Characterisation of a novel matrix metalloproteinase inhibitor on pancreatic adenocarcinoma cells *in vitro* and in an orthotopic pancreatic cancer model *in vivo*

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Abstract. Matrix metalloproteinases (MMPs) play a central role in tissue maintenance, inflammation and during tumour invasion and metastasis. The impact of MMPs in cancer has encouraged the development of novel MMP-inhibitors without adverse effects on the cell viability. We describe here the synthesis and characterisation of a triazine-derivative as a highly potent MMP-inhibitor. The new compound Triazin 17-2 was developed on the basis of a triazine backbone as a well known and well tolerated chemical scaffold. It was *de novo* synthesized and tested for MMP inhibition in a cell free assay. *In vitro* characterisation included tests for cell viability, protein expression and MMP activity in PancTu-1 cells. Effectivity of MMP inhibition was analysed *in vitro* by invasion assay. Triazin 17-2 was investigated *in vivo* using an orthotopic pancreatic ductal adenocarcinoma (PDAC) xenograft model in SCID/bg mice. Triazin 17-2 proved to have no adverse effects on cell viability *in vitro* at concentrations effectively inhibiting MMPs in an invasion assay. Application of Triazin 17-2 *in vivo* in the orthotopic PDAC model in SCID/bg mice showed a significant reduction of primary tumour weight using conservative therapy and inhibition of metastasis in adjuvant therapy. The MMP-inhibitor Triazin 17-2 was developed and characterised *in vitro* and *in vivo*. The new compound has no intrinsic activity to kill cells but is very effective in inhibition of MMPs. *In vivo* testing revealed that MMP-inhibitors are useful tools in anticancer therapy reducing tumour size and invasion even without direct effects on cell survival.

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Introduction

Several forms of human cancer, e.g. of the pancreas, ovaries and lung, show significant resistance towards standard cytotoxic chemotherapy (1-4). Considering the poor prognosis of pancreatic cancer the development of new therapeutic approaches is mandatory (5). Growth inhibition by cytostatic drugs represents the first approach towards therapy, inhibition of tumour metastasis and invasion represents a further aspect of therapeutical intervention (6). Thus matrix metalloproteinases (MMPs) responsible for evasion of tumour cells from primary tumour masses and invasion of those cells into distant tissues, were investigated (1,7,8).

Metalloproteinases are a superfamily of enzymes whose numbers have increased in recent years. Based on structural and functional considerations they have been classified into families and subfamilies (9,10). MMPs are zinc-dependent endopeptidases involved in the degradation and remodelling of connective tissues (11). Members of these MMPs are present in various cell types that reside in or are associated with connective tissue, such as fibroblasts, monocytes, macrophages, endothelial cells, and invasive or metastatic tumour cells (12). MMP expression is stimulated by growth factors and cytokines in the local tissue environment, where these enzymes act to specifically degrade protein components of the extracellular matrix, such as collagen, proteoglycans, fibronectin and laminin (13,14). These ubiquitous extracellular matrix components are present in the linings of joints, interstitial connective tissues, basement membranes, cartilage and other sites (15-17).

Excessive degradation of extracellular matrix by MMPs is implicated in the pathogenesis of many diseases; here we concentrate on tumour metastasis, invasion and growth, and present data on a novel compound inhibiting MMPs (18,19).

The design and use of synthetic metalloproteinase inhibitors (MMP-Is) are the subject of numerous publications and patent applications (20). Recent efforts by a number of laboratories have provided several classes of MMP-Is which have been extensively reviewed (21). In general, each class contains a zinc ligand attached to a small peptide fragment or peptide mimetic which is capable of binding to specificity pockets of the MMP enzymes. Hydroxamates as the zinc ligand provides for the most effective MMP-Is. However, hydroxamates are often found to be biologically labile which has prompted additional efforts toward the discovery of new chelating groups suitable for use in MMP-I templates. The value of triazines as inhibitors of MMPs has not hitherto been recognized. They provide potency for high selectivity against individual MMPs and potentially are novel, nonpeptidic alternatives to peptide-mimetic hydroxamate MMP-Is.

Various MMP-Is developed since showed effectiveness *in vitro* as well as in animal models of various tumours (22-25). However, in several phase II and III clinical trials none of these newly developed MMP-Is was highly effective and without severe side effects (26). This implicates the need for new therapeutic approaches to tumour invasion and metastasis with new inhibitory compounds. We report here the development of a new class of MMP-Is based on a triazine backbone, the characterisation in cell free systems, studies on cells *in vitro* and in an orthotopic pancreatic xenotransplant cancer model *in vivo* as a new promising approach to therapy.

Materials and methods

All materials for *de novo* synthesis of Triazin 17-2 were of highest purity (p.a. grade) and purchased from Sigma-Aldrich. Solvents are generally obtained from Merck and Baker (all Germany).

Monoclonal antibodies directed against MMP-2, MMP-9, MT1-MMP (MMP-14) and uPA (urokinase-type Plasminogen activator) were purchased from Oncogene and Chemicon (Germany) respectively. All other antibodies and p.a. grade chemicals, unless otherwise stated, were purchased from Sigma.

Laboratory animals. Four-week-old female SCID/bg mice weighing 14-19 g were purchased from Harlaan-Winkelman (Germany). The animals were allowed to become accustomed for one week in a sterile environment, where bedding, food and water were autoclaved.

Synthesis of Triazin 17-2. In general all reactions were performed in oven-dried glassware under a positive pressure of argon and were stirred magnetically. Sensitive liquids and solutions were transferred via syringe or cannula and were introduced into reaction vessels through rubber septa.

