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New insights in the treatment of chondrosarcomas; role of the hydroxy-methyl-glutaryl-CoA reductase inhibitors "*in vitro*"

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Chondrosarcoma is a very aggressive chemo- and radio-resistant tumor of the adulthood. The search of an effective treatment for this muscle-skeletal tumor has not been successfully achieved. Consequently, the investigation of new drugs for chondrosarcomas is still a priority in bone tumors. Statins are lipid-lowering agents, with pleiotropic effects on the mevalonate pathway, the products of which are vital for a variety of key cellular functions. These drugs have been tested as chemotherapeutic agents in some types of tumors, however there is no evidence of its use in chondrosarcomas. The aim of this work was to evaluate the usefulness of statins treatment *in vitro* in chondrosarcoma cell lines.
Rat chondrosarcoma cell lines (LTC and 422) were grown under conventional conditions. The following parameters were studied after administration of simvastatin at different doses (vehicle, 0.1, 0.3, 1.0, 3.0 and 10 μM) and during different times (24, 48 and 72 hours): cell growth rate by cell counting; cell viability by Trypan blue cell exclusion assay and by MTT; morphologic changes; apoptotic response studied by DAPI, flow cytometry with PI/annexine V and by DNA laddering; cell cycle alterations were analyzed by flow cytometry with PI; and cell motility was studied by cell wound assay. Data was presented as mean ± SEM of at least three different experiments. A rejection level of $p < 0.05$ will be considered significant. It was observed that statins induced: 1. cell rounding and decreased ability of cell adhesion to the substrate; 2. decreased cell viability, assessed by MTT assay, with IC_{50} between 1–2 μM; 3. induction of time- and dose-dependent cell apoptosis; 4. cell growth arrest in G1 and G2/M phases with decreased S phase; 5. alteration of cell motility.

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Dual tyrosine kinase inhibitor PKI 166 alters the contractile structure and function of rat cardiomyocytes

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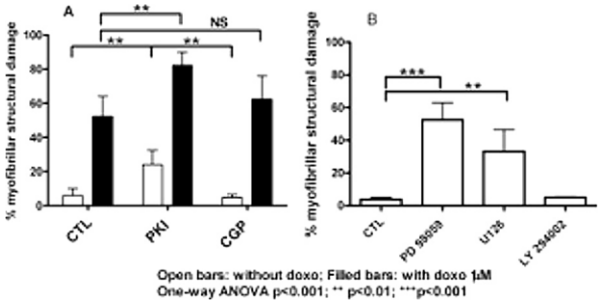
Background: Treatment of selected breast cancer patients with the anti-ErbB2-antibody Herceptin® improves survival but can cause significant cardiac dysfunction, particularly in combination with doxorubicin (doxo). Newer data suggest that combined blockage of ErbB1/EGF- and ErbB2-receptors with dual tyrosine kinase inhibitors (TKI) might further improve anti-cancer efficacy but cardiac safety is unknown. We therefore investigated the effect of two selective, reversible TKI on cardiomyocyte survival and contractile function.
Material and methods: We tested the effect of an ErbB1-(CGP059326-CGP;1 μM/48h) and a dual ErbB1/ErbB2-(PKI166-PKI;1 μM/48h) tyrosine kinase inhibitor in an in-vitro system of cultured (10 days) adult rat ventricular cardiomyocytes and assessed the effect on myocyte 1. LDH release and MTT assay, 2. TUNEL, 3. integrity of the contractile apparatus (immunofluorescence) 4. MAPK- and PKI3-signaling and 5. contractile function (video-edge detection; electrically paced at 2 Hz).



