Cytostatic Activity of a 5-Fluoro-2'-deoxyuridine–Alendronate Conjugate against Gastric Adenocarcinoma and Non-malignant Intestinal and Fibroblast Cell Lines

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Abstract. *Background: 5-Fluoro-2'-deoxyuridine (5-FdU), a drug against gastric cancer, was covalently linked via its nucleobase with the amino-bisphosphonate alendronate (Ale), resulting in a new antimetabolite-bisphosphonate conjugate (5-FdU-Ale), designed for bone-targeting. Materials and Methods: The cytostatic effect of 5-FdU-Ale was evaluated in vitro compared to monomers and mixtures using CASY Technologies and the human gastric adenocarcinoma cell lines 23132/87 and MKN-45, in comparison to the intestinal CCL-241 and dermal fibroblast NHDF neonatal cell lines. Results: The adenocarcinoma cell lines demonstrated a slightly higher sensitivity, with respect to the cell lines CCL-241 and NHDF, to incubation with 5-FdU-Ale. In comparison to 5-FdU, 5-FU and an equimolar mixture of Ale+5-FdU and Ale+5-FU, the cytostatic activity of the 5-FdU-Ale was markedly reduced. Conclusion: 5-FdU-Ale was only partially or not at all metabolized to a mixture of cytostatic metabolites in vitro. Therefore an in vivo evaluation of the conjugates is indicated.*

Bone metastasis and peritoneal seeding are known as poor prognostic factors in patients with gastric cancer (1, 2). There is a high demand for the development of effective therapeutics against tumor-induced bone diseases. In patients with metastatic gastric cancer, palliative combination chemotherapy including 5-fluorouracil (5-FU) is a widely used regime (3-9). A drug targeting 5-FU or its nucleosides (5-FdU) to enhance and prolong the 5-FdU concentration locally in the microenvironment of bone after systemic administration could be an efficient strategy for the treatment of bone metastasis. *Bis*phosphonates (BPs) are bone-specific drugs that seek their target site and exert their pharmacological activities preferentially on the bone (10, 11), under significant accumulation in regions of cancer metastasis, in comparison with healthy bone. Cytostatic agents conjugated with BPs represent a new class of cytostatic agents in which the BP moiety seems to target the linked drug to osseous tissue. BP conjugates may become promising therapeutic agents, which would increase the local concentrations of the cytotoxic agents without increasing systemic toxicity. Antitumor agents such as methotrexate (12), doxorubicin (13) and cisplatin (14) were, for example, directly covalently linked to the terminal amino group of the amino-BP pamidronate using an appropriately bioreversible amide bond. The obtained conjugates should survive in the circulation, bind to the hydroxyapatite matrix of the bone and subsequently release the BP as well as the coupled cytostatic agent into the bone microenvironment by cleavage of the amide linkage of the conjugate. The published cytostatic activities of the BP conjugates evaluated *in vitro*, as well as in any animal models, are modest. The choice of the sufficient linkage is an important factor for determining the pharmacokinetic and pharmacological properties of the BP conjugates. Recently, another conjugation of the BPs etidronate and medronate with the 5'-monophosphates of nucleoside analogues, resulting in nucleoside-5'-triphosphate analogues, in which the β- and γ-phosphorous atoms are linked *via* a carbon instead of an oxygen atom, was described (15, 16). The therapeutic effects of the anhydride formed between arabinocytidine-5'-phosphonate and etidronate were evaluated *in vivo* using breast cancer and multiple myeloma

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Key Words: Bisphosphonates, 5-fluoro-2'-deoxyuridine-alendronate, gastric cancer cell lines, intestinal cell lines, fibroblast cell lines.

