HDAC Inhibitor-loaded Bone Cement for Advanced Local Treatment of Osteosarcoma and Chondrosarcoma

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Abstract. *The treatment of osteosarcoma, especially wide resection, is challenging. An additional local drug therapy after resection using anti-neoplastic bone cement (Polymethylmethacrylate (PMMA)) could help improve the outcome of therapy. In this study, we evaluated the effects of PMMA loaded with valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) on the cell activity of a SaOs-2 cell culture, as well as the elution rate of the drugs out of the bone cement. Materials and Methods: In our experiments, we used the SaOs-2 osteosarcoma and the SW1353 chondrosarcoma cell line. Bone cement clots (5 g) were prepared and loaded with different drug concentrations of VPA (25 mg and 50 mg) and SAHA (1 mg, 2.5 mg and 5 mg). Two control groups were established, one with a native cement clot, the other with human mesenchymal stem cells, in order to evaluate toxicity on non tumor-cells. Cell activity was measured using an Alamar Blue assay on days 1, 2, 3, 4 and 7. The cement clots were additionally examined in a material testing unit for biomechanical and structural changes. Results: Tumor cells showed a significant and complete reduction of activity under therapy with VPA and SAHA. Drug release of VPA was extensive between days 0 and 3 and decreased progressively to day 7. Cumulative drug concentration in the medium continuously increased. Biomechanical testing of the cement clots showed no differences in stability and architecture compared to the control group. SaOs-2 and SW1353 cells with medium from native cement clots without drug therapy presented a cell activity of 100% in all groups and during all measurements. Human mesenchymal stem cells were not significantly affected during therapy with VPA and low concentrations of SAHA. In contrast, cell activity of human mesenchymal stem cells was significantly reduced under therapy with higher concentrations of SAHA, with an approximately linear decrease between days 0-3 and a rapidly decreasing activity between days 4-7. Conclusion: A local cytotoxic therapy in the treatment of osteosarcoma and chondrosarcoma might improve the rate of metastasis and survival of patients. Our results present an encouraging approach to loading PMMA with anti-neoplastic drugs.*

Osteosarcoma and chondrosarcoma are very aggressive neoplasms and the most common types of bone cancer. Most often (more than 50% of all cases) osteosarcoma affects the distal femur and proximal tibia. The overall survival has improved dramatically during the last centuries. Comparing the 1960s, with a survival rate of about 15%, actually approximately 70% of all patients with osteosarcoma survive nowadays (2). The metastatic disease is the most challenging difficulty in treatment. The presence of metastasis at diagnosis reduces the survival rate to 30% (1, 2) Chondrosarcoma often affects the lower extremity and the pelvic bone and has a peak of incidence in the second half of life. The survival rate for patients with high-grade chondrosarcoma is below 50%, with a high risk (approximately 70%) of pulmonary metastasis. Traditional radiotherapy and chemotherapy is not very effective (1, 2).

Biopsy as well as therapy should be carried out in specialized oncological centers. Surgery with a wide resection of the tumor is still the most important step in therapy. Wide resection reduces local and systemic metastasis significantly (2, 3). Surgery is combined with a multi-agent chemotherapy. Several agents, such as cisplatin,

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doxorubicin, methotrexate and ifosfamide, are well-examined and are active against osteosarcoma (4). However, postoperative treatment with cytotoxic agents is rarely curative and is accompanied by severe side-effects such as bone marrow depression, cardiotoxicity or nephrotoxicity.

Targeted therapy is a new part of chemotherapy. Tumor cells often achieve their resistance towards apoptosis by epigenetic deactivation of apoptosis-regulating genes. In this context histone deacetylase (HDAC) inhibitors is a promising group of medications. Treatment with these inhibitors causes chromatin condensation and down-regulation of gene expression which is necessary for carcinogenesis and tumor growth (4-6). Several *in vitro* and *in vivo* studies have demonstrated a tumor response to HDAC inhibitor (4-6). HDAC inhibitors have actually undergone clinical assessment (7). Therefore, two HDAC inhibitors, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA), were used in this study.

