, 10,000–20,000 cells were plated in each well of a 96-well plate containing treatment media and incubated for 0-72h. Following the incubation period, media were discarded and replaced by 100 µl of MTT solution (1% serum medium containing 0.2 mg/mL MTT) for 1h. Thereafter, the formazan formed was solubilized in 100 µL DMSO and absorbance at 540 nm was measured with a Victor X4 spectrophotometer.

2.4 Cell Counts

5,000–10,000 cells were plated in 96-well plates and treated with increasing concentrations (+)catechin:lysine 1:2 for 0-72h. The cell number under each treatment was obtained by counts facilitated by Spectramax. The change in cell number following each day of treatment are expressed as ratios to day 0 controls.

2.5 Cell Migration

Cell migration was assayed in a 48-well micro-chemotaxis chamber (Neuroprobe AP48) with polycarbonate porous membrane (8 µm diameter, Neuroprobe PFB8) according to the manufacturer’s instructions. Briefly, 20,000 MDA-MB-231 cells of each treatment condition were seeded in the upper compartment in standard culture media deprived of FBS, while 0.2% (v/v) FBS-containing medium was used as chemo-attractant. Cells well allowed to migrate to the membrane overnight (16h) and the next day, the membranes were washed with PBS, fixed in methanol and stained with Crystal Violet (0.23% v/v), observed and pictured under an Anxiovert microscope equipped with a Mrc camera (Zeiss). Analysis of the covered – by migrating cells – area was performed using the Image J software (Java).

2.6 Cell Invasion

Invasion assays were performed in a 5% serum gradient using BD BioCoat Matrigel transwells. The BD matrigel serves as a reconstituted basement membrane. Briefly, the matrigel was applied to the bottom of the transwell for at least 2 hours prior seeding of cells within the transwell. MDA-MB231 cells
previously treated with (+)-catechin:lysine 1:2 for 48h were seeded in a density of 200,000 cells per transwell, in serum-deprived standard culture media and were allowed to invade the matrigel overnight. The following day, the transwells were washed and the invading cells were stained with Crystal Violet (0.23% v/v), observed and pictured under an Anxiovert microscope equipped with a Mrc camera (Zeiss). Analysis of the covered – by invading cells – area was performed using the Image J software (Java).

2.7 Protein expression analysis (Western blotting)

Total protein was extracted MDA-MB231, SiHa-F3 and B16M4 cells using the modified protein extraction buffer (RIPA) (150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L Na₂EDTA, 1% (v/v) Triton-X 100, 0.25% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulphate (SDS)) containing protease inhibitor cocktail. Following centrifugation at 10,000 g for 30 min at 4°C, supernatants were collected and assayed for protein content using the Bradford assay. 30–60 μg of protein was loaded into each lane of a 10% polyacrylamide gel, in the presence of SDS, and proteins were allowed to separate at 120 V for 90 min. Transfer of proteins to PVDF membranes was performed at 4°C at 100 V for 80 min. The membranes were then blocked using 5% (w/v) powdered milk for 1 h and incubated with the primary antibodies overnight at 4°C. All antibodies were obtained from Cell Signaling Technology, unless otherwise stated. The primary antibodies used and final concentrations (which were based on manufacturer’s recommendations) were: rabbit anti-pyk2, 1:1,000 dilution; rabbit anti-p-pyk2 (Tyr 402; Santa Cruz Biotech) 1:500 dilution; rabbit antiGRP78, 1:1,000 dilution; mouse anti-β-actin (Sigma-Aldrich), 1:1,000 dilution; rabbit p-ERK1,2, 1:1,000 dilution; rabbit anti-p-p38 (p-Thr180/Tyr-182), 1:1,000 dilution; rabbit anti-p-p38, rabbit anti-p53, 1:1,000 dilution; rabbit anti-E-cadherin, 1:1,000 dilution, rabbit anti-Akt, 1:1,000 dilution; rabbit anti-p-Akt (p-Ser473), 1:1,000 dilution; rabbit anti-SNAIL, 1:1,000 dilution; rabbit anti-Caspase-3, 1:500 dilution. Immunodetection was performed at room temperature for 1 h using an appropriate secondary peroxidase-coupled antibody diluted 1:5000 in Tween-Tris buffered saline containing 1 or 5% (w/v) milk or BSA. Visualization of protein bands was performed using an ECL Plus chemiluminescent detection system (GE Healthcare RPN 2209). Densitometric analysis was performed using Image J (Java) and the results were normalized using β-actin measures.