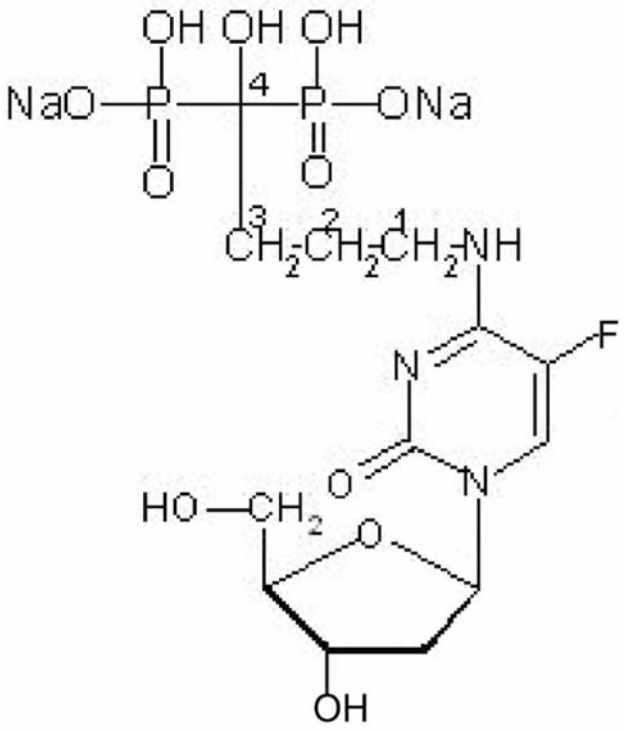

Figure 1. *Chemical structure of the antimetabolite-bisphosphonate (5-FdU-Ale).*

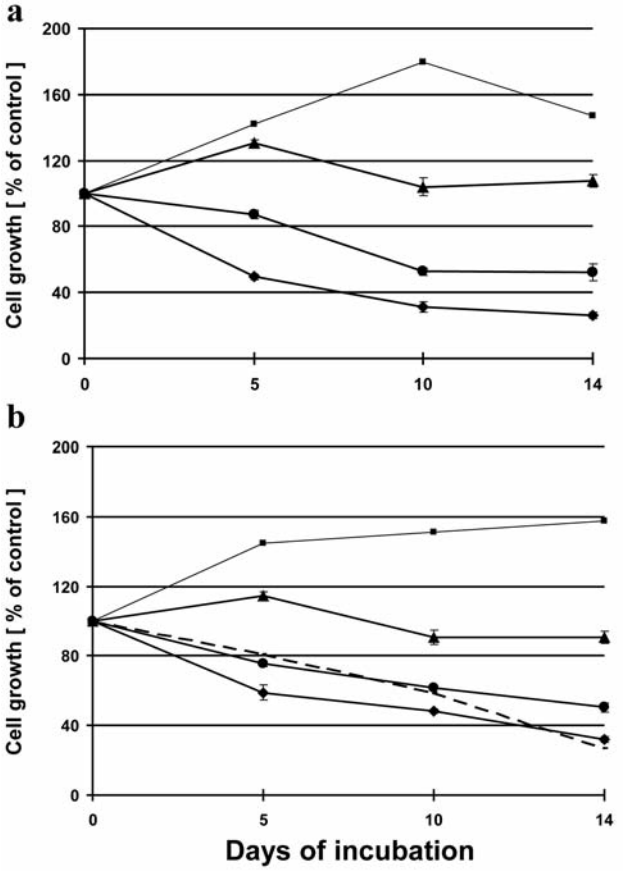

Figure 2. *Dose- and time-dependent growth inhibition of human adenocarcinoma cell lines 23132/87 (a) and MKN-45 (b) in comparison to the control (PBS treatment instead of drug (■) during 14 of days incubation with 106 μM (▲), 318 μM (●), 954 μM (◆) alendronate, or 49 μM (- - - -) 5-FU. Values are the means obtained from triplicates of each experiment. PBS-control is marked with a weak line.*

mouse models and produced very encouraging results. However, the synthesis of nucleoside-5'-triphosphate analogues was limited because the coupling of the more active amino-BPs such as alendronate and zoledronate cannot be performed according to the described procedure. Additionally, the direct coupling of 5'-nucleotide with one of the two terminal phosphonate groups of BPs can reduce the affinity for hydroxyapatite, resulting in reduced bone targeting. We have developed a conjugation of amino-BPs with cytotoxic pyrimidine nucleoside analogues resulting in new chimeric BPs, whose alkyl chain is terminated by an antimetabolite residue, whereas both phosphonate groups are underivated (17). Figure 1 shows the structure of the selected chimeric 5-FdU-alendronate (5-FdU-Ale), in which the antimetabolites 5-FdU and alendronate are linked *via* the nucleobase of 5-FdU. A preliminary ATP tumor-chemosensitivity assay demonstrated different sensitivities of breast and ovarian cancer cell lines to 5-FdU-Ale (18). 5-FdU-Ale should also be effective against gastric cancer cells because the linked 5-FdU residue is a highly potent drug against gastric cancer. In the current article, the *in vitro* activities of 5-FdU-Ale against different cell lines, in comparison to the single-drugs 5-FdU, 5-FU, alendronate and mixtures are described.