Bone cement is a commonly used material in surgery. It can be applied for direct stabilization of bone (*e.g.* pathological fractures, bone defects/voids), or as support for osteosynthesis or endoprosthesis. The cement is a compound consisting of 90% polymethylmethacrylate (PMMA) and 10% of contrast media crystals. The structure of the bone cement is made out of two substances, small particles of pre-polymerized PMMA being supplied as a white powder, and a liquid monomer of MMA. Both substances are mixed and glued together in a polymerization reaction. Microscopically this reaction leads to formation of a net-like structure in which the PMMA particles are embedded. The polymer net also enables the bone cement to be loaded with other substances such as antibiotics (8, 9).

A possible extension in the treatment of sarcoma would be to add local drug therapy to the surgical therapy. A benefit might be the possibility of achieving a high local drug concentration, reducing the number of free tumor cells, as well as micrometastases. Local control of the tumor as well as the risk of metastasis might be improved. For local drug delivery, certain material properties are necessary. The chemotherapy agent should elute a sufficient local drug concentration constantly over a period of several days. Additionally, the stability of the bone cement should not be affected by the added drug.

A reasonable approach would be to use bone cement as a carrier for a local chemotherapy. PMMA is already commonly loaded with antibiotics and there is much clinical and experimental experience in the therapy of bone infection (10-13). Research in local therapy of sarcoma using drug-loaded PMMA is in early stages (14). No clinical data are available.

This *in vitro* study was carried-out as a new approach to examine the effects of anti-neoplastic drug-loaded PMMA on the cell activity of osteosarcoma and chondrosarcoma cells. The interaction between bone cement and tumor cells, as well as the drug elution rate and the biomechanical changes of the PMMA, were evaluated. Two different HDAC inhibitors were examined. Local cytotoxic effects were tested in tumor cells, as well as in human mesenchymal stem cells (hMSCs), to evaluate the effects on non-tumoral cells.

Materials and Methods

Bone marrow-derived hMSCs, SaOs-2 cells and SW1353 cells. hMSC cultures were generated from bone marrow. After hip replacement, pieces of stromal bone marrow were excised from the femoral head with a medical forceps. The femoral head was considered to be excess material after surgical treatment and would otherwise have been discarded. Informed consent was obtained from the donating patient.

The pieces of stromal bone marrow were washed several times with phosphate buffered saline (PBS) by mixing vigorously until the supernatant remained clear. The PBS from the washing steps was centrifuged and the supernatants were discarded. The cells were cultivated in 6-well plates with an initial density of 1×10^6/ml in Dulbecco's Modified Eagle's Medium (DMEM)/F12-GlutaMAX™ (Invitrogen Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS). The medium was changed every 2-3 days and non-adherent cells were discarded.

The osteosarcoma cell line SaOs-2 and the chondrosarcoma cell line SW1353 were purchased from Cell Line Service (Eppelheim, Germany). The cells were cultured in DMEM/F12-GlutaMAX™ supplemented with 10% FCS.

PMMA, VPA and SAHA. A standard bone cement without antibiotic load was used in this trial (VertebroX; Somatex Inc., Berlin-Teltow, Germany). The cement procedure started with the mixing of the PMMA and the drugs in different concentrations. A standard vacuum-assisted mixing device was used (SOMIX; Somatex Medical Technologies). A standard cement clot of 5 g each with the shape of a tablet was produced (Figure 1). The drug concentration used was chosen to be high in order to achieve a high local concentration. The amount of PMMA-loaded chemotherapy followed the usually applied concentration for systemic therapy and was chosen in this experiment to be between 25- and 50-times higher than that used in systemic therapy. This approach is known from the loading of PMMA with antibiotics (10, 15). The 5 g cement clots with different drug concentrations were embedded in 10 ml of DMEM/F12-GlutaMAX™ each. On day 1, medium from the cement clots was removed and stored at −20˚C until administration to the hMSC, SW1353 and SaOs-2 cells. After removal, the cement clots were immediately embedded again with 10 ml cell culture medium due to local tissue effects being taken into account. This procedure was repeated on days 2, 3, 4 and 7. Drug concentration eluted into the medium was measured each day for VPA. Drug concentration of SAHA could not be measured due to there being no measurement procedure.

Cell activity assay. For evaluating cell activity and cytotoxic effects, SaOs-2, SW1353 and hMSCs were placed in cell culture plates with 96 wells. Each culture plate contained 2×10^4 cells and 100 µl of DMEM/F12-GlutaMAX™ per well. The cells were seeded out 24 h prior to the assay to ensure adherence.