2.8 Animals

All animal experiments involved mice purchased from Charles River Laboratories and were approved by the UCL Ethical Committee in Agreement with Belgian and European laws. All animals were housed in 12 h light/12 h conditions, fully accessible to chow and water. For the pharmacokinetic
experiments, 8-weeks old female BALB/cAnNCrl mice were used. For the xenograft-bearing mouse models of breast cancer, 6-8 week old immunodeficient female SCID mice were used.

2.9 Pharmacokinetics of (+)-catechin:lysine 1:2 after oral administration

BALB/cAnNCrl mice when reached age of 8 weeks received by oral gavage increasing concentrations of (+)-catechin:lysine 1:2 dissolved in drinking water. Two hours following drug administration, animals were anesthetized using a ketamine/xylazine solution and blood was collected via puncture of the facial vein (cheek). Animals were then sacrificed by cervical dislocation. Plasma was isolated from blood following centrifugation of the samples at 3,500 rpm for 10 min, and was sent to CER Marloie for (+)-catechin:lysine 1:2 determination.

2.10 Mouse model of breast cancer (xenograft)

Two separate experiments, one of acute oral administration and one of low dose long lasting treatment, were performed on a mouse model of breast cancer (xenograft), testing the effects of (+)-catechin:lysine 1:2 on the primary tumors and metastasis incidence.

For the acute oral administration experiment, MDA-MB-231-LUC-D3H2LN human breast cancer cells expressing firefly luciferase were orthotopically injected either to the I4 inguinal mammary fat glands of immunodeficient 8-week old female SCID mice at approximately 1 x 10^6 cells/injection. 30 days post initial implantation of the tumor cells, at the point where tumors had reached, at least in some of the animals, 0.5 cm in diameter, the animals were assigned into 2 different groups. Each group contained mice with comparable tumor sizes. Animals then received daily oral gavage of either vehicle (water) or (+)-catechin:lysine 1:2 (500 mg/kg) for up to 16 days. During this period, tumor size (volume) was measured every three days with a caliper as indicated by the formula: Volume_{tumor}=(length_{tumor} \times width_{tumor}^2)/2. At the end of the experiment, blood was collected via puncture of the facial vein, animals were sacrificed, and the lungs were removed and examined for ex vivo bioluminescence.

For the low dose-chronic administration experiment, 6-week old SCID mice (18-21g) received an orthotopic injection of MDA-MB-231-LUC-D3H2LN human breast cancer cells expressing firefly luciferase (1x10^6 cells/site) at the T2 mammary fat pad. The tumor xenograft was inoculated for 14 days, until it reached an average diameter of 0.5 cm, at the point of which drug administration begun. Animals were sorted into two groups; one receiving vehicle [0.5% v/v carboxy-methyl-cellulose (CMC) in drinking water]
and one receiving (+)-catechin:lysine 1:2 (80mg/kg) dissolved in vehicle solution (calculated as 80mg/kg to be dissolved in 3 mL of treatment solution per mouse), on a daily basis. The 80 mg/kg dose was calculated to be administered per 3 mL of average drinking volume per mouse. The treating solutions were prepared fresh every day and were given to the animals over a 38-day period. At day 22-25 post-tumor implantation, the animals were operated and primary tumors were resected due to the reach of a size of over 1 cm in diameter. Drug administration continued as normal until the end of the experiment, when the animals were anesthetized and sacrificed and their lungs were processed for bioluminescence and histopathological analyses.

