Bone tumours may recur locally even after wide surgical excision and systemic chemotherapy. Local control of growth may be accomplished by the addition of cytostatic drugs such as methotrexate (MTX) to bone cement used to fill the defect after surgery and to stabilise the reconstructive prosthesis. We have studied the elution kinetics of MTX and its solvent N-methyl-pyrrolidone (NMP) from bone cement and their biological activities in five cell lines of osteosarcoma and in osteoblasts, and compared them with the effects of the parent compounds alone and in combination.

Our findings show that MTX is released continuously over months at concentrations highly cytotoxic to osteosarcoma cells and suggest that the impregnated bone cement would be effective in the long term. Proliferating osteoblasts, however, were much less sensitive towards MTX. The dose-response relationship for NMP and experiments with MTX/NMP-mixtures show that the eluted concentrations of solvent are not toxic and do not influence the effects of MTX.

We suggest that bone cement containing MTX dissolved in NMP releases the drug in a suitable and effective way and may be of value in the treatment of bone tumours.


Despite systemic chemotherapy local recurrence is a considerable problem in the treatment of bone tumours. Local control may be achieved by the addition of cytostatic drugs to the bone cement filling the defect after surgery to stabilise the reconstructive prosthesis. Bone cement containing antibiotics has been used successfully as a local drug-delivery system for the treatment and prevention of musculoskeletal infections. It has been shown that chemotherapeutic drugs such as methotrexate (MTX) added to bone cement do not alter the biomechanical properties in a concentration of up to 2 g MTX per 40 g of cement and are biologically active on tumour cells in vitro and in vivo after elution from the cement. Eluted MTX inhibits the proliferation of fibrosarcoma cells in vitro and reduces the growth of mammary carcinoma and osteosarcoma in vivo. In these studies MTX was added as powder to the components of the cement. A polymethylmethacrylate bone cement containing MTX dissolved in 1-methyl-2-pyrrolidone (N-methyl-pyrrolidone, NMP) has been developed. The homogeneous distribution of the drug and the progressive exchange of NMP by water during elution improve the kinetics of release and give more reproducible results. The avoidance of the formation of MTX dust during preparation and application of cement is safer and easier.

We have examined the elution kinetics of MTX and its solvent NMP from bone cement and their biological activity on five cell lines of osteosarcoma and compared them with those of the parent compounds alone and in combination. To assess the effects of MTX and NMP on normal bone cells, osteoblasts were incubated with eluates. Dose-response curves were prepared.

Materials and Methods

In vitro release of cement compounds into culture medium. Test tablets of bone cement (Palacos-R; Kulzer, Wehrheim, and Merck, Darmstadt, Germany) were prepared by mixing 20 ml of monomer and 2 ml of MTX (250 mg MTX; Orion Pharma GmbH, Hamburg, Germany) in NMP (Merck, Darmstadt, Germany) with 40 g of cement powder at room temperature. The paste was placed in a number of cylindrical hollows. After polymerisation the tablets (10 mm in height, 24 mm in diameter) were removed and
the weight of each was determined (mean 5.63 ± 0.14 g). Two types of control cement were prepared: Palacos-R with sodium carbonate and Palacos-R with sodium carbonate and NMP. Preliminary experiments showed that sodium carbonate, which is added to cement to improve the release kinetics of MTX, did not affect the proliferation of osteosarcoma cells. Each group consisted of three tablets.

The tablets of cement were placed in sterile vials filled with 20 ml of RPMI-1640 culture medium (PAA Laboratories, Linz, Austria) containing 10% fetal calf serum (FCS; PAA Laboratories) and 2 mM of L-glutamine, 100 U/ml of penicillin G, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B (Gibco, Grand Island, New York) and incubated at 37°C in 5% CO₂; the medium was removed and replaced every 24 hours for a period of ten days. Then elution was continued with 66.7 mM phosphate buffer for 56 consecutive days. Concentrations of the eluted compounds (MTX, NMP) were determined by high-performance liquid chromatography (Merck-Hitachi, Darmstadt, Germany).