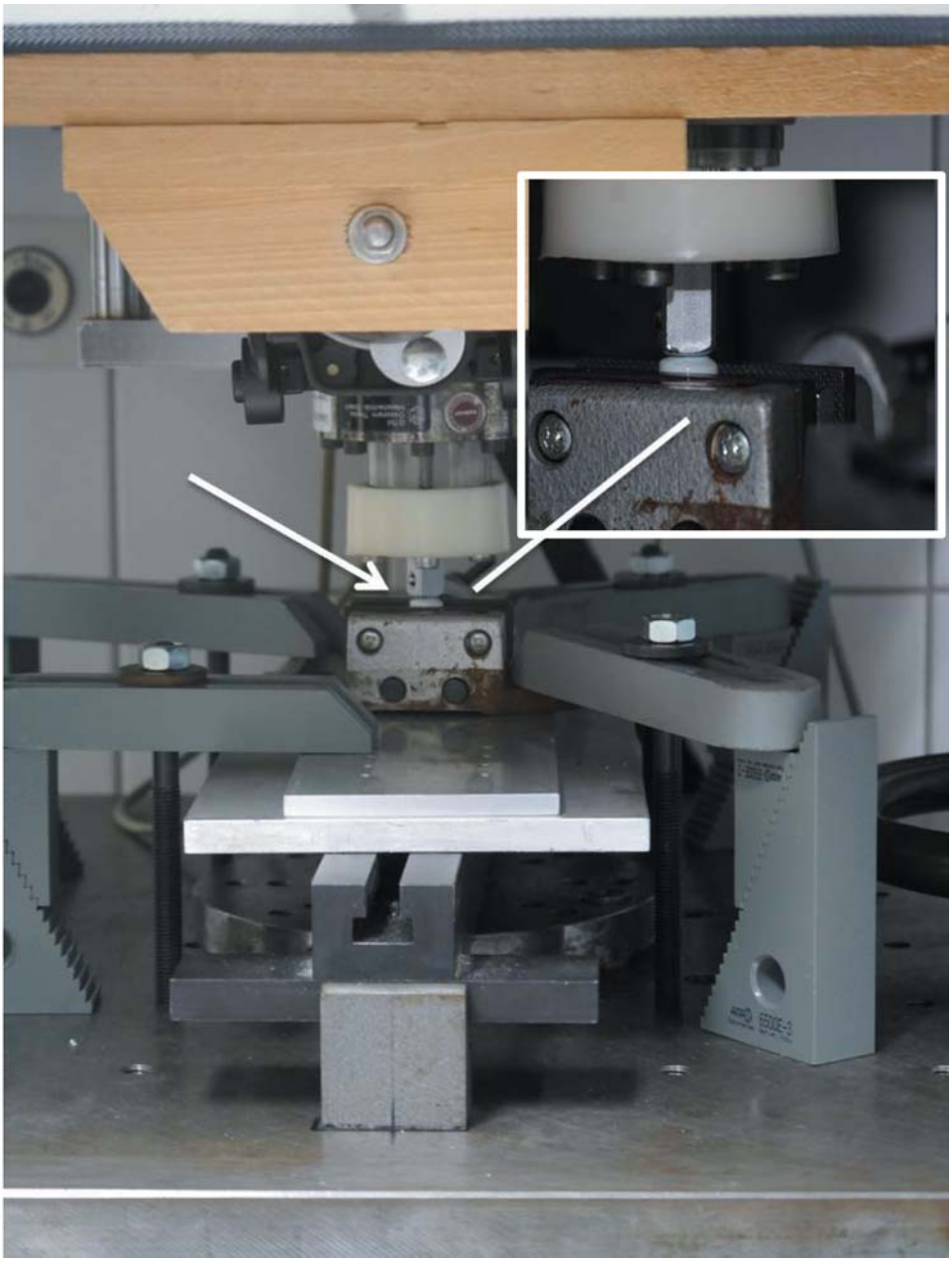

Figure 1. *Biomechanical test unit. White arrow: Clamped bone cement clot with 5 g of Polymethylmethacrylate (PMMA). Inset: Enlarged image showing detail of the cement clot.*

On day 0, the supernatants were removed and cell activity was measured using an Alamar Blue assay (Sigma Aldrich, Munich, Germany). Alamar Blue solution was mixed with DMEM/F12-GlutaMAX™ at a final concentration of 10% (v/v). One hundred milliliters of this mixture was added to each well. After 1-2 h, the Alamar Blue assay solution supernatants were removed into a new 96-well plate and the absorbance was measured at 540 and 630 nm.