2.11 Bioluminescence and analysis

Anesthetized mice were injected intraperitoneally with 2.5 mg of D-Luciferin (Xenogen) in saline. 10 minutes after injection, animals were sacrificed and their lungs were inflated with 2.5 mg/mL D-luciferin, removed and placed in D-luciferin solution. Bioluminescence images were then acquired with the IVIS Imaging System (Xenogen). Acquisition times at the beginning of the time course started at 60 seconds and were reduced in accordance with signal strength to avoid saturation. Analysis was performed using LivingImage software (Xenogen) by measurement of photon flux (measured in photons/s/cm²/steradian) with a region of interest (ROI) drawn around the bioluminescence signal to be measured. In order to correlate metastasis incidence to primary tumor size, bioluminescence data were divided by the primary tumor sized resected from the respective animal at day 22-25 post-tumor implantation.

2.12 Histological analysis of the xenografts

All of the reagents used in the procedure were purchased from BD Bioscience. Following sacrifice of animals, the xenografts were resected, fixed in 10 % buffered formalin, embedded in paraffin, sectioned and processed with a conventional hematoxylin and eosin (H&E) staining protocol for the visualization of the tissue. Percentage of necrotic area (pink) in each xenograft image was calculated by Image J (Java) with the equation: Area (necrotic) / Area (total xenograft) × 100 %.

2.13 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) for n independent observations (replicates) as indicated. N is the number of independent experiments. Statistical differences between mean values of groups were determined using either non-paired t-tests for comparison of two means or one way analysis of variance (ANOVA) followed by Dunnett's post-significance test for comparison of
multiple means, with commercially available software (Graphpad Prism, version 4.0, Graphpad Software, San Diego, CA, USA). The level of significance was set at P<0.05. On dot plots, each dot represents a single mouse.

3. Results

3.1 (+)-catechin:lysine 1:2 is well tolerable in all cancerous cell lines tested, affecting cellular viability and number only after 48-72h treatments in doses over 200μmol/L

We aimed to determine in vitro the sublethal concentrations of the compound in order to utilize these concentrations for the later experiments to exclude toxic events while assessing anticancer effects. (+)-Catechin:lysine 1:2 appeared to affect cell number and metabolic activity only after 48h of treatment. Specifically, the complex was well tolerable in concentrations of up to 200 μmol/L in human cervical cancer cells, squamous cell carcinoma, glioma and breast cancer cells, as well as in mouse breast cancer cells, as direct cell counts revealed (Fig. 1). The results of 24h treatment periods are not shown. Consistent to the results of cell counts, the metabolism of cells, as evident by MTT assays, was not greatly affected in human MDA-MB231 cells after 48h treatment with (+)-catechin:lysine 1:2 (Fig. S1A), while in 72h treatments, the metabolic activity of human breast cancer cells and metastatic variants was dropped to nearly 70% under the 200 μmol/L dose, when healthy breast epithelial cells (MCF-10A) were not affected at all (Fig. S1B). These results suggest that the complex is affecting preferentially cancer cells rather than healthy cells, possibly due to their greater dependency to ROS for proliferation. In further support of this event, the metabolism of the highly metastatic mouse melanoma B16M4 variant was shown to be affected more than the parental melanoma B16F10 cell line by (+)-catechin:lysine 1:2 under 72h treatments in concentrations over 200 μmol/L (Fig. S1C).

3.2 (+)-catechin:lysine 1:2 dose-dependently inhibits mouse breast cancer cell migration and invasion, by affecting the expression of -relevant to these actions- proteins

The determination of sublethal conditions of (+)-catechin:lysine 1:2 treatment indicated above allowed the investigation of the antimigratory and anti-invasive properties of the complex. Indeed, 48h treatment of MDA-MB231 cells with (+)-catechin:lysine 1:2 resulted in dose-dependent decrease of migration (Fig. 2A) and invasion (Fig. 2B) ability of these cells through matrices. Additional validation of this result came from the similar response observed in highly metastatic human MCF-10C1A1 cells treated with (+)-catechin:lysine 1:2 for 72h (data not shown). Western blot analysis against candidate proliferation, viability and metastatic markers revealed that these antimigratory and anti-invasive effects
were accompanied by increased levels of p38 phosphorylation and GRP78, indicating a possible involvement of the ER stress response (Fig. 2C-E). It should be noted that p38 phosphorylation is considered as an event of cell cycle arrest and pro-apoptotic signaling in breast cancer [22, 23]. Further, there was a notion of decreased viability (as evident by reduced phospho-Akt levels) and proline-rich tyrosine kinase 2 (pyk2) phosphorylation, the latter being considered as a metastatic marker [24] (Fig. 2C-E). In addition, the expression levels of SNAIL, a protein associated with the establishment of breast cancer migration and invasion properties [25] were found to be downregulated in response to 72h treatment with (+)-catechin:lysine 1:2 (Fig. 2C-E).