Synthesis of Triazin 17-2 (2-[4-butylsulfonyl)phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazin-6-carbonitril): A solution of butylamine (17 mmol) in ether (50 ml) was added dropwise to stirred slurry of 4-nitrophenylsulfonylchloride (18 mmol) in ether (150 ml). After the mixture was stirred for 3 h at reflux, the sulfonylamide was removed by filtration and washed with 6 N hydrochloric acid (HCl) and recrystallised in ethanol. A solution of $SnCl_2{}^*2H_2O$ (290 mmol) in 13 ml of conc. HCl was added to a stirred slurry of sulfonamide (73 mmol) in acetone. After 2 h at reflux, the mixture was poured into 100 ml of water with stirring. After 3 h, the solid was separated by filtration and washed repeatedly with water. An aqueous suspension was made alkaline with 30% sodium hydroxide (NaOH) solution and then extracted with chloroform. After drying over sodium sulphate (Na_2SO_4) removal of the solvent yielded the 4-aminophenylsulfonylamide. A mixture of N-cyanoacetylurethane (27 mmol) and 30 g sodium acetate in 900 ml water was stirred gentle at 0˚C while a solution of diazonium salt was added dropwise during 20 min. The diazonium salt was prepared by adding a solution of 1.4 g sodium nitrite in water to a cooled (0˚C) solution of 4-aminophenylsulfonylamide (20 mmol) and 10 ml conc. HCl in 200 ml water. After stirring the mixture for 15 min, 15 g sodium acetate in 40 ml water were added.

The whole mixture was left standing overnight; the resulting solid was collected, washed repeatedly with water and recrystallized repeatedly from ethanol. The carbamate (2.5 mmol) was cyclised by reflux in a solution from 25 mmol sodium carbonate in 21 ml water for 10 min. The hot solution was filtered, acidified with acetic acid and cooled. The separated solid was washed with water and recrystallized from a mixture of ethanol/water.

Determination of MMP inhibition. The enzymatic activity was measured using a modified version of a resonance energy transfer fluorogenic assay as described (11). Progress curves were monitored by following the increase in fluorescence at 393 nm (λ_{ex} = 328 nm), induced by the cleavage of the (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitro-phenyl]-L-2,3-diamino-propionyl)-Ala-Arg-NH_2 (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) fluorogenic substrate (Bachem, Switzerland) by MMPs. The fluorescent MCA-group is quenched by resonance energy transfer to the 2,4-dinitrophenyl group. MMPs cleave this substrate at the Gly-Leu bond. Cleavage results in the loss of energy transfer and a large increase in fluorescence of the MCA group.

Enzyme inhibition assays were carried out in MRB which consisted of 50 mM HEPES/NaOH, pH 7.0, 10 mM $CaCl_2$ and 0.02% (w/v) PEG 8000 at 25˚C. A standard screening procedure was used to determine the inhibitory activity. Measurements were carried out with a fluorescence spectrometer LS 50B (Perkin-Elmer, Germany) in 10-mm cuvettes containing 2 ml buffer including 0.5-1% DMSO vehicle, a final substrate concentration of 4-10 μM MCA-peptide, ~0.3-5 nM MMP and variable inhibitor concentrations. From a 30-mM stock (100% DMSO) the inhibitors were serially diluted with MRB to 75, 30, 15, 10, 5, 3, 1, 0.6, 0.3, 0.1, 0.05, 0.015 and 0.005 μM final assay concentration. After preincubation for 30 min at 25˚C the reaction was started by addition of substrate. Each measurement was done in triplicate to ensure statistically significant results. The experiment was further controlled for background fluorescence of the substrate, for fluorescence of fully cleaved substrate and for fluorescence quenching or augmentation from solutions containing the test compounds. The response of inhibition of Triazin 17-2 was determined for various inhibitor concentrations by comparing the amount of hydrolysis (fluorescence units generated >30 min of hydrolysis) of cuvettes containing Triazin 17-2 to cuvettes containing fully active enzyme without

inhibitory compound. Using the program GraFit (Erithacus Software Limited, USA) a four parameter logistic fit to the dose-response data was applied to calculate IC_{50} values.

For each MMP, initial rate measurements in the absence of inhibitor were made for eight different substrate concentrations. From these data, K_m values were determined by non-linear fit using the program GraFit. Assuming competitive inhibition K_i values were calculated automatically for each enzyme tested based upon the equation previously described by Cheng and Prusoff: $K_i = (K_m \times IC_{50})/(K_m + [S])$ (27).

Cell culture. Cells used in this study were PancTu-1 human pancreatic adenocarcinoma cells (28). Cells were routinely cultured in RPMI-1640 medium (Invitrogen, Germany) supplemented with 10% heat inactivated fetal calf serum (PAA-Systems, Austria), 2 mM glutamine and 1 mM sodium pyruvate (Invitrogen). Cells were maintained in monolayer culture at 37˚C in a humid atmosphere with 5% CO_2. Human foreskin fibroblasts KiF-5 were cultivated as described before (29).

Colorimetric cell vitality assay (EZ4U®-Assay). Cells were plated in 96-well culture dishes (Nunc, Germany) at a density of 20,000 cells/well in standard culture medium. Eight wells were left free of cells for control measurements with medium or reagent. After 24- or 48-h treatment, 10 μl of substrate solution (EZ4U, Biomol, Germany) were added to each well. After 3-h absorption was measured at 450 nm with 620 nm as reference with a microtiter plate reader (AnthosII, Anthos Labtec, Germany). For analysis the mean absorption value of reagent-only control was subtracted from measured values of treated cells.

DNA-Synthesis (Methyl-[^{3}H]-thymidine Incorporation Assay). Cells were plated in 96-well dishes at a density of 5,000 cells/well in standard culture medium. After 24 h medium was replaced by medium containing only 0.5% FCS (starvation medium) and incubated for another 24 h. Cells were than treated in starvation medium for 24 or 48 h. During the last three hours of incubation time 7.4 MBq Methyl-[^{3}H]-thymidine (Amersham, Germany) per well were added. Cells were harvested using a cell-harvester (Skaton, Norway) and incorporated radioactivity was determined by liquid scintillation counting.