The cells were cultured with appropriate supernatants of the cement clots from day 1. The measurement of cell activity and subsequent cultivation with the appropriate cement clot supernatants was repeated on days 2, 3, 4 and 7.

Materials testing unit. In this study, we used a testing unit (Instron 1195; Wolpert Group, Bretzfeld, Germany) consisting of a reference block for cement clot fixation, a pressure sensor (Sensor WS10; ASM Group, Moosinning, Germany), a pressure piston and a computer-based measurement and control module (Cadman 4.0; Hottinger & Baldwin, Darmstadt, Germany). Five cements clots for

each drug concentration, as well as 10 native cement clots, were tested (35 in all).

All cement clots were clamped in the reference block (Figure 1). Axial pressure with a maximum of 1000 N was administered; this force was chosen because this is a critical pressure force which is even higher than bone cement has to withstand in the human body. Microscopic examination of the cement clots followed in order to evaluate structural changes in the material. The pressure was measured with a sensor between the piston and the syringe and was digitally recorded.

Statistical evaluation. We performed univariate analysis of independent variables with the *t*-test for independent samples for quantitative variables. All tests were two-sided and a *p*-value less than 0.05 was considered to be significant. All analyses were conducted with SPSS statistical software for Windows 14.0 (SPSS, Chicago, IL, USA).

Results

In addition to surgical resection of osteo- and chondrosarcoma, local treatment with chemotherapeutic drugs might enhance the therapeutic effect. Therefore, bone cement clots were loaded with VPA, an inhibitor of class I and II HDACs, incubated in cell culture medium and the supernatants were tested for their growth-inhibitory effect. Biomechanical testing of the cement clots loaded with chemotherapeutic drugs showed no changes in stability and architecture compared to the control group without chemotherapy (Figure 2).

For an improved simulation of the *in vivo* situation, supernatants were changed every day and the cell lines SaOs-2 and SW1353 were incubated with the corresponding supernatants on consecutive days. The supernatants from the cement clots containing a high concentration of VPA induced already on day 1 a significant reduction of cell vitality in both osteo- and chondrosarcoma cell lines (Figure 3). On day 3, no cell vitality was measurable for the osteosarcoma cell line SaOs-2. The complete reduction of cell vitality of the SW1353 chondrosarcoma cell line was achieved on day 7. The lower concentration of VPA (25 mg in 5 g bone cement) caused only a reduction in cell vitality of 40% in the chondrosarcoma cell line and a complete cell death of SaOs-2 cells on day 7.

In order to determine the release kinetics from bone cement clots, the concentrations of VPA of the supernatants from each day were measured (Figure 4). As expected, the highest release of valproic acid was measured on day 1. On day 2, the release of VPA was clearly reduced to one-third compared to day 1. Even on day 7, a VPA-release from the bone cement clots is measurable in the supernatants.

To increase the spectrum of treatment with HDAC inhibitors SAHA, a more hydrophobic HDAC inhibitor additionally affecting class IV HDACs, was included in bone cement clots. As described above, the cell lines SaOs-2 and SW1353 were incubated with the supernatants of cultured cement clots including SAHA at three different concentrations. Even the supernatants from the clot with the lowest concentration of SAHA (1 mg SAHA in 5 g bone cement) caused a strong reduction of cell vitality in both cell lines, with a 100% reduction on day 3 (SaOs-2) or day 4 (SW1353). No significant differences in reduction of cell vitality were observed between the different concentration groups. Nevertheless, local