Materials and Methods

Chemicals. The drugs 5-FU and 5-FdU were obtained from Sigma (Taufkirchen, Germany). Alendronate and 5-FdU-Ale were synthesized according to published procedures (16). All diluted drug aliquots were stored as solutions at –20˚C. Dilutions were made with phosphate-buffered saline (PBS, pH 7.4) (Invitrogen, Germany). Concentrations of stock solutions were the following: 5-FU, 6 mg/ml; 5-FdU, 15 mg/ml; alendronate, 20 mg/ml; and 5-FdU-Ale, 20 mg/ml. Combinations were made up by adding two drugs in an equimolar ratio.

Cell lines. Two human gastric adenocarcinoma cell lines 23132/87 and MKN-45 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were seeded in 150-cm^2 culture flasks (Falcon) and maintained in RPMI-1640 plus GlutaMax™-I Invitrogen culture medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. The human normal intestinal cell line (CCL-241) was obtained from the American Type Culture Collection (Rockville, MD, USA) and

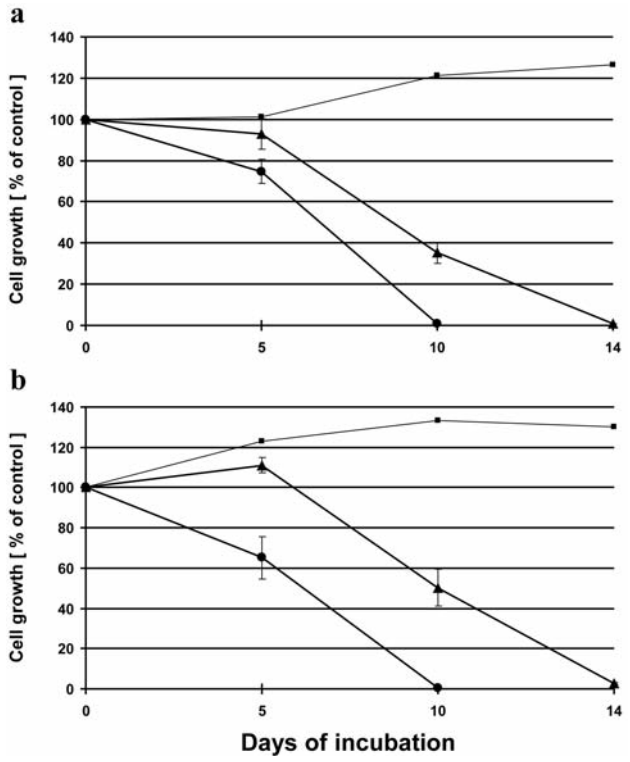

Figure 3. *Dose- and time- dependent growth inhibition of the human adenocarcinoma cell line 23132/87 over 14 days of incubation with equimolar mixtures [106 μM (▲), 318 μM (●)] of alendronate plus (5-FdU) (a) and alendronate plus nucleobase (5FU) (b). (■) Control (PBS treatment instead of drug). Values represent the means of three independent experiments.*

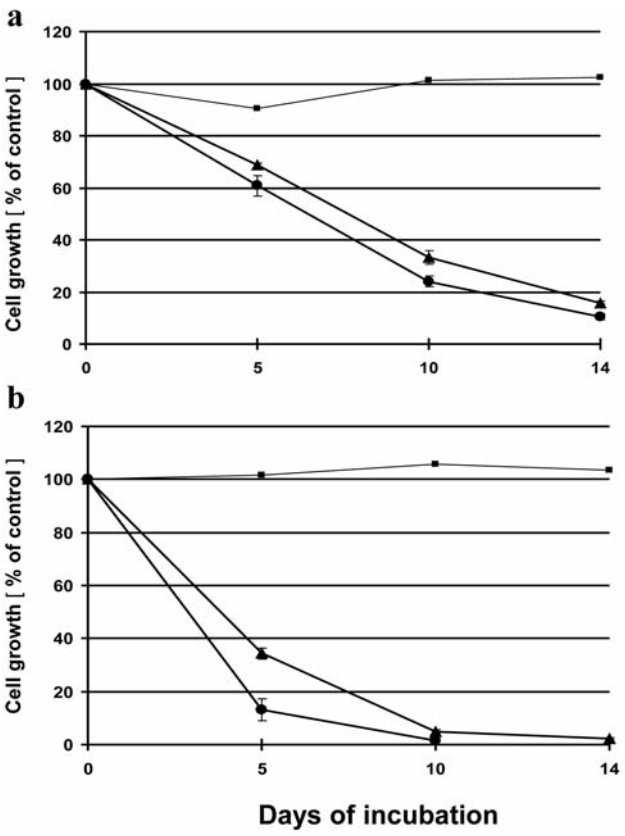

Figure 4. *Dose- and time-dependent growth inhibition of the human adenocarcinoma cell line MKN-45 over 14 days of incubation with equimolar mixtures [106 μM (▲), 318 μM (●)] of alendronate plus (5-FdU) (a) and alendronate plus (5FU) (b). (■) Control (PBS treatment instead of drug). Values represent the means of three independent experiments.*