3.3 Effects of acute administration of (+)-catechin:lysine (500mg/kg) to primary breast tumors developed in mice

Before assessing the anticancer properties of (+)-catechin:lysine 1:2 in vivo, we aimed to determine the drug concentrations reached in the plasma of mice following administration of increasing drug doses by oral gavage. The results of this pharmacokinetics experiment revealed that a concentration of (+)-catechin:lysine 1:2 as high as 500 μmol/L can be achieved in the plasma of mice, 2 hours after administration of a 2,000 mg/kg dose (Fig. 3A).

Having matched in vitro conditions with in vivo realistic plasma concentration values, we then performed an acute toxicity experiment, whereas immunocompromised SCID mice bearing xenograft MDA-MB231 tumor implants were treated for either 10 or 16 days with 500 mg/kg (+)-catechin:lysine 1:2. The size of the primary tumors of animals receiving the drug unexpectedly demonstrated a trend of increase (Fig. 3B). Western blot analysis of lysates of the primary tumors revealed interesting patterns of expression of candidate markers. Animals receiving (+)-catechin:lysine 1:2 sacrificed at both time points showed a common pattern of increased levels of p53 and phospho-p38, and reduced levels of phospho-Akt in their primary tumors (Fig. 3C-F), coming to agreement with in vitro results. Paradoxically, the levels of the EMT markers SNAIL and pyk2 were increased in the tumors of drug-treated animals, although without affecting E-cadherin levels (Fig. 3C-F). Caspase-3 and p21 levels appeared reduced in tumors of drug-treated animals compared to those of control animals (Fig. 3C-F).

The paradoxical increase in tumor size, together with the unexpected alterations of the levels of EMT markers in tumors of (+)-catechin:lysine 1:2-treated animals prompted us to perform histological analysis on the tumors based on H/E staining. Interestingly, the core of the tumors of drug-treated animals demonstrated a larger necrotic area compared to control (Fig. 3G), partly explaining the diminished
caspase-3 (apoptotic marker) levels, as necrosis seems to be prevailing apoptosis in response to treatment.

3.4 Effects of chronic mild administration of (+)-catechin:lysine (80mg/kg) to the primary breast tumors developed in mice and to lung metastasis incidence

Based on the efficacy of low concentrations of (+)-catechin:lysine 1:2 against the metastatic potency of melanoma cells in vivo, previously demonstrated by our group [19], we performed a chronic administration scheme of (+)-catechin:lysine 1:2, whereas the drug was administered to female SCID mice bearing MDA-MB231 xenografts at a concentration of 80mg/kg for a period of 38 days. During this period, water consumption (in which the complex was dissolved) was monitored and was not different between vehicle-treated and drug-treated groups of mice (data not shown). The incidence of lung metastasis was shown not to be affected by the treatment, as there were no differences between the secondary lung lesion bioluminescence signal (Fig. 4A), number of lung metastases (Fig. 4B) or area covered by metastatic loci (Fig. 4C) between vehicle-treated and (+)-catechin:lysine 1:2-treated mice. However, a similar pattern as to the acute toxicity experiment was observed, whereas the primary tumors of drug-treated mice showed a trend of increase in size (Fig. 1E) and of the necrotic area of the core (Fig. 1F).

Although the metastatic potency of MDA-MB231 was not affected by the treatment, the results analysis of expression levels of certain markers agreed to those of the acute toxicity experiment. Specifically, p53 expression was increased in the primary tumors of (+)-catechin:lysine 1:2-treated animals, while E-cadherin levels remained unchanged compared to the control group (Fig. 5A-B). Of note, chronic administration of the treatment seemed to switch the necrotic pathway towards apoptosis, as evident by increased PARP cleavage and caspase-3 levels (Fig. 5A-B).