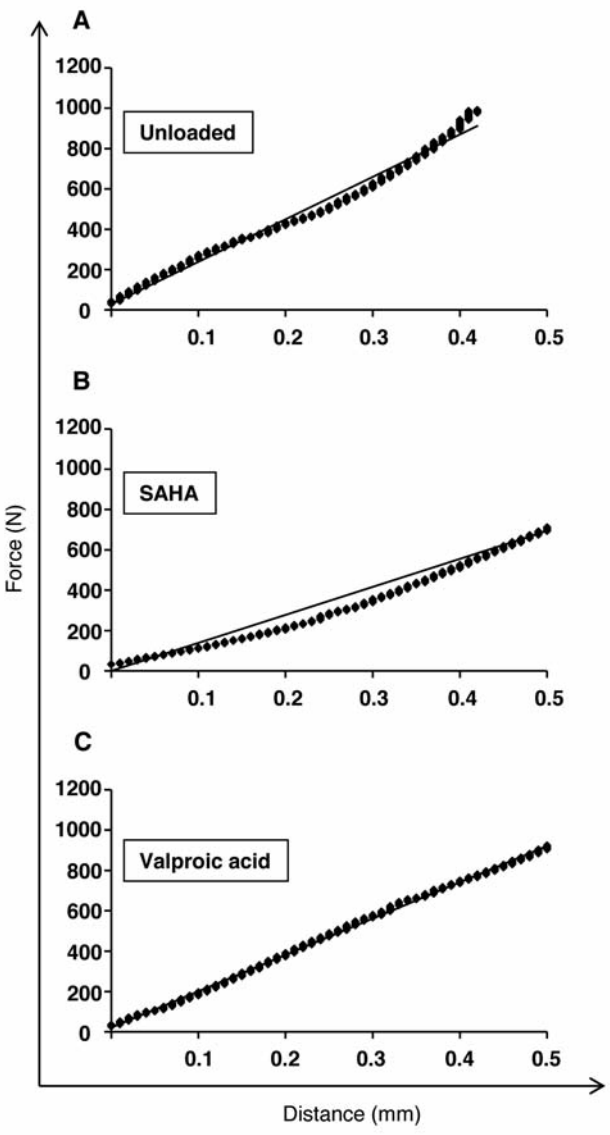

Figure 2. *Biomechanical testing of the bone cement clots without chemotherapeutic drugs (A), of the suberoylanilide hydroxamic acid (SAHA) group (B) and the valproic acid (VPA) group (C). There is a constant plastic deformation of the bone cement due to increasing pressure of the piston. A fracture or a significant change of the architecture of the cement would lead to an abrupt rise in the movement of the piston. This was not seen in any group. The results in this figure show that loading bone cement with chemotherapeutic drugs does not change the properties of the material.*

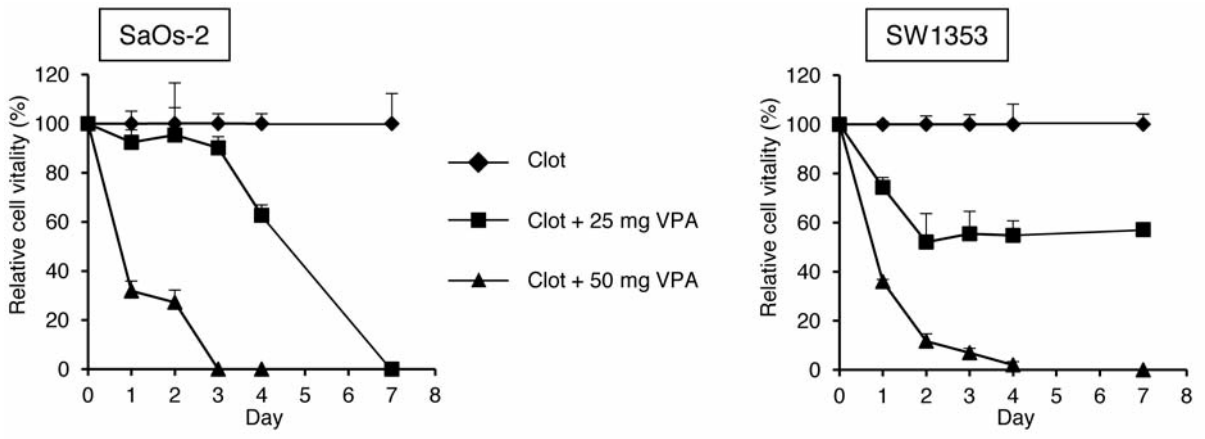

Figure 3. *Valproic acid (VPA) released from cement clots severely reduces vitality of osteo- and chondrosarcoma cells. Five grams of bone cement were mixed with either 25 mg or 50 mg VPA. After hardening, the cement clots were cultivated. On days 1, 2, 3, 4 and 7, the medium was changed and supernatants were collected. Cultivated osteo- and chondrosarcoma cell lines were incubated with the supernatants. Before adding the clot medium from the appropriate day, relative cell vitality was measured by the Alamar Blue assay. The Alamar Blue reagent was mixed 1:10 with culture medium and added to the wells. After 1-3 h of incubation, the absorbance was measured at 560 nm (600 nm reference wavelength). Experiments were performed in triplicates (data are the mean±SD).*