maintained in Dulbecco's Modified Eagle Medium (DMEM) plus GlutaMax™-I (4.5 g/l D–glucose; 25 mM HEPES; Invitrogen) culture medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 30 ng/ml epidermal growth factor (EGF). Normal human dermal fibroblast cell line (NHDF neonatal) was obtained from Bioproducts Boehringer Ingelheim, Leimen, Germany and maintained in DMEM plus GlutaMax™-I (4.5 g/l D–glucose; 25 mM HEPES; Invitrogen) culture medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin.

Cell culture and drug treatment. The cells were grown at 37˚C in a humidified atmosphere of 5% CO_2. The malignant cell lines were plated at a density of 4×10^5 cells/well, the non-malignant cell lines were plated at a density of 2×10^5 cells/well in 12-well culture plates (Corning) and experiments were started at 90-100% confluence. Cells were incubated with different concentrations of drugs over a period of two weeks and a replacement of the medium with the same fresh drug concentration every three days. Controls were allowed to grow in the presence of PBS instead of drug for the same period of time as the treated cells. At day 0, 5, 10 and 14 of experiments, the cells were washed once with 1 ml PBS followed by trypsinization with 300 μl of 0.05% trypsin-EDTA (Invitrogen). Images at day 14 were taken using a phase-contrast microscope (Axiovert 25; Zeiss, Oberkochen, Germany) equipped with a Canon EOS 500D camera and twenty-fold magnification. The trypsinization was stopped by the addition of 1.7 ml complete medium. The cell suspension was transferred to a 2-ml polyethylene vial and was shaken in an overhead rotator at low speed until cell counting. Cell suspension (100 μl) was diluted with 10 ml of CASYton (Schärfe System, Reutlingen, Germany) and then analyzed with an automated cell counting system.

Cell counting. Cell growth was evaluated using CASY Technologies. To determine the number and viability of tumor and normal cells, a CASY® Model TT cell counter and analyser system (Schärfe System, Reutlingen, Germany) was employed. To determine the number of cells in the 12-well culture plates, the system was operated in the 3×400 μl mode, *i.e.* 400 μl of the diluted cell suspension (dilution 1: 100 in CASYton; Schärfe System) were drawn through the apparatus and the mean of three measurements was stored. The effect of the drugs was evaluated as the difference in percentage of cell growth between the starting value and the following days of cell counting. Determination of cell parameters were carried-out with different optimized programs for the two carcinoma cell lines, as described elsewhere