Discussion

There is plenty of evidence that catechins can provide an additional tool for the treatment of cancer. Whether they could serve as a therapeutic regime on their own or be used complementarily to current therapies such as chemotherapy remains to be clarified by clinical studies ongoing. However, it has been well established that catechins have diverse modes of anticancer action based primarily on their ability to reduce intracellular ROS levels. Cancer cells maintain large intracellular ROS pools due to their high metabolic rates and oncogene activation and account to their increased capacity to proliferate. While there is still a debate whether ROS scavenging or induction instead is the proper way to combat cancer [26], ROS in cancer cells are considered oncogenic as they trigger oncogenic DNA-damage, but can also
promote anoikis resistance, thereby enabling metastasis [27]. Based on their antioxidant activity, catechins have been shown to exert anti-estrogenic [28], antiangiogenic [29] [30], anti-proliferative [8] and pro-apoptotic [31] effects in breast cancer models.

Cancer cell migration and invasion constitute key steps for the process of metastasis. Catechins have been shown to partially block all these events in various breast cancer in vitro models. For instance, EGCG was shown to suppress the EMT process and inhibit angiogenesis, cell migration and invasion in vitro [10]. While there is plenty of evidence of the in vitro antimetastatic effects of catechins, there is limited literature on in vivo antimetastatic ability of breast cancer cells, as also highlighted by a recent review on the topic [32]. In MDA-MB231 xenograft model, EGCG was shown to inhibit primary tumor growth but authors did not provide any evidence for metastasis inhibition [33]. Rather, we have previously shown that (+)-catechin:lysine 1:2 inhibits melanoma cell metastasis in mice but the cells were treated with the drug prior infusion to the bloodstream of mice, thus deviating from the pharmacological view of treatment. For breast cancer, the most relevant studies revealing antimetastatic actions of polyphenols were focusing on combined polyphenol supplements, such as green tea [11] and grape [34] extracts, rather than individual catechins alone. Thus, any additional data regarding isolated catechin effects on breast cancer metastasis are pivotal for the understanding of their benefits against this process of cancer progression.

Since mitochondrial superoxide is viewed as the factor for metastasis initiation [18], investigation of anticancer properties of (+)-catechin moieties complexed to lysine residues are of increasing interest, as these can selectively remove mitochondrial superoxide [17]. The evidence of the effects of these complexes come mainly from our lab and collaborators. Specifically, (+)-catechine:lysine complexes were shown to be more effective superoxide scavengers than EGCG, based on studies using DCFDA probes in human cervical cancer cells [19]. Similar results we obtained in MDA-MB231 cells by flow cytometric analysis using MitoSOX probes which are specific mitochondrial superoxide detectors, thus ensuring that the complex is acting within mitochondria (data not shown). Recent data from collaborator’s lab indicated that (+)-catechin:lysine 1:2 is inhibiting migration of MDA-MB231 breast cancer cells, by mechanisms involving inhibition of JAK/STAT pathway and Wnt signaling, as well as promoting apoptosis. In that study, the concentration of (+)-catechin:lysine 1:2 that proved effective was the 1mmol/L dose for 24 hours. Here, we present a complete scan of a range of concentrations and we demonstrate that our effective dose of (+)-catechin:lysine 1:2 is 5- to 10-fold less of that study whereby excluding any possible toxic effects of the compound to cells, and that this concentration proved reliable in terms that this can be
reached in the plasma of mice orally-dosed with the complex. Inhibition of Wnt signaling and JAK/STAT pathway has been reported in response to catechin treatments [31, 35] and may represent a common effect of catechins in breast cancer cells.