Cell culture. We used the following osteosarcoma cell lines: MG-63, MNNG-HOS, OST, SaOS and U2OS (ATCC, Rockville, Maryland). These were cultured in RPMI-1640 medium supplemented with 2 mM of L-glutamine, 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B and 10% FCS. The cultures were incubated in humidified 5% CO₂ at 37°C. At confluence, cells were placed in 25 cm² tissue-culture flasks (Falcon Plastics, Oxnard, California) after brief exposure (5 min) to 0.05% trypsin/0.02% EDTA. For testing the cytotoxic effects, assays of viability were performed in 96-well flat-bottomed microtitre plates at densities of 2000 cells/well in 100 µl of culture medium. In addition, osteoblasts were seeded at a density of 60 000 cells/well. After two to three days the medium was discarded and replaced with eluates or MTX- and NMP-containing media. After the indicated time periods 10 µl of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, München, Germany) in phosphate-buffered saline (PBS) was added to each well and the cultures were incubated for another four hours at 37°C. The medium was aspirated and formazan crystals solubilised in 100 µl of 50% N,N-dimethylformamide in 10% sodium dodecyl sulphate (Sigma). The plates were read at 550 nm (test wavelength) and 630 nm (reference wavelength) using a Dynatech MR7000 microplate reader (Dynatech Germany, Denkendorf, Germany).

Results

In vitro release of MTX and NMP on osteosarcoma cells. As shown in Figure 1 the release of MTX and NMP from bone cement followed a similar pattern. After an initial phase with a sharp decrease in the rate of discharge...
on day 1 from 48 µM of MTX and 3.6 mM of NMP to 5.0 µM of MTX and 0.53 mM of NMP on day 7, the release of both compounds then decreased only slightly. After 56 days of elution the concentrations of MTX and of NMP were 2 µM and 0.27 mM, respectively.

**Effects of MTX and NMP on osteosarcoma cell growth: dose-response curves.** For estimating the extent of the influence of the eluate on the proliferation of osteosarcoma cells, the effect of various concentrations of MTX and NMP (10^{-10} to 10^{-3} M of MTX and 10^{-8} to 1 M NMP) were determined at 48, 96, 144 and 196 hours. At 96 hours of incubation, all the cell lines of osteosarcoma were affected at concentrations of MTX higher than 0.1 M, but displayed different sensitivities depending on their rate of growth (Fig. 2a). The fast proliferating cell lines MG-63 and MNNG-HOS were much more affected by MTX after incubation for 48 hours than the slower-growing cell lines SaOS, OST and U2OS (Fig. 2b). Of all cell lines tested, OST was the least sensitive to MTX even after incubation for 196 hours. The concentrations released from the bone cement after 56 days are in the range of the maximum effect of MTX.

Sensitivity to NMP was similar for all cell lines. Cell proliferation was not affected by NMP at concentrations ranging from 10^{-9} M to 10^{-2} M. Concentrations of NMP higher than 10^{-2} M showed cytotoxic effects (data not shown).

**Effects of eluates from bone cement on osteosarcoma cells.** Eluates of MTX were found to inhibit proliferation of osteosarcoma cells (Fig. 3). The proliferation of OST cells was only inhibited but the other four cell lines were killed by the eluates. The slow-growing cell lines (OST, SaOS, U2OS) had a proliferation profile which could be predicted from the dose-response curves: eluates from days 2 and 10 were equally effective in these cell lines. Eluate from day 1 was shown to have an unexpected high cytotoxic effect on MG-63 and MNNG-HOS cells (shown for MG-63 in Figure 3a).

Two types of cement (Palacos-R with sodium carbonate and with or without NMP) served as controls for MTX-
cement. Sodium carbonate, which is added to cement to improve release of MTX, did not affect proliferation of osteosarcoma cells. As shown in Figure 4, eluates of control cement with and without NMP had hardly any effect on slow-growing U2OS but NMP-containing control 1-day eluate had a cytotoxic effect in fast proliferating MG-63 cells. These data suggest that NMP promotes the release of cytotoxic cement component(s) other than MTX.

**Effects of MTX/NMP-mixtures on osteosarcoma cells.** To investigate whether NMP may influence the cytotoxicity of MTX on the proliferation of osteosarcoma cells in MTX/NMP mixtures at concentrations equal to those in MTX-cement 1-day eluates were determined. As shown in Figure 5, MTX-mediated inhibition of proliferation of osteosarcoma cells was not modulated when NMP was also present, suggesting that NMP alone at the indicated concentration has no cytotoxic effect and does not interact with MTX.