DNA fragmentation assay (JAM-Assay). Cells were plated in 96-well culture dishes at a density of 5,000 cells/well in standard culture medium. After 24 h medium was replaced by starvation medium and incubated for 24 h. Cells were then metabolically labelled by addition of a total amount of 7.4 MBq Methyl-[^{3}H]-thymidine per well for three hours. Afterwards, cells were washed with PBS and subsequently treated in starvation medium for 24 or 48 h and processed as described above.

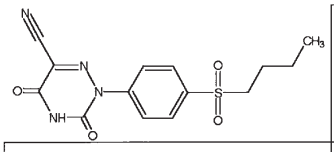
Invasion Assay. A model system to quantify the ability of PancTu-1 pancreatic carcinoma cells to invade monolayers of fibroblast cells was developed using a modification of the protocol described by Casey *et al* (30). KiF-5 cells (10,000 cells/well) were seeded to 24-well culture dishes (Nunc) and grown to confluency for 48-72 h in RPMI-1640 medium containing 10% FCS. Confluence was controlled by light microscopy. The fibroblast monolayer were rinsed twice with PBS, permeabilized with 250 μl dimethyl-sulfoxide (DMSO, Sigma, Germany) for 50 min at room temperature, rinsed twice with PBS and with RPMI-1640 media. PancTu-1 cells (15,000 cells/well) were resuspended in RPMI-1640 medium and added to the permeabilized fibroblast monolayer. For long-term incubation, treatment medium was changed every 24 h. For evaluation wells were washed gently with PBS and subsequently 500 μl 0.2% trypan blue solution (Sigma) was applied to each well for 9 min and finally washed with PBS. Wells were investigated using an inverted microscope, equipped with a digital camera (Axio-Vison, Zeiss, Germany) to document proteolytic areas. The size of the proteolytic areas was determined using Scion Image Systems software (Scion Corporation, USA).

Gelatin zymography. Conditioned media were prepared by plating 300,000 cells/well in 6-well culture dishes (Nunc) in standard culture medium. Cells were grown over night before treatment in serum-free medium. The supernatants were collected, centrifuged for 5 min in a microfuge at maximum speed to remove cell debris and stored in aliquots at -80˚C. For zymography 10 μl of supernatant was combined with an equal amount of 2-fold concentrated SDS sample buffer and run on 12.5% polyacrylamide gels containing gelatin under non-reducing conditions (all from Invitrogen). After electrophoresis, gels were washed twice in renaturation buffer for 20 min. Then the gels were incubated in developing buffer (all from Novex, Germany) overnight at 37˚C. Finally, gels were stained with Coomassie Brilliant Blue solution. Lysis of gelatine by active enzymes is detectable as white bands in an otherwise blue background.

Western blot analysis. For preparation of cell lysates, cells were plated at a density of 300,000 cells/well in 6-well plates in standard culture medium and grown overnight before treatment in serumfree medium. Cells were washed twice with PBS and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate), containing protease inhibitors (Complete, Roche, Germany). Protein concentration was determined by BCA-Protein-Assay (Pierce, USA). Protein (20 μg) were resolved by denaturing SDS-PAGE (Anamed, Germany), and subsequently transferred to a nitrocellulose Hybond C membrane (Schleicher and Schuell, Germany). The membrane was blocked with 5% dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature (RT) and then washed in PBS-T. Primary antibodies were incubated for various times at RT, followed by washing and incubation with secondary peroxidase coupled antibodies. Detection was carried out by chemiluminescence (ECLplus, Amersham Pharmacia Biotech, Germany) and exposure to X-ray film (Amersham Biotech). The blots were reprobed with antibodies against ß-actin to show equal loading.

Orthotopic xenograft model. The human pancreatic adenocarcinoma cell line PancTu-1 forms a highly invasive pan-

Table I. K_m and K_i - values for different tested MMPs.[a]

	cdMMP-2	MMP-7	cdMMP-8	cdMMP-9	cdMMP-12	cdMMP-13	cdMMP-14
K_m [µM] (for assay substrate)	9.1	1.9	5.9	1.8	27.3	7.5	6.8
K_i [µM] (for inhibitor)	28.00	0.05	4.00	10.80	14.90	2.50	0.22

[a]The chemical structure is shown in the left upper field.

creatic adenocarinoma when injected orthotopically into SCID/bg mice, as has been described before (31). General anaesthesia was induced using midazolam (1 mg/kg/bw), fentanyl (0.05 mg/kg/bw) and medetomidin (0.5 mg/kg/bw). A median laparotomy was performed and the pancreas identified. Tumour cells (1,000,000) resuspended in Matrigel® were injected into the body of the pancreas. The abdominal wall was closed in layers with Vicryl 6-0 (Ethicon, Germany). The recovery was carefully supervised with red light re-warming and volume substitution. Tumour formation and growth was allowed for seven days.

For therapy, Triazin 17-2 was dissolved in DMSO and applied 120 mg/kg/bw as a single dose per day. In the control group DMSO was administered. Therapy regime included three 5-day intervals. Finally mice were sacrificed by carbon oxide intoxication. Mice were also redeemed if they showed any adverse effects or symptoms of disease. The tumours were resected and weighed. For adjuvant therapy, primary tumours were resected seven days after orthotopic inoculation, as described before (32). Therapy regime was as described above, starting two days after resection.

The study was conducted in accordance with the standards established by the guidelines for the Care and Use of Animal experiments of the University of Kiel and approved by the local veterinary committee. The appropriate government institution has provided permission for animal experiments under no. V 742-72241.121-7 (48-6/04).