We also demonstrate that (+)-catechin:lysine 1:2 inhibits MDA-MB231 cell invasion together with migration, in a dose-dependent manner, indicating potential markers responsible for these events, such as induction of the ER stress response and p38 signaling and reduced viability, as evident by reduced phosphorylation of Akt. Similar results in breast cancer cells have been obtained in response to EGCG treatments [36, 37]. We have previously demonstrated that ER stress signaling can induce JNK and p38-induced apoptosis [38] and this could be likely the case here, an assumption that awaits verification. Further, the fact that in prolonged (72h) treatment with (+)-catechin:lysine 1:2 the GRP78 upregulation event is lost (Fig. 2C,2E) suggests that ER stress may be an initial event under the treatment, and later the cells have entered the apoptotic pathway, without necessity to upregulate GRP78.

In addition, under the in vitro treatments of the MDA-MB231 cells, we found that markers associated to metastasis were deregulated, as pyk2 phosphorylation and SNAIL levels were reduced. At the same time, p38 phosphorylation was greatly increased. Of note, pyk 2 (also called focal adhesion kinase 2; FAK2) is a ROS-sensitive cytoplasmic tyrosine kinase that plays a role in cellular adhesion and is conceived as a metastatic marker in various forms of human cancer [39], including breast cancer in humans [40]. Similarly, SNAIL, is a transcription factor that can be induced under DNA damage responses in breast cancer and trigger metastasis [41], although metastasis induction in MDA-MB231 cells is primarily driven by SLUG [42]. In addition, although the stress-inducible p38 MAPK has highly debatable effects on tumor progression [43], it has been shown that it can inhibit breast cancer metastasis [44]. Without having measured the direct effects of (+)-catechin:lysine 1:2 directly on DNA-damage parameters, all our in vitro data, taken together, allows the assumption to be made that the complex triggers p38-mediated (induced either by ER stress or DNA-damage) proliferation inhibition and decrease in metastatic potential of MDA-MB231 cells.

In attempts to validate the effects of (+)-catechin:lysine 1:2 on primary tumor growth following acute drug administration, we observed a robust increase of p53 and p38 phosphorylation levels, associated with decrease of the viability marker p-Akt and larger necrotic areas at the core of the tumors. The results of chronic administration experiment with lower (+)-catechin:lysine 1:2 doses revealed that the necrotic pattern gives its place to apoptosis, with also apparent p53 increases. P53 is a tumor suppressor protein [8] which can mediate antimetastatic effects on breast cancer cells [45], also being
able to inhibit EMT via inhibition of β-catenin pathway [46]. For instance, we previously demonstrated that (+)-catechin:lysine 1:2 inhibits Wnt signaling in MDA-MB231 cells [20], thus this could be attributed to the enhanced p53 levels reported here. The absence of an effect of the drug on lung metastasis, together with the unexpected findings of increased levels of the metastatic markers pyk2 and SNAIL, without however an effect on E-cadherin levels, prompts us to suspect that p53 plays a key role in these observations. Interestingly, both pyk2 [47] and SNAIL [48] have been demonstrated to directly inhibit p53 to promote cancer progression and metastasis and we therefore presume that p53 elevations are a stress response signal to the tumors that causes pyk2 and SNAIL upregulation to overcome stress. SNAIL elevations, on the other hand could account for p21 inhibition as previously described [49] and could likely be the case for the relevant observation in the acute administration experiment. Of note, inhibition of apoptosis is the best known oncogenic function of p21. Up to date research directions encompass a therapeutic scheme based on wild type p53 overexpression with p21 downregulation [50], and the results of our study demonstrate that (+)-catechin:lysine 1:2 follows that pattern. At this point, it is hard to predict whether is mutated or wild-type the p53 protein for which we are showing the elevations, and how this affects the overall response of the tumor.

The work described here provide the first attempt in characterizing the anticancer effects of (+)-catechin:lysine 1:2 in a xenograft model, revealing that although in vitro it suppresses metastatic processes and cell growth, in vivo metastasis inhibition is absent. It is important to note certain limitations of this study. These include the selected dosing concentrations of the animals, the duration of the in vivo experiments and the time-points for lung metastases detection. Alterations of these parameters may have great impact on the results. Further, we did not test metastasis detection to other organs, such as liver and bone, sites whereas polyphenol treatment may preferably affect in MDA-MB231 xenograft models [34], rather than lungs. Lastly, in our study, we did not get into detail in assessing all metastatic markers, as the primary aim was to elucidate whether the complex exerts direct antitumor and antimetastatic activity in vivo. More accurate conclusion can be made upon a complete scan of metastasis markers, DNA-damage assays and analysis of MAPK signaling using inhibitors. So far, however, we can conclude that a p53-mediated pyk2/SNAIL/p21 axis explains the positiveness of outcomes with regards to primary tumor and also the lack of metastasis inhibition by (+)-catechin:lysine 1:2 and we point out research focus on catechins towards that direction.
References