Results: *Myocyte survival*: Neither CGP nor PKI induced necrosis or apoptosis. In contrast, doxo led to a dose-dependent increase of both necrosis and apoptosis.
Myofibrillar Structural Damage (MSD): Since ErbB2-antibodies, especially in combination with anthracyclines, induce MSD to the cardiac contractile apparatus, we tested if CGP or PKI alone or in combination with doxo (1 μM) had a similar effect. PKI but not CGP alone caused MSD (figure A), which was significantly worsened by the addition of doxo (1 μM). Inhibition of MAPK/ERK1/2- (PD98059 50 uM and U126 5 μM), but not of PI3/AKT-signaling (LY294002 10 μM), led to a similar MSD (Figure B).
Signaling: CGP and PKI reduced phosphorylation of AKT (CGP by 40% (NS) and PKI by 30%, $p < 0.05$ vs. CTL, $N = 3$), whereas only PKI reduced ERK 1/2 phosphorylation (30% vs. CTL, $p < 0.05$, $N = 3$).
Contractility: PKI but not CGP attenuated fractional shortening (FS) by 25% (NS). PKI and doxo combined further reduced FS by 70% ($p < 0.0001$; $n = 400$ cells)
CONCLUSIONS: The dual ErbB1/ErbB2-TKI PKI166 can cause MAPK-dependent structural damage to the myocyte contractile apparatus which leads to contractile dysfunction. However, it does not induce myocyte death, suggesting a different pathophysiological mechanism than doxorubicin-induced cardiotoxicity. Only combination of PKI 166 with doxorubicin leads to an additive cardiotoxic effect. Clinical studies with ErbB2-blocking TKI should carefully monitor for cardiac dysfunction.

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Nutritional calcium regulates synthesis of the tumor-preventing steroid hormone 1, 25-dihydroxyvitamin D3 in the mouse colon

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Epidemiological data suggest a protective role of calcium and vitamin D against colorectal tumor pathogenesis. It has been demonstrated previously that colonic epithelial cell hyperproliferation and hyperplasia induced in mice fed a Western-style diet could be reversed by the addition of calcium to the diet. We propose that nutritional calcium in the intestinal lumen can act as a growth-regulator and may prevent cancer by direct reduction of colonocyte proliferation. This could occur by modulating colonic vitamin D synthesis. We investigated a possible interaction between dietary calcium and the colonic 1, 25-D_3-synthesizing machinery. Synthesis of 1.25-D_3 from its precursor 25-hydroxyvitamin D_3 is catalyzed by the mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D_3-1alpha-hydroxylase (CYP27B1), which is present also in human colon carcinoma cells. 25-hydroxyvitamin D_3-24-hydroxylase (CYP24) is the enzyme responsible for the first step in 1.25-D_3 catabolism.
We quantified mRNA levels by real time RT-PCR using the comparative $\Delta\Delta C_T$ method. CYP24, p21, and the proliferating cell nuclear antigen (PCNA) protein levels were measured by revealed by immunohisto-chemistry.
We investigated the concentration-dependent action of dietary calcium on colonic expression of vitamin D receptor (VDR), CYP27B1, CYP24, and on proliferation as shown by PCNA and p21 expression in a mouse model. Mice were fed a modified AIN-76 diet containing 20% lactose and 0.9%, 0.1% or 0.04% calcium. We measured calcium content of the feces and found a significant reduction of fecal calcium content in animals fed low calcium diet. Low fecal calcium concentration had a promitotic effect on crypt cells, increasing PCNA protein expression. Quantitative evaluation of the cyclin dependent kinase inhibitor p21 showed less sensitivity to lowered fecal calcium: only the diet containing 0.04% calcium was able to reduce p21 expression. While low dietary calcium did not affect expression of VDR, we observed a moderate but insignificant increase in CYP27B1 mRNA expression. Low calcium diets significantly augmented CYP24 mRNA expression in the ascending colon, without affecting it at all in the descending colon. We confirmed our claim that low fecal calcium content is the regulator of colonic CYP24 expression by showing *in vitro*, in the Caco-2 colon cancer cell line, that 0.0 mM calcium in a medium without any 1, 25-D_3 indeed increased CYP24 mRNA levels.
We conclude that low dietary and thus luminal calcium concentrations increase CYP24 levels, which may result in reduced colonic accumulation of 1, 25-D_3, decreased p21 expression leading to hyperproliferation and unhindered tumor progression.
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Publication
Basic science

216 PUBLICATION

Nitric oxide reverse arginine deiminase (ADI) induced anti-proliferative activity through NFKB and BCL-XL