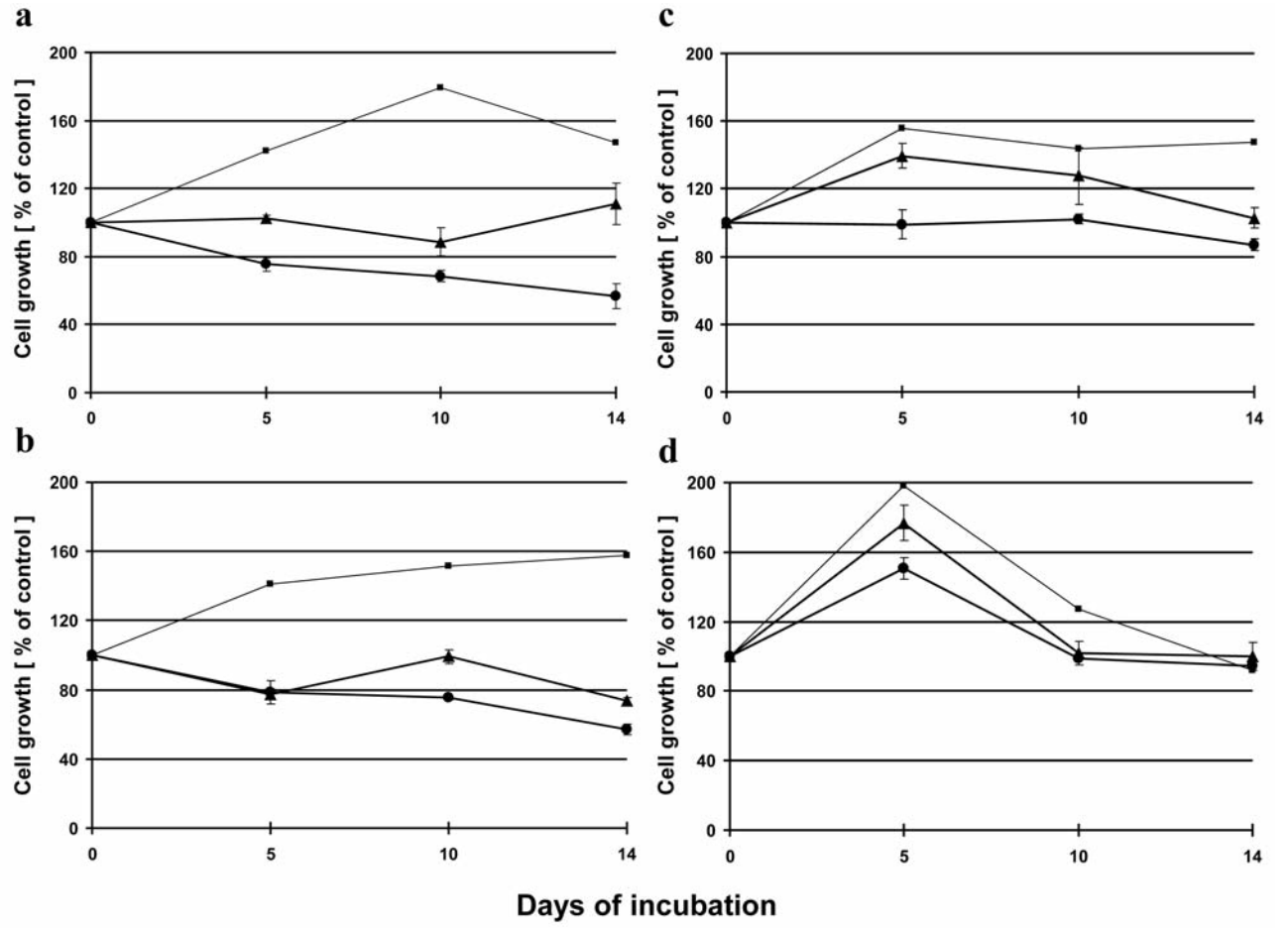

Figure 5. *Dose- and time-dependent growth inhibition of the human adenocarcinoma cell line 23132/87 (a), MKN-45 (b) and the human non-malignant cell line NHDF (c), and CCL-241 (d) during 14 days incubation with 636 μM (●), 318 μM (▲) (5-FdU–Ale) conjugate. (■) Control (PBS treatment instead of drug). Values represent the means of three independent experiments.*

(19). In the case of the normal cell line CCL-241, the parameters for evaluation cursor (x-axis) were set to 9.90-30.00 μm and the norm cursor was set to 7.65-30.00 μm, while for the NHDF normal cell line, the parameters for evaluation cursor were set to 10.80-30.00 μm and the norm cursor was set to 7.65-30.00 μm. All experiments were repeated three times. Statistical analysis was performed with SigmaStat 3.5 statistical analysis software (2006, Systat Software, Inc).

Results

Sensitivity of cell lines against single drugs. The *in vitro* testing of alendronate and the single drug 5-FU showed growth reduction of the gastric adenocarcinoma cell lines 23132/87 and MKN-45 from 100% to about 30% by incubation with 954 μM alendronate over an incubation period of 14 days (Figure 2a). For a comparable growth inhibition of the MKN-45 cell line, a 19-fold lower molar dose (49 μM) of 5-FU, with respect to alendronate, was needed (Figure 2b). The moderate activity of alendronate in gastric cancer cell lines corresponded to published *in vitro* cytostatic effects on other cell lines (18, 20).