Statistical analyses. Significance was estimated by Student's t-test. A $p<0.05$ was considered as significant.

Results

Effect of Triazin 17-2 in cell free systems. Activity of Triazin 17-2 was tested in a cell-free assay using isolated MMPs expressed either as a truncated version consisting the catalytic domain (cd), as for MMP-2, MMP-8, MMP-12, -13 and -14 or in the full length protein as for MMP-7 (obtained from Calbiochem, Germany) or isolated according to Tschesche *et al* (33-35). The results of the respective tests are presented in Table I. MMP-9 and MMP-7 have the lowest binding efficiency for the substrate used in this assay, though the inhibition of substrate cleavage by MMP-9 is in the micromolar range, while MMP-7 activity is already inhibited at nanomolar concentrations of Triazin 17-2. MMP-2 is inhibited at a concentration of 28 μM Triazin 17-2, which represents the highest concentration determined for the inhibitor in this test panel. Next to MMP-7, MMP-14, i.e. MT1-MMP, is inhibited in the nanomolar range, and showed medium binding of the assay substrate. Other MMPs tested were the catalytic domains of MMP-8 and 13, i.e. collagenase 2 and 3, respectively, which were also effectively inhibited at low micromolar concentrations of Triazin 17-2. These cell-free assays revealed that the design of Triazin 17-2 offers the opportunity for enzyme specificity depending on concentrations used, with highest inhibitory efficiency in the nanomolar range on MMP-7 and MMP-14.

Effects of Triazin 17-2 on cell viability in PancTu-1 cells in vitro. An assessment of general toxicity of the novel compound Triazin 17-2 was performed *in vitro* on PancTu-1 cells. Three different assays were employed: a general cell proliferation test, determination of DNA-synthesis and DNA-fragmentation as a measure of apoptosis. A broad range of concentrations of Triazin 17-2 was used starting at 30 μM up to 60 μM, 300 μM and 3 mM were tested, all well above the concentrations determined for MMP inhibition in cell-free assays. In a colorimetric proliferation test using formation of formazan by intact mitochondria of the cells, which corresponds directly to cell number, Triazin concentrations of 30, 60, and 300 μM proved to be without effect on cell proliferation, as depicted in Fig. 1A. Only the highest dose of Triazin 17-2 at 3 mM showed a significant reduction of vitality to 50% of control after 48-h treatment. This effect was independent of cell density and presence of FBS, as a source of growth factors (data not shown). In a second test, influence

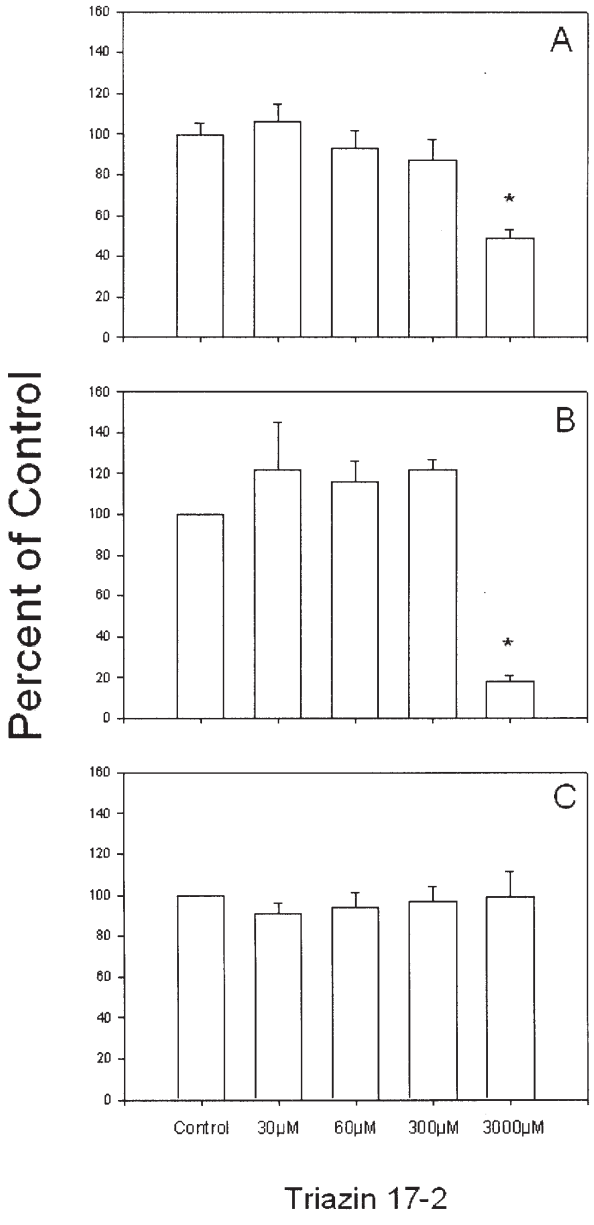


Figure 1. Test for toxicity of Triazin 17-2 against PancTu-1 cells. (A) Cell vitality test using 20,000 cells/well, incubation with Triazin 17-2 in concentrations indicated for 48 h. Only treatment with 3 mM Triazin 17-2 produced a significant reduction of vitality (*p=0.001, t-test, n=3). (B) DNA synthesis was tested on 5,000 cells/well for 24 h. Only treatment with 3 mM Triazin 17-2 produced a significant inhibition of DNA synthesis (*p<0.001, t-test, n=4). (C) DNA fragmentation was tested as a sign for apoptosis on 5,000 cells/well after 24-h incubation. There were no significant changes, even at highest concentration (n=3).