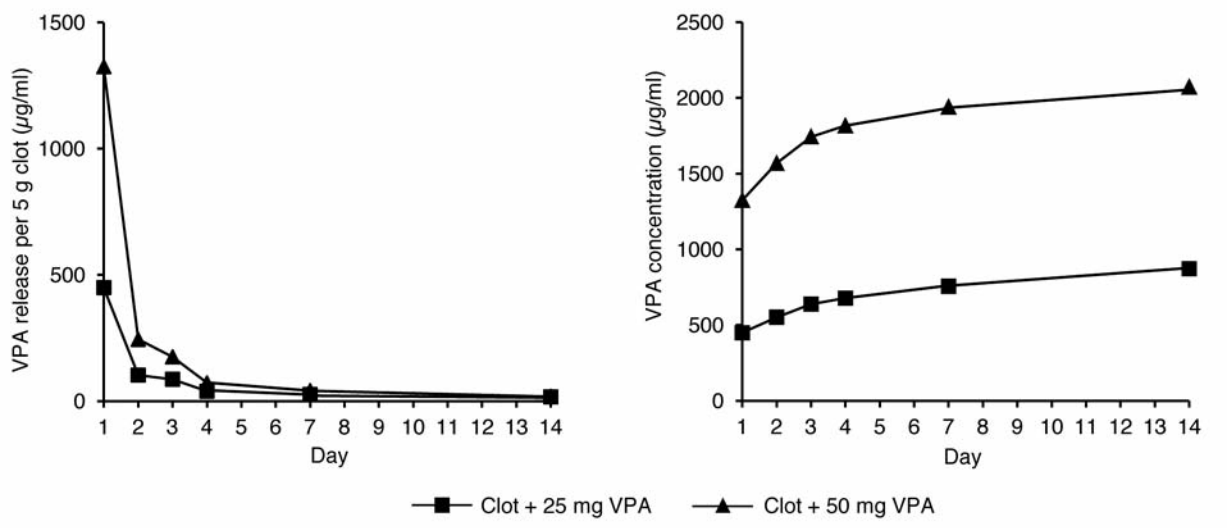

Figure 4. *Cement clots loaded with valproic acid (VPA) continuously release the histone deacetylase (HDAC-) inhibitor in the presence of culture medium. Five grams bone cement were mixed with either 25 mg or 50 mg VPA. After hardening, the cement clots were placed in 10 ml culture medium. On days 1, 2, 3, 4, 7 and 14, the media were changed, supernatants were collected and the concentration of VPA in the supernatants was measured.*

treatment with SAHA was more effective than treatment with VPA, a member of the same drug group.

To investigate whether the vitality of non-tumor cells can also be affected by local treatment with HDAC inhibitors, hMSCs generated from bone marrow were incubated with the supernatants from the HDAC-loaded bone cement clots. The supernatants of the VPA-loaded cement clots, regardless of concentration, had no effect on the vitality of hMSCs (Figure 6). The supernatants from the cement clots loaded with the highest and middle concentration of SAHA caused even in hMSCs a strong reduction of cell vitality until day 7. The supernatants from the cement clots loaded with the lowest concentration of SAHA induced only a reduction in cell vitality of 20% until day 7. More experiments are required to determine the best concentration of SAHA at which cell vitality of non-tumoral cells is almost unaffected