Sensitivity of cell lines to mixtures of alendronate plus 5-FdU, and alendronate plus 5-FU. The cytostatic potential of an equimolar mixture of 5-FdU plus alendronate was evaluated as a model for the assessment of metabolites which could be expected due to the hydrolytic cleavage of 5-FdU-Ale resulting in a mixture of 5-FdU and alendronate. The mixture of 318 μM 5-FdU or 5-FU plus 318 μM alendronate totally inhibited the cell growth of the gastric adenocarcinoma cell line 23132/87 on 10 days of incubation (Figure 3). With 106 μM 5-FdU, or 5-FU and 106 μM alendronate, total growth inhibition was achieved in 14 days. Under corresponding conditions, MKN-45 cells were less sensitive compared to the 23132/87 cell line (Figure 4). Cell growth of the MKN-45 cells, when incubated with the

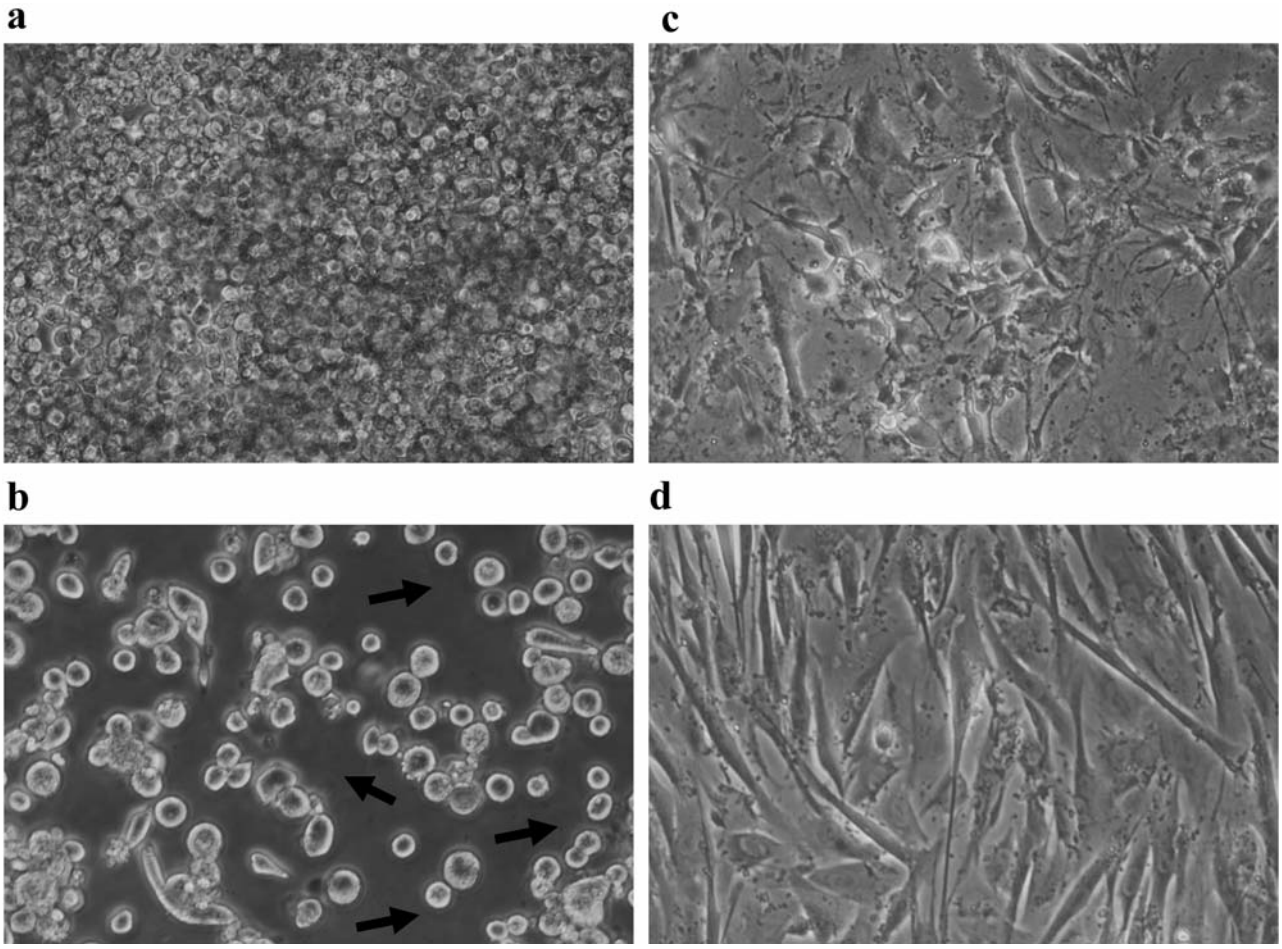

Figure 6. *Cell culture images of malignant cell line MKN-45 (a, b) and non-malignant cell line CCL-241 (c, d). Images (a) and (c) show the cell monolayer after 14 days treatment without drug (PBS, control). Images (b) and (d) show the cell monolayer after 14 days treatment with 636 μM (5-FdU–Ale). The cell-free areas (black arrows) in image (b) are caused by much higher cytotoxicity of the drug towards the MKN-45 cell line. All pictures were taken with a phase-contrast microscope (Axiovert 25; Zeiss, Oberkochen) equipped with a Canon EOS 500D camera and twentyfold magnification.*