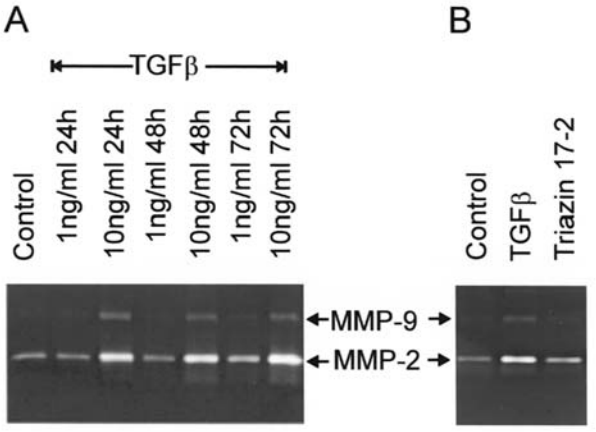


Figure 2. Zymography of cell supernatants in gelatin gels. (A) PancTu-1 cells were stimulated with human recombinant TGFß1 at the indicated times and concentrations. The lower band corresponds to the molecular weight of MMP-2, while the upper (fainter) band reflects the molecular weight of MMP-9. (B) PancTu-1 cells were incubated either with DMSO as vehicle only (control) or with 2 ng/ml TGFß1 or with 100 μM Triazin 17-2 for 24 h. Cell supernatant (15 μl) was tested. Only a faint band for MMP-2 is clearly visible, while MMP-9 is barely detectable (representative gel of three different experiments).

of Triazin 17-2 on DNA-synthesis was analysed (Fig. 1B). At low doses after 24 h, DNA-synthesis appears to be stimulated by Triazin 17-2 while at 3 mM concentration; DNA-synthesis is reduced to 20% of controls. To test whether this effect was due to apoptosis, a DNA-fragmentation assay was performed (Fig. 1C). There is no DNA fragmentation detectable by JAM-assay at any concentration of Triazin 17-2. These results show that Triazin 17-2 does not kill pancreatic tumour cells *in vitro* even at non-physiological concentrations of 3 mM.

Expression of MMPs in PancTu-1 cells. A general test for expression of MMPs in PancTu-1 cells was carried out using a commercially available RT-PCR kit (SuperArray, Bioscience Corp., USA). At mRNA level we found expression of MMP-2, 7, 9, 10, and 12. Membrane-bound MMPs-13 and 17 were also detected, as well as expression of TIMP-1 and TIMP-2 (data not shown).

To test for MMP secretion we performed zymography in gelatin gels. Fig. 2A shows PancTu-1 cells after various times of stimulation with TGFß1. Under control conditions PancTu-1 cells secreted detectable levels of MMP-2 but only very little MMP-9. After 24 h, MMP-2 secretion was not enhanced by incubation with 1 ng/ml TGFß1, but clearly upregulated by 10 ng/ml TGFß1. Similar results were obtained for MMP-9. Only after 72 h we found visibly enhanced secretion of MMP-2 and MMP-9 after stimulation with 1 ng/ml TGFß. Thus, stimulation of secretion of MMP-2 and MMP-9 by TGFß was time- and dose-dependent. We tested whether the MMP-I Triazin 17-2 had any effect on secretion of MMP-2 and -9. Fig. 2B shows that after 24-h incubation with 100 μM Triazin 17-2, MMP-2 secretion and activity were unaltered, as compared to control conditions while incubation with 2 ng/ml TGFß for 24 h slightly induced activity, thus secretion. MMP-9 was less pronounced but showed the same pattern as MMP-2. To address protein expression in general, we used Western blot analysis on cell lysates. We tested for MMP-2, MMP-9, MT1-MMP (MMP-14), and the serine protease uPA; ß-actin was used to test for equal loading. Fig. 3 shows a representative result of several analyses performed. We were unable to detect MMP-2 in whole cell lysates of PancTu-1 cells. Since we have shown MMP-2 was secreted into the cell supernatant as detected by its enzymatic activity, we thus conclude that MMP-2 is synthesised and rapidly secreted and the sensitivity of Western blot analysis is insufficient for MMP-2 protein detection in cell lysates. MMP-9 on the contrary was detected more readily by Western blot analysis and protein levels

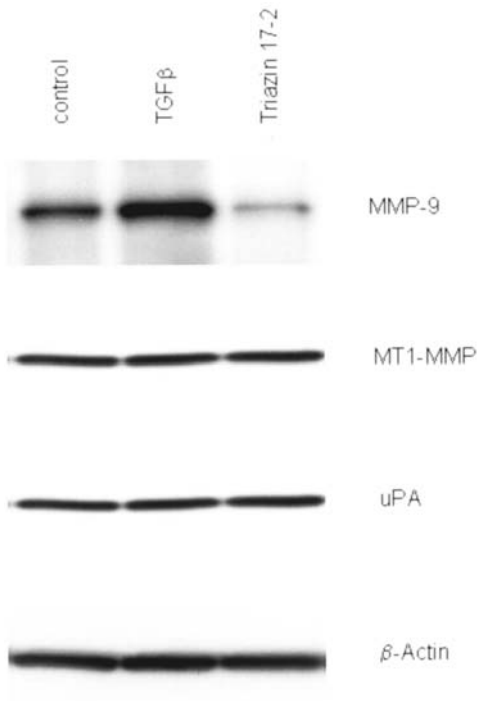


Figure 3. Western blot analysis of MMPs in PancTu-1 cells. PancTu-1 cells were treated either with DMSO (control) or 2 ng/ml TGFß1 or 100 μM Triazin 17-2 for 24 h. Protein (20 μg) were resolved per lane and tested for MMP-9. MT1-MMP, uPA and ß-actin, as indicated (a representative experiment out of 3).

were enhanced in cell lysates by incubation of PancTu-1 cells with 2 ng/ml TGFß for 24 h. Incubation with 100 μM Triazin 17-2 appeared to reduce detectable MMP-9 protein levels in cell lysates. The membrane bound MT1-MMP (MMP-14) was identified by Western blot analysis, but did not show regulation by TGFß, nor was affected by Triazin 17-2. Similar results were obtained for Western blot analysis of uPA expression. Analysis of ß-actin not only revealed equal loading but also showed that Triazin TF17-2 had no general effects on protein expression in PancTu-1 cells.