List of figures

Figure 1

A

SiHa

B

SiHa-F3

C

SOD9

D

T98G

E

U373

F

MCF-10 A

G

MCF-10 A1

H

MCF-10-C1A1

WITHDRAWN

see manuscript DOI for details
WITHDRAWN

see manuscript DOI for details
Figure 2

A

B

(+) catechin:lysine 1:2 (µmol/L)

Covered area of migratory cells (AU)

% Invasive cells

Coveryad of fmigra
ty ce(ls (A

WITHDRAWN

WITHDRAWN

see manuscript DOI for details

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C

(+)-catechin: lysine 1:2 (μmol/L)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM (+)-catechin:lysine 1:2</td>
<td><img src="image1" alt="Images of Western Blots" /></td>
<td><img src="image2" alt="Images of Western Blots" /></td>
</tr>
<tr>
<td>100 μM (+)-catechin:lysine 1:2</td>
<td><img src="image3" alt="Images of Western Blots" /></td>
<td><img src="image4" alt="Images of Western Blots" /></td>
</tr>
<tr>
<td>200 μM (+)-catechin:lysine 1:2</td>
<td><img src="image5" alt="Images of Western Blots" /></td>
<td><img src="image6" alt="Images of Western Blots" /></td>
</tr>
</tbody>
</table>

**D**

fold change in expression levels

- p-Akt
- p-P38
- GRP78
- p-pyK2
- pyK2
- Snail
- p-ERK1,2

**E**

fold change in expression levels

- p-Akt
- p-P38
- GRP78
- p-pyK2
- pyK2
- Snail
- p-ERK1,2

- 0 μM (+)-catechin:lysine 1:2
- 100 μM (+)-catechin:lysine 1:2
- 200 μM (+)-catechin:lysine 1:2
Figure 4

A

Vehicle (0.5% CMC)

(+) catechin:lysine 1:2
(80 mg/kg)

B

Bioluminescence signal (p/s/cm²/sr)

Vehicle (0.5% CMC)

(+) catechin:lysine 1:2 (80 mg/kg)

n.s.

C

# lung metastases

Vehicle (0.5% CMC)

(+) catechin:lysine 1:2 (80 mg/kg/day)
Figure 5

A

Vehicle (+) catechin: lysine 1:2

E-cadherin
PARP uncleaved cleaved
p-akt
p53
p-p38
p38
Casp3
actin

B

protein expression fold change

vehicle
catechin: lysine 1:2

pp38 p38 E-cadherin cleaved PARP Casp3

* ***
Figure S1

A

![Graph A showing MTT absorbance at 540 nm for MDA-MB-231 cells.](image)

B

![Graph B showing MTT absorbance at 540 nm for MCF10A, MDA-MB231, MCF10-CA1, and MCF10-AT1 cells.](image)

C

![Graph C showing MTT absorbance at 540 nm for B16F10 and B16M4 cells.](image)
Figure legends

**Figure 1. (+)-Catechin:lysine 1:2 does not greatly affect cell number up to 0.2 mmol/L treatment dose.**

Cell number change over a 48h treatment with (+)-catechin:lysine 1:2 was determined for human cancerous cell lines, including cervical cancer cells (A, B), squamous cell carcinoma (C), glioma (D, E), breast cancer cells (F-I), as well as for mouse breast cancer (K) and melanoma (L, M) cells. Data represent mean ± SEM; * P < 0.05, ** P < 0.01, *** P < 0.005 compared to cell number change of untreated (UT) control. N=3-4, n=9-51.