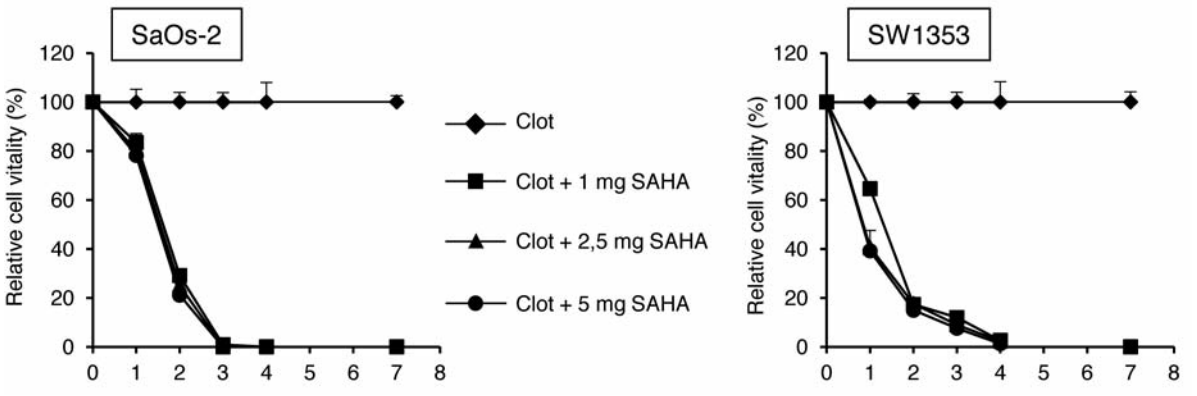

Figure 5. *Suberoylanilide hydroxamic acid (SAHA) released from cement clots severely reduces vitality of osteo- and chondrosarcoma cells. Five grams of bone cement was mixed with either 1 mg, 2.5 mg or 5 mg SAHA. Hardened cement clots were cultivated in 10 ml culture medium. On days 1, 2, 3, 4 and 7, media were changed and supernatants were collected. Cultivated osteo- and chondrosarcoma cell lines were incubated with the supernatants. Prior to cultivation of the cells with the appropriate supernatant, relative cell vitality was measured by Alamar Blue assay. The Alamar Blue reagent was mixed 1:10 with culture medium and added to the wells. After 1-3 h of incubation, the absorbance was measured at 560 nm (600 nm reference wavelength). Experiments were performed in triplicates (data are the mean±SD).*

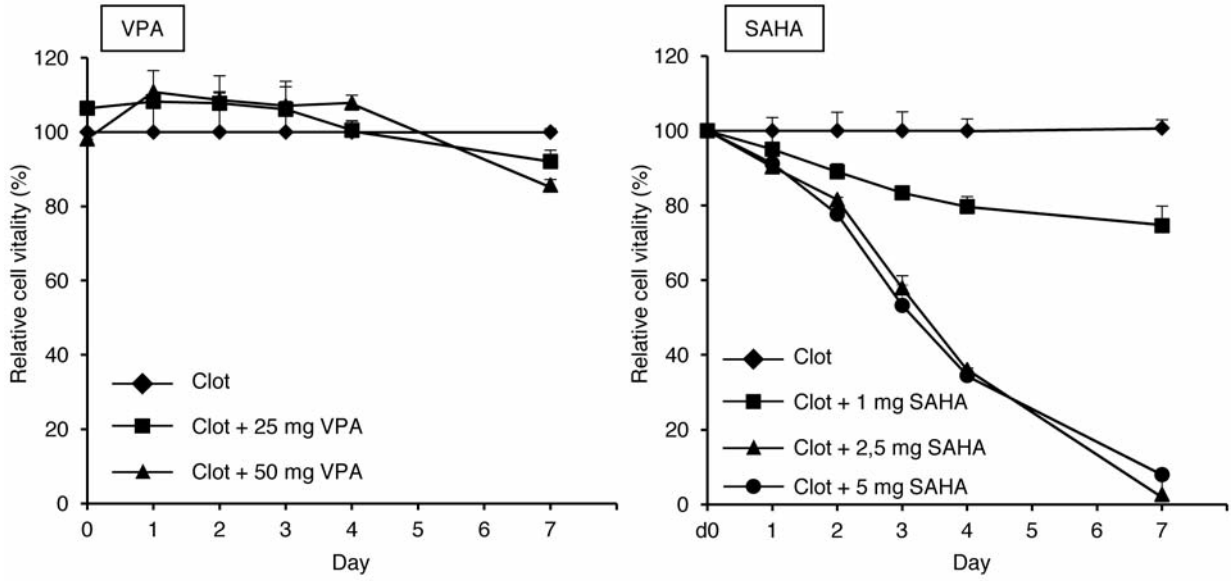

Figure 6. *Influence of histone deacetylase (HDAC-) inhibitors on the cell vitality of non-tumoral human mesenchymal stem cells. Human mesenchymal stem cells were cultivated with the supernatants from cement clots cultured with SAHA or VPA as described in the Materials and Methods. Prior to cultivation of the cells with the appropriate supernatant, relative cell vitality was measured by Alamar Blue assay. Experiments were performed in triplicates (data are the mean±SD).*

and the vitality of osteo- and chondrosarcoma is clearly reduced to a minimum.