Triazin 17-2 inhibits invasion of PancTu-1 cells in vitro. To establish that Triazin 17-2 inhibits MMPs *in vitro* the invasion assay adapted from Carey *et al* was employed (30). This assay is based on the invasion of tumour cells into a confluent layer of incapacitated fibroblasts and is accompanied by protein degradation. Invasion is detected by formation of proteolytic areas devoid of staining that surround uninhibited tumour cells. The size of these areas is determined as a measure for proteolytic degradation. Fig. 4 shows a representative experiment of an invasion assay. On the left hand side (A-C) integrity of the permeabilised fibroblast monolayer in the absence of tumour cells throughout the incubation time is shown. The middle column of micrographs (D-F) shows PancTu-1 cell activity after incubation for 24 h (D), 48 h (E) and 72 h (F) in the absence of inhibitor. Notably, large proteolytic areas are seen after 48 and 72 h (E and F). Treatment with 300 μM Triazin 17-2 is seen in the right hand panel (G-I) after 24-, 48-, and 72-h incubation, respectively. No proteolytic areas are visible in the fibroblast monolayer, where invasive activity of PancTu-1 cells has been blocked effectively by a concentration of 300 μM Triazin 17-2. To test for reversibility of Triazin 17-2 activity and to prove that PancTu-1 cells are not affected by incubation with inhibitor, we performed a wash-out experiment. PancTu-1 cells were

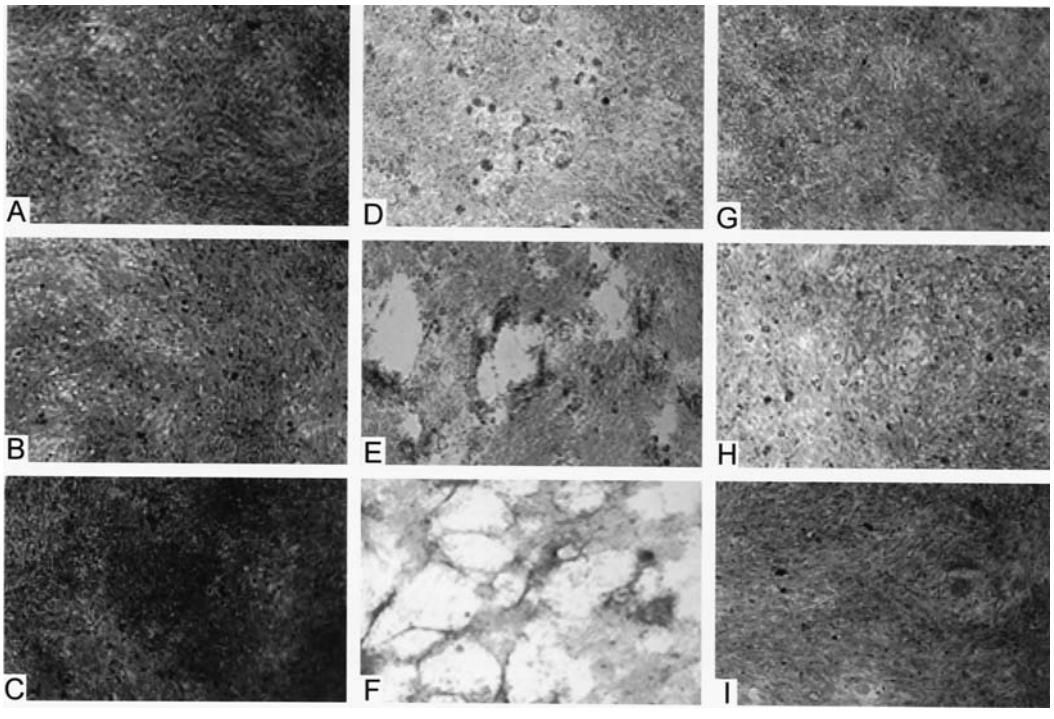


Figure 4. Invasion of PancTu-1 cells *in vitro*. PancTu-1 cells were plated onto incapacitated monolayers of fibroblasts and left either untreated (D-F) or were incubated with Triazin 17-2 at a concentration of 300 μM for 24 h (D and G), 48 h (E and H) or 72 h (F and I). Micrographs A-C show intact monolayers of fibroblasts incubated without PancTu-1 cells for 24, 48 and 72 h, (A,B and C, respectively) to test for detrimental effects of time and temperature on the substratum (a representative experiment out of 3).

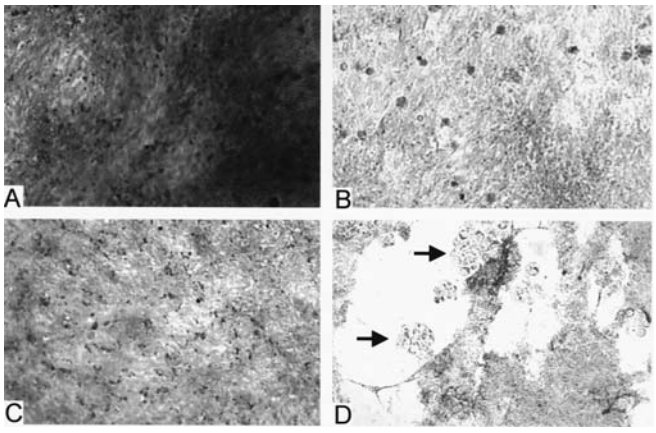


Figure 5. Recovery of PancTu-1 cells from Triazin 17-2 treatment. PancTu-1 cells were plated onto incapacitated monolayers of fibroblasts and incubated for 72 h with Triazin 17-2 at a concentration of 3 mM (B). Subsequently, Triazin 17-2 was washed out and PancTu-1 cells were incubated for 48 h in standard culture medium without inhibitor (D). Arrows in D point towards PancTu-1 cell agglomerates in proteolytic areas. Micrographs A and C show intact monolayer of fibroblasts incubated without PancTu-1 but in the presence of 3 mM Triazin 17-2 for 72 h (A) and subsequently in standard culture medium (C) for another 48 h (a representative experiment out of 3).