**Figure 2. (+)-Catechin:lysine 1:2 dose-dependently inhibits breast cancer cell migration and invasion via modulation of certain migratory and invasive protein markers.** Human MDA-MB-231 breast cancer cells were treated for 48h with (+)-catechin:lysine 1:2 and were allowed to migrate towards a serum gradient and invade through matrigel (A) migration representative images and analysis of MDA-MB231 cells following 48h treatment with (+)-catechin:lysine 1:2; (B) invasion representative images and analysis of MDA-MB231 cells following 48h treatment with (+)-catechin:lysine; (C) representative Western blot against certain protein markers in MDA-MB-231 cell lysates, previously treated for 48-72h with (+)-catechin:lysine; (D) Densitometric analysis of protein expression levels following a 48h treatments of cells with (+)-catechin:lysine; (E) Densitometric analysis of protein expression levels following a 72h treatments of cells with (+)-catechin:lysine. Data represent mean ± SEM; * P < 0.05, ** P < 0.01, *** P < 0.005 compared to untreated (UT) control. N=3-4 for all experiments described.

**Figure 3. (+)-Catechin:lysine 1:2 is readily absorbed in plasma and short term acute administration promotes primary tumor necrosis, possibly via p53- and p38-mediated mechanisms.** (A) Plasma absorption of (+)-catechin:lysine 1:2 at 2h following gavage of BALB/c mice with increasing doses of the compound; (B) The effect of (+)-catechin:lysine 1:2 (500mg/kg) on the growth of the primary tumor of MDA-MB-231 tumor-bearing SCID mice (n=9-19 mice/group); (C) Western blot image against certain protein markers in primary tumor protein lysates of animals treated with (+)-catechin:lysine 1:2 (500mg/kg) for 10 days; (D) Densitometric analysis of protein expression levels in the primary tumor lysates of animals treated with (+)-catechin:lysine 1:2 (500mg/kg) for 10 days (n=5 mice/group); (E) Western blot image against certain protein markers in primary tumor protein lysates of animals treated with (+)-catechin:lysine 1:2 (500mg/kg) for 17 days (n=5 mice/group); (F) Densitometric analysis of protein expression levels in the primary tumor lysates of animals treated with (+)-catechin:lysine 1:2 (500mg/kg) for 17 days; (G) Measures of the necrotic areas of the primary tumors (PT) of mice treated...
with/without (+)-catechin:lysine 1:2 (500mg/kg) for 10-17 days (n=10 mice/group). Data represent mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared to vehicle-treated (control) mice.

**Figure 4.** Chronic mild administration of (+)-Catechin:lysine 1:2 does not have a major effect on metastasis incidence or size. (A) Lung bioluminescence of MDA-MB-231 tumor-bearing SCID mice treated with/without (+)-catechin:lysine 1:2 (80mg/kg) for 38 days; (B) Analysis of the bioluminescence signal obtained for individual lungs (n=8-9 mice/group); (C, D) Histopathologic analysis of lung metastases (n=8-9 mice/group); (E) The effect of (+)-catechin:lysine 1:2 (80mg/kg) on primary tumor growth (n=9-10 mice/group); (F) Histopathologic analysis of primary tumor (PT) necrotic areas (n=9-10 mice/group).

**Figure 5.** Chronic mild administration of (+)-Catechin:lysine 1:2 promotes primary tumor apoptosis. (A) Western blot image against certain protein markers in primary tumor protein lysates of animals treated with (+)-catechin:lysine 1:2 (80mg/kg) for 38 days; (B) Densitometric analysis; Data represent mean ± SEM; * $P < 0.05$, *** $P < 0.005$ compared to vehicle-treated (control) mice. n=6-7 mice/group.

**Supplementary Figure 1.** (+)-Catechin:lysine 1:2 does not greatly affect cancer cell metabolism up to 0.2 mmol/L treatment dose. Cellular metabolism was determined by the MTT assay following treatment with (+)-catechin:lysine 1:2 for (A) 48h for the human breast cancer cell line MDA-MB-231 and (B) for 72h for the human breast cancer MCF-10 and MDA-MB-231 cells and (C) mouse melanoma cells. Data represent mean ± SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared to untreated (UT) control. N=3-4, n=8-32.