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Arginine deiminase (ADI) is an inducer of apoptosis *in vitro* and has an anti-tumor effect *in vivo*. And, nitric oxide (NO) has been reported to be an

anti-apoptotic mediator in cancer cells. Furthermore, ADI causes arginine depletion enzymatically, and thus ADI is an inhibitor of NO synthesis by arginine substrate deprivation. However, the mechanisms of NO in ADI treated cell lines have not been previously elucidated. Here, we analyzed the mechanisms of NO in an ADI treated cell line.
We selected the Ramos human lymphoma cell line, a known ADI sensitive cell line. Having determined the optimum ADI concentration for experimentation, the cells were divided into several groups based on SNP (an NO donor) treatment levels (i.e., a ADI and SNP untreated control, ADI without SNP, ADI with 10 μM/ml SNP, ADI with 50 μM/ml SNP, and ADI with 100 μM/ml SNP). The MTT assay was used to determine cell survival fractions, nitric oxide assays to determine NO levels, and Western blot analysis to determine the expressions of NO mediators, such as NFκB and *Bcl-X_L* antibody.
The optimal ADI experimental concentration was 0.001 U/mL. Surprisingly, SNP treatment reversed ADI induced cell growth inhibition. Furthermore, we found that NFκB and *Bcl-X_L* expressions were induced by SNP.
We believe that ADI-induced Ramos cell growth inhibition is reversed by the NO donor SNP, and that this is mediated by NFκB and *Bcl-X_L*.

217 PUBLICATION

Phase I of intermittent chronomodulated oral therapy with capecitabine in patients with advanced and/or metastatic cancer

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Background: Capecitabine is an orally administered pro-drug of 5-fluorouracil (5-FU) which goes through the intestinal mucosal membrane as an intact molecule. It is subsequently activated by a cascade of three enzymes resulting in the preferential release of 5-FU at the tumor site. In phase I study capecitabine was administered twice daily as outpatient therapy, each cycle administered for 2 weeks followed by 1 week of rest. The recommended phase II dose was 2510 mg/m^2 daily. The rational of capecitabine administration especially in nocturnal hours, as performed in the present report, is just based on the attempt to mime 5-FU chronomodulated infusion.
Material and Methods: The aim of this study was to determine the maximum tolerated dose (MTD) of capecitabine when administered in a chronomodulated way according to the following schedule: 1/4 dose at 8:00 a.m.; 1/4 dose at 6:00 p.m. and 1/2 dose at 11:00 p.m. each day for 14 consecutive days followed by 1 week of rest. A total of 24 pts (17 female, 7 male), aged 49–88 yr (median 75) with a variety of solid tumors (11 breast, 7 colorectal, 2 pancreas, 1 gastric, 1 renal, 1 hemangiopericytoma and 1 unknown primary) have been treated. The starting dose level in our phase I trial was 1500 mg/mq daily. The subsequent dose levels were: 1750, 2000, 2250, 2500, 2750 mg/m^2 daily. The capecitabine dose was escalated when almost 3 patients in a cohort had completed two cycles of treatment. Dose-limiting toxicities (DLT) were determined on the basis of toxicity from the first two cycles. The MTD was defined as the dose level at which no more than one of six patients experienced a DLT. The MTD represents the dose recommended for further studies. All patients except five had been pretreated for cancer.
Results: No DLT occurred at doses of 1500, 1750, 2000, 2250 and 2500 mg/m^2 in any of the almost 3 patients included at each level. At 2750 mg/m^2, 1 of 6 patients experienced DLT (fatigue grade 4 and diarrhoea grade 3). Another 6 patients are being evaluated at the capecitabine 2750 mg/m^2 level, with an ongoing evaluation of cumulative (all cycles) toxicity and efficacy. The other toxicities have been generally mild or moderate in nature with only one case of severe hand–foot syndrome being observed at the fourth cycle in one patient. All these toxicities resolved upon treatment interruption with patients restarting on the chronomodulated schedule where appropriate. The recommended dose for further studies is 2750 mg/m^2 daily for 14 consecutive days followed by 1 week of rest. In terms of response, we have observed 5 PR in breast cancer and 2 PR in colorectal cancer.
Conclusions: In conclusion, chronomodulated capecitabine treatment seems to be a feasible approach which has demonstrated promising clinical activity.