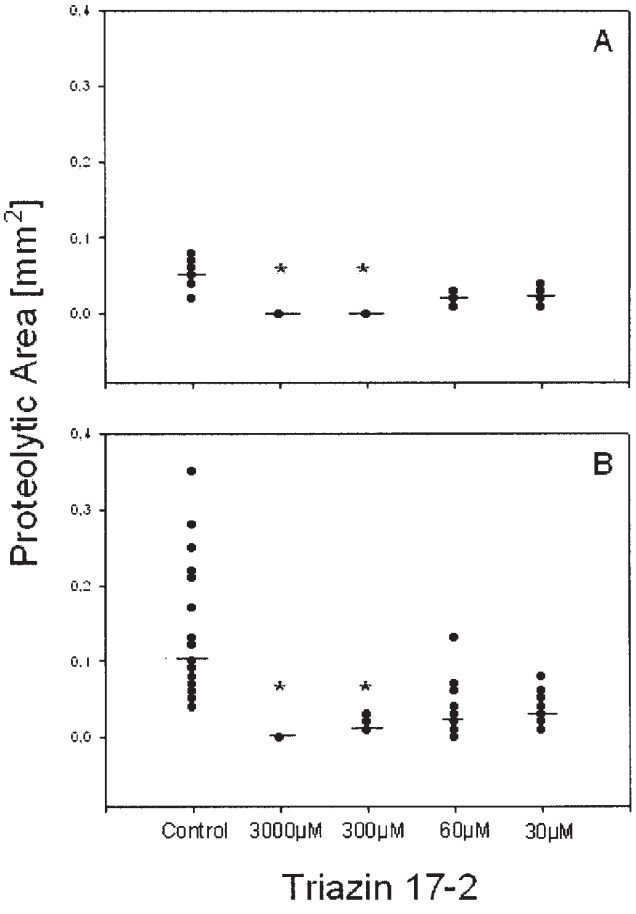


Figure 6. Quantitative analysis of invasion assays. (A) Measurement of proteolytic areas in untreated controls after 48 h, or in the presence of different concentrations of Triazin 17-2, as indicated. (B) Measurement of proteolytic areas in untreated controls after 72 h, or in the presence of different concentrations of Triazin 17-2, as indicated (n=3, *p<0.001 significant, Wilcoxon rank sum test).

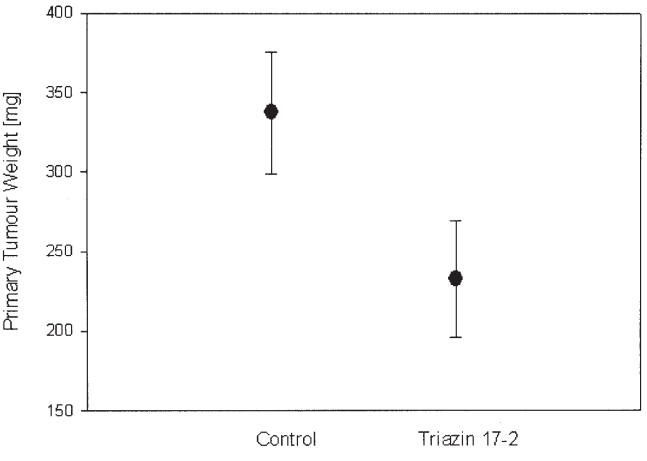


Figure 7. Effect of Triazin 17-2 *in vivo*. Conservative treatment of established tumours in an orthotopic PDAC model in SCID/bg mice. Animals received 120 mg/kg/bw Triazin 17-2 or DMSO only in control groups (n=6 for each group, p=0.002 t-test).

plated onto incapacitated monolayers as described and incubated in the presence of 3 mM Triazin 17-2 for 72 h, then cells were washed with PBS and standard culture medium was applied for 48 h. Fig. 5 shows the results of this experiment; integrity of the permeabilised fibroblast monolayer after 72 h (A) and 72+48 h (B) was tested to prove that no lysis occurs during prolonged incubation times even in the absence of tumour cells. After 72-h incubation of PancTu-1 cells with 3 mM Triazin 17-2, the fibroblast layer remains mostly intact (C) after wash out of inhibitor and 48 h of incubation under standard conditions (D) PancTu-1 cells, visible as agglomerates of cells in proteolytic areas (see arrows in D), were still viable and retained their ability for proteolytic cleavage of the fibroblast monolayer. Quantitative evaluation of invasion assays is seen in Fig. 6. After 48 h (A) Triazin 17-2 fully inhibited formation of proteolytic areas at 3 mM and 300 μM concentrations. At lower concentrations of 60 and 30 μM proteolysis was seen, but still lower levels than in untreated controls. After 72 h, the median values of proteolytic areas in untreated controls doubled (B), most likely due to tumour cell proliferation, though Triazin 17-2 at highest concentration significantly inhibits proteolysis of the fibroblast monolayer. At lower, therapeutical concentrations proteolytic areas were still smaller than in uninhibited controls.