mixture of 318 μM alendronate and 318 μM 5-FdU, was reduced to approximately 20% in 10 days (Figure 4a). The mixture of 318 μM alendronate plus 318 μM 5-FU reduced the growth of the cell line to approximately 5% (Figure 4b). The cytostatic potential of the 5-FdU plus alendronate against MKN-45 cells (30-40% growth inhibition), was markedly lower than that of 5-FU plus alendronate (70-90% growth inhibition) during a short incubation time (5 days).

Sensitivity of the cell lines to the 5-FdU-Ale conjugate. Comparison of the cytostatic effect of 5-FdU-Ale towards the gastric adenocarcinoma cell lines compared to the non-malignant cell lines CCL-241 and NHDF, demonstrated a slightly higher sensitivity of the cancer cell lines under comparable conditions. 5-FdU-Ale (636 μM) reduced the growth of both cancer cell lines during a 14-day incubation period from 100% to 60% (Figure 5a and b), whereas the growth of the non-malignant cell lines were only reduced to about 90% (Figure 5c and d). It is remarkable that the MKN-45 cells were slightly more sensitive to 5-FdU-Ale in comparison to the 23132/87 cells. 5-FdU-Ale at 318 μM did not reduce the growth of 23132/87 cells during the 14-day incubation (Figure 5a), whereas the growth of MKN-45 cells was reduced from 100% to about 70% (Figure 5b). The cytostatic activity of 5-FdU-Ale was slightly lower *in vitro* but comparable to that of the single-drug alendronate. However, compared to 5-FU-alone, the activity of 5-FdU-Ale was about 19-fold lower. The higher sensitivity of the gastric adenocarcinoma cell lines MKN-45 compared to that of the non-malignant CCL-241 cell line, for example, can be seen in Figure 6. The cell-free areas seen in Figure 6b were caused by the incubation of cells with 636 μM 5-FdU-Ale. Under the

same conditions, the CCL-241 cell line (Figure 6d) exhibited no sensitivity towards 5-FdU-Ale. The treatment of both gastric adenocarcinoma cell lines with 954 μM of alendronate or 5-FdU-Ale led to a slow decrease of viability with increasing incubation time (Figure 7). Thereby, the cytostatic effect of alendronate was slightly higher than that of 5-FdU-Ale (Figure 7a). The cytotoxicity of 5-FdU-Ale remained constant from incubation day 10; however, MKN-45 cells exhibited a decreasing sensitivity towards alendronate with increasing incubation time (Figure 7b).