Discussion

Osteosarcoma and chondrosarcoma are very aggressive neoplasms and irrespective of advances in therapy, a large proportion of patients does not survive. Wide resection is essential and significantly reduces local and systemic metastasis (2, 3). The quality of surgery has a strong influence on the survival of the patient. However, the advances in surgery are much slower than in chemotherapy. Orthopaedic implants are already highly developed. Local therapy might improve the surgical outcome. Efforts have

been expanded in local radiotherapy and photodynamic therapy. Interstitial brachytherapy has certain risks for wound complications and osteoradionecrosis (16, 17). Photodynamic therapy has undergone only minor clinical assessment and is a complex technique (18). Bone cement might be a better alternative for local therapy. It is easy to administer and has a long history of development and usage. PMMA, a solid material with good biocompatibility, was invented by Otto Röhm in 1902. Edward Haboush and Sir John Charnley were the first to document the use of bone cement in orthopaedic surgery in the 1950s. At the end of the 1950s, Buchholz came up with the idea of adding an antibiotic to the cement to reduce the incidence of infection. Since then, more than one generation of scientists have shown that adding antibiotics to the bone cement ensures a significant and continuing local release without systemic toxicity (10, 11, 19-21). Additionally it was shown that adding an amount of antibiotics of less than 10% of the weight of the bone cement does not change the mechanical properties of the material. This is only validated for powder-like substances and not for liquids (10, 19). This is a limitation because some chemotherapy agents are only available as a liquid substance. The 10% margin for adding agents to the bone cement is not a difficulty because the dosage of the chemotherapy is generally below this limit. In our study, all bone cement clots showed a strong and constant elution of the chemotherapy drug, with a high local concentration (Figure 3). No changes in the mechanical properties of PMMA were seen in any group (Figure 2).

The amount of medium obtained from the cement clots was refilled every 24 h and therefore a certain reduction of drug concentration due to local tissue effects (clearance) needed to be taken into account. This is a weak point of an *in vitro* study design. Local tissue effects and influences on local drug application are complex and difficult to understand. *In vitro* designs, therefore, have limitations in simulating those effects. Experiences of studies using antibiotics in PMMA were used in this context (10, 19).

Strong cytotoxicity was seen in all groups. VPA caused a nearly 100% reduction of tumor cell activity from the first day of measurement when using 50 mg of VPA per clot. In contrast, 25 mg VPA caused a progressive reduction of cell activity, with around 80% cell activity during the first three days and 0% activity at day 7. These results suggest that a concentration of 25 mg sufficiently reduces tumor cell activity. A high concentration of 50 mg is not necessary and therefore additional damage to local non-cancer tissue can be avoided by using lower concentrations. Regardless of the concentration of VPA, hMSCs did not suffer additional damage during therapy. A different situation was found during therapy with SAHA. There was a potential cytotoxicity towards hMSCs, showing that possible side-effects on healthy tissue might be stronger during therapy with SAHA compared to VPA (Figure 6). Comparing the results in the SAHA groups, it seems to be more efficient to use a lower drug concentration.

Our results also show that the drug release from bone cement was very high at the beginning and decelerated in the course of the therapy. Drug elution tests of VPA showed that about 80% of the drug concentration was released from the bone cement during the first four days. These results are quiet similar to the tests using antibiotics in PMMA (10, 11, 19-21).

Conclusion

Local cytotoxic therapy in the treatment of osteosarcoma and chondrosarcoma might reduce local recurrence and improve the rate of metastasis and thereby the overall survival of patients. Our results present an encouraging approach loading PMMA with anti-neoplastic drugs. Adding chemotherapeutic drugs does not change the mechanical properties of the bone cement. Further animal studies are required to validate the results *in vivo* and to carry-out examinations on local drug release and systemic drug effects.

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