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The investigation of phosphatidylinositol 3-kinase (PI3k) isoforms which express by human prostate cancer cell lines, PC-3 and DU-145

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Matrix metalloproteinases (MMPs) are the most important enzymes which not only degrade basement membranes but also involve in angiogenesis and neovascularization; making possible cellular migration. Phosphatidylinositol 3-kinase (PI3K) is involved in modulating MMPs activities. PTEN is a tumor suppressor gene whose primary function is lipid phosphatase. By dephosphorylating phosphatidyl inositol 3, 4, 5-triphosphate, PTEN antagonizes the PI3K activity. Prostate cancer is one of the most prevalent cancers all over the world. Two highly invasive and metastatic cell lines from prostate cancer, PC3 and DU145, are not the same in respect to PTEN expression status. While DU145 express PTEN mRNA and its protein, PC3 is null for PTEN gene. Nevertheless PC3 is also invasive and metastatic, it was isolated from prostate cancer metastasis to the bone marrow. It was interesting for us to know if there was any difference in PI3K isoforms expression patterns between these two cell lines. For this reason the mRNA content from the cells was analyzed using RT-PCR method.
Surprisingly our data showed that both of the cell lines express identical isoforms. Here, we introduce P110α catalytic subunit and P85 adapter protein from classIA, PI3K-C2 from classII and Vps34p from classIII of PI3K super family as PI3K isoforms which expresse by PC3 and DU145 cells. Now to address any inequality in PI3K isoforms expression, using Real-Time RT-PCR we are going to quantify each isoform mRNA individually.

219 PUBLICATION

Expression and activity of signal transducer and activator of transcription (STAT) pathways in gastric adenocarcinoma

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Objective: Gastric cancer is initiated and progressed through a number of signaling pathways. In the present study, we investigated the expression and activity of signal transducers and activators of transcription (Stat) in gastric cancer cell line as well as in tissues and their relationship with clinicopathological parameters.
Methods: We have obtained 62 tissue specimens from 16 patients of surgically resected gastric adenocarcinoma and AGS gastric cancer cell line. Western blotting of gastric cancer tissues, adjacent normal tissues and AGS cell line were used to detect the expression of Stat1, Stat3 and Stat5. The expression intensity of phosphorylated STAT protein in gastric cancer tissues and adjacent normal tissues were measured by immunohistochemical stains.
Results: Of 16 patients with gastric adenocarcinoma, 14 were male and 2 were female, and median age was 66 years (range, 37 to 80). Eleven patients were at stage III or IV without distant metastasis, while 5 were at stage I or II. Activations of Stat1, Stat3, and Stat5 were observed in AGS cells, gastric cancer tissues and adjacent normal tissues. No significant difference in Stat activity was found between gastric cancer tissues and adjacent normal tissues. Furthermore, STAT activity did not correlate with stage, tumor penetration and nodal spread.
Conclusions: Expressed in gastric cancer tissues and adjacent normal tissues, Stats may play a critical role for development and adjacent penetration in gastric adenocarcinoma.

220 PUBLICATION

Bisphosphonates down-regulate the GAPDH gene expression in prostate and breast cancer cell culture: is the GAPDH a housekeeping or a new target gene?

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The employment of the RT-PCR method has been widely used for the analysis of gene expression in many systems, including tumor samples. The GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) has been commonly considered as a constitutive housekeeping gene to normalize the specif gene expression. However the GAPDH has been shown to be upregulated in cancer. Bisphosphonates (BPs), synthetic analogs of pyrophosphate, are potent inhibitors of bone resorption and recently an antitumor effect has been shown *in vitro* and in animal models by inhibition of the mevalonate pathway. Furthermore BPs has been shown to modulate many gene expression not only in osteoclasts but also in cancer cells.
The aim of this study was to evaluate GAPDH gene expression by real time RT PCR (Applied Biosystems) in different breast (MCF-7 and T-47D) and prostate cancer cell lines (PC-3 and DU-145) lines (purchased from ATTC Rockville, MD, USA). treated with amino and non-amino bisphosphonates (clodronate, pamidronate, alendronate and zoleronate) to exclude, if any,