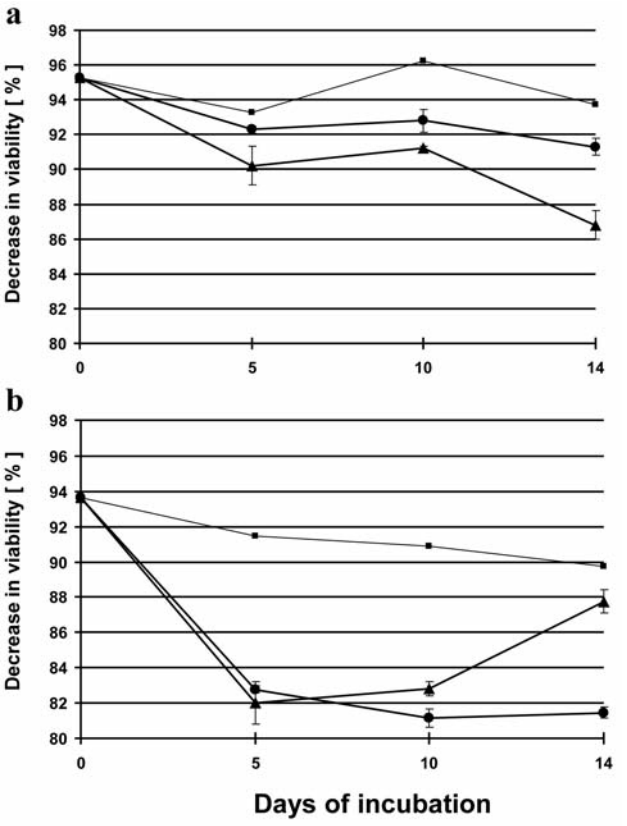

Figure 7. *Cell viability of the human adenocarcinoma cell line 23132/87 (a) and MKN-45 (b) during 14 days incubation with 954 μM alendronate (▲) and 954 μM (5-FdU–Ale) (●). Values are the means obtained from triplicates of each experiment. (■) Control (PBS treatment instead of drug).*

Discussion

For an effective treatment of metastatic gastric cancer with bone metastases, a sufficient therapeutic dose of systemically applied antitumor agents in the microenvironment of bone is needed, along with minimized systemic adverse effects. The modification of cytostatic drugs with BPs, bone-seeking agents, is a promising strategy for antitumor bone targeting, as well as for the reduction of adverse effects. The proposed new concept for the preparation of BP-nucleoside conjugates by linking the pyrimidine base of 5-FdU, a well-known antitumor drug, with amino-BP, resulted in 5-FdU-Ale, a new chimeric cytostatic molecule. The intact molecule 5-FdU-Ale represents on the one hand a new amino-BP, whose alkyl-chain is terminated by the nucleobase of the linked 5-FdU instead of its amino group, while on the other hand, 5-FdU-Ale is a new 5-fluoro-2'-deoxycytidine derivative, whose nucleobase is N^4-alkylated with the BP-(ale)-residue. The BP moiety of 5-FdU-Ale preserves its high affinity to hydroxyapatite, the premise of the desired bone-targeting. The adsorption of 5-FdU-Ale to hydroxyapatite was about six-fold higher compared to that of 5-FdU (17). The *in vitro* test demonstrated, on the one hand, that intact 5-FdU-Ale was not more effective compared to alendronate alone. On the other hand, the moderate cytostatic activity indirectly indicated a very high hydrolytic stability *in vitro*, which prevents the metabolization of 5-FdU-Ale during its circulation. By linking alendronate to the nucleobase of 5-FdU, the cytostatic activity of 5-FdU drastically decreased whereas the bone-targeting of the alendronate residue remained (hydroxyapatite adsorption). When 5-FdU-Ale is cleaved *in vitro* into a mixture of 5-FdU and alendronate, growth inhibition of the cell lines, comparable to that of the evaluated mixture of 5-FdU and alendronate, should be observed. However, the activity of the mixture was drastically higher compared to the *in vitro* activity of 5-FdU-Ale, indicating that the conjugate was not metabolized to a sufficient amount of 5-FdU and alendronate under the *in vitro* conditions. The cytostatic activities evaluated by cell assays provide only orientating data for *in vivo* effects. It can be postulated that 5-FdU-Ale represents a pro-drug of a mixture of 5-FdU and alendronate and other cytostatic metabolites thereof. Cell assays for the evaluation of pro-drugs are limited, especially when the whole activity of a pro-drug only unfolds after its metabolism. For such drugs, *in vivo* testing is essential. It should be borne in mind that over the *in vitro* cell incubation of 14 days, the conjugates can only be metabolized for a time span of three days, since the medium in this cell assay was changed every third day. In this way, 5-FdU-Ale was replaced by the same amount of new intact conjugate. The intended very slow metabolism of 5-FdU-Ale over a longer period of time, desirable in bone, cannot be realized in an *in vitro* cell assay. We hypothesize that intact 5-FdU-Ale survives as a pro-drug in the circulation and binds to the hydroxyapatite matrix of bone. The bone-targeting of 5-FdU-Ale allows a locally high concentration of the pro-drug and causes a depot in the regions of cancer metastasis because they are the areas of significant preferential BP accumulation. During slow metabolism of 5-FdU-Ale, a mixture of 5-FdU plus alendronate or other cytostatic metabolites together with alendronate, can be subsequently released into the bone microenvironment. The high therapeutic potential of 5-FdU or 5-FU plus alendronate, enriched in the bone

microenvironment, could be very effective against metastatic gastric cancer. 5-FdU-Ale could contribute to more successful polychemotherapeutic strategies against metastatic gastric cancer and merits further evaluation.

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Received June 22, 2012
Revised August 7, 2012
Accepted August 9, 2012