Effect of Triazin 17-2 on orthotopic pancreatic ductal adenocarcinoma in mice. After we established that Triazin 17-2 did not have adverse effects on PancTu-1 cells *in vitro*, but revealed its effectiveness in inhibition of invasion *in vitro*, we tested the inhibitor *in vivo*, using an orthotopic PDAC model in SCID/bg mice (31,32). Triazin 17-2 was applied at a dosis of 120 mg/kg/bw for three cycles of five consecutive daily applications followed by two days without therapy. Conservative treatment of established tumours yielded a median weight of resected tumours at 227.0 mg. Tumour weight of the control group was 332.0 mg, thus the treatment group showed significantly (p=0.002) less tumour weight

Table II. Effect of adjuvant treatment on resected PDAC in mice.

	Total tumour–weight (mg) MW ± SD	Occurrence/animals	
		Local recurrence	Metastases
Control animals treated with DMSO	171.7±105.1	5/6	4/6
Adjuvant treatment with Triazin 17-2	44.0±49.9	4/6	3/6

increase. Metastasis or other changes in the consistency of the parenchymatous organs were not observed (Fig. 7). Conservative treatment of animal with Triazin 17-2 was well tolerated. Control groups, which received the solvent DMSO only, did not show adverse effects.

In a second approach, we tested for adjuvant therapy. Established tumours were resected and treatment with Triazin 17-2 was carried out as described above starting two days after re-laparotomy. Untreated control groups developed a median total tumour load of 343.2 mg, while the treatment group showed only a median total tumour load of 134.8 mg, representing a 60.8% reduction. With resection only, one animal was without relapse and metastasis, while resection and treatment with Triazin 17-2 resulted in two animals without local recurrence and metastasis. Table II shows the results of adjuvant treatment. While local recurrence was not generally inhibited, it was reduced in size and numbers. Incisional metastases, a general phenomenon after resection of pancreatic tumours in mice (32), were also reduced in size and numbers compared to untreated resected animals.

Discussion

This study reports on the development and characterisation of a novel MMP-I. The new type of MMP-I was structurally based on a triazine scaffold of which compounds have been used for other quite well tolerated therapeutics. A major aim was to develop a compound with higher metabolic stability and reduced side effects on cells apart from the inhibition of MMPs. Interesting derivates of the triazines are pyrimidino-[5.4-e][1,2,4] triazines, which are structural components of natural antibiotics (36,37). Intense studies for the use of derivates of triazines have been performed for the treatment of human immune deficiency virus disease (HIV), as a ligand for the thyroid hormone receptor and as an inhibitor of interleukin-5 production (38-43).

Triazin 17-2 showed nanomolar inhibitory potential against classic MMPs in free cell systems and was assessed for general toxicity on human cells *in vitro*. Cell vitality testing using a colorimetric assay showed a wide concentration range with neglectable influences on the proliferation of the pancreatic tumour cells and other cell types (data not shown). Only the very high concentration of 3 mM, not applicable under physiological conditions to animals and humans, some retarding effects were observed *in vitro*. However, the reduction of proliferation as depicted by formazan formation and DNA synthesis in monolayer cells at this concentration appears negligible due to the fact, that cells *in vivo* grow as three dimensional cell formations, and not as monolayer. We investigated cells growing as spheroids and found them less sensitive against adverse effects of Triazin 17-2 at high concentrations (data not shown). We did not find substantial apoptotic effects as monitored by DNA fragmentation. These observations are confirmed by recovery experiments. Using an invasion assay, it was shown that viability and invasive potential of the PancTu-1 cells after removal of Triazin 17-2 was unimpaired.

Most of the other synthetic MMP-Is described before have adverse effects such as cytokine or interleukin modulation (44,45). First experiments carried out to address an effect on Triazin 17-2 on the activity of ADAM10 have shown no inhibition by Triazin 17-2 (data not shown) (46). Further investigations are necessary to test for an effect of Triazin 17-2 on the so-called sheddases (47,48). Experiments presented here, regarding effects on protein expression in general i.e. Western blot and zymographic analysis, showed no effect of Triazin 17-2 for MMP-2, MMP-9, MT1-MMP and uPA. These proteases were selected because of their significance in the activation cascades for MMPs and as effectors.

Application of Triazin 17-2 *in vivo* in an orthotopic pancreatic adenocarcinoma model in SCID/bg mice was chosen to test for toxicity and effect in animals. In general this model is well established and standardized for the applied tumour cell line PancTu-1 (49). The reduction of primary tumour weight by 30% during conservative therapy serves as a proof of principle. In general, Triazin 17-2 showed no intrinsic cytostatic effect, thus, it has no fatal effect on tumour cells *per se*. The observed effect on tumour growth could be explained by inhibition of MMPs. Using Triazin 17-2 in adjuvant therapy, revealed that Triazin 17-2 did not generally inhibit formation of local recurrence, suggesting that relapse up to a certain size might be independent of MMPs. Formation of metastases, thus invasion and settlement in incisional injuries, representing distant tissues in the model used here, which would most likely depend on MMPs, was effectively inhibited or largely reduced in size (31,32). These data support the notion that the use of an MMP-I as singular therapy against various cancer entities, though often applied, has to be considered inappropriate (26). It actually appears to be a general error during establishment of the MMP-Is in anti-cancer therapy to use them as a single drug (26). Since their mode of function underlies certain restrictions regarding application and dosage, as well as their principally non-cytotoxic nature, MMP-I alone can not be expected to be an effective agent against aggressively growing cancer. MMP-Is are most likely to facilitate a limitation of invasion thus improving the potential of other cytostatic drugs. Well aware of these limitations, Triazin 17-2 was tested as a single drug for proof of principle and toxicity *in vivo*, but further investigations in combination with other drugs are necessary to show synergistic effects (22,50). The era of synthetic MMP-Is was thought to be finished, this appeared to be

valid at first sight, but their use to limit cancer invasion is of persisting interest (26). It is therefore necessary to test new classes of substances for MMP inhibition, as well as to test combinations with cytostatic drugs to improve the efficiency of the anti-cancer therapy.

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