**Effects on osteoblasts: incubation with eluates and various MTX and NMP concentrations.** To assess the effects of MTX and NMP eluted from loaded bone cement on normal bone cells, cultures of proliferating (seeding density, 2000 cells/well) and confluent (seeding density, 60 000 cells/well) osteoblasts were incubated with 10-day eluates for 96 hours (Fig. 6). In addition, dose-response curves of MTX (Fig. 2a) and NMP were recorded. Whereas eluates from control cement and from NMP-containing control cement did not affect proliferation of osteoblasts, that from MTX-cement (5 µM MTX) reduced proliferation by about 30% (Fig. 6). Inhibition of the proliferation of osteosarcoma cells was more than 90% in four of the five cell lines. Only cell line OST showed moderate sensitivity to the eluate of MTX-cement with approximately 55% inhibi-

---

**Figure 4a** The effect of eluates from control cement on the proliferation of osteosarcoma cells exemplified by U2OS (a) and MG-63 (b) (1d, 10d, eluates from control cement (i.e., Palacos-R with sodium carbonate); 1d-NMP, 10d-NMP, NMP-containing eluates of control cement from days 1 and 10 of elution). Each point denotes the mean of eight replicates from two representative experiments. The standard deviation of the mean value was less than 10%.

**Figure 5** Effect of MTX/NMP-mixtures on proliferation of osteosarcoma cells exemplified for MG-63. The concentrations of MTX and NMP are equal to those of eluates of MTX-cement from day 1 (47.8 µM MTX, 3.6 mM NMP) (NMP, NMP-containing medium; MTX, MTX-containing medium; NMP + MTX, medium with MTX and NMP). Each point denotes the mean of eight replicates from two representative experiments. The standard deviation of the mean values was less than 10%.

**Figure 6** Cytotoxicity of bone cement 10-day eluates in osteoblasts (OB) in comparison to osteosarcoma cells. Cells were incubated with eluates from control cement (contr. cem.), NMP-containing control cement (NMP-cem.) and MTX-impregnated cement (MTX/NMP-cem.) for 96 hours. Proliferating osteoblasts (OB) were seeded at the same density as osteosarcoma cells (2000 cells/well). Osteoblast cultures confluent at the start of incubation (OB confluent) were seeded at a density of 60 000 cells/well. Each point denotes the mean of six (osteoblasts) or eight (osteosarcoma) replicates from two representative experiments. The standard deviation of the mean values was less than 10%.
tion, but was still more affected than osteoblasts. Confluent osteoblast cultures were not affected by the eluates. Dose-response curves showed that concentrations of MTX higher than 1 µM reduce osteoblast proliferation of osteoblasts by approximately 30% confirming the results obtained from eluate-incubation experiments. By comparison, osteosarcoma cells are more sensitive to this cytostatic (≥0.1 µM; Fig. 2a). No cytotoxic effect of MTX was observed in confluent osteoblast cultures up to concentrations of 0.1 mM MTX.

Dose-response curves for NMP showed that this solvent had similar cytotoxic effects in osteoblasts and osteosarcoma cells. NMP at concentrations present in the eluates was ineffective, whereas concentrations higher than 0.1 M NMP were found to be cytotoxic. In contrast to MTX, cells of confluent osteoblast cultures were affected in the same way as proliferating cells.

Discussion

The application of bone cements loaded with antibiotics in the treatment and prevention of musculoskeletal infections has suggested that cement could be used as a drug-delivery system for the control of local tumour growth. Studies in vitro have indicated that bone cements impregnated with antimitotic drugs such as MTX may be suitable as a supporting vehicle for local chemotherapy. It has been reported that the addition of up to 2 g of MTX per 40 g of cement did not alter the biomechanical characteristics of polymethylmethacrylate cement and that the incorporated MTX was not affected by the heat evolved during polymerisation. In these studies, MTX was added as powder to the components of the cement, but in our study we prepared the bone cement with the direct addition of MTX dissolved in NMP. Preparation of bone cement using this mixture has some important advantages. The formation of MTX dust during the preparation of the cement is eliminated and the elution kinetic of MTX is improved; this is highly reproducible due to the homogeneous distribution of the drug and progressive exchange of the solvent by water. Our results showed that the release of MTX and its solvent NMP from bone cement has similar kinetics. After an initial phase with a sharp decrease in the rate of discharge the release of both compounds decreased only slightly. We showed that MTX is released continuously over months which agrees with the findings of studies carried out using MTX as a powder. The aim of our study was to determine the effects of MTX and NMP eluted from the bone cement prepared by this new procedure on the proliferation of osteosarcoma cells in vitro. We have shown that eluates from different elution phases were biologically effective and inhibited the proliferation of osteosarcoma cells. Dose-response curves using native MTX indicated that the drug is released at concentrations showing maximum effects (≥0.1 µM). Even after 56 days the eluted MTX concentration (2 µM) was found to be highly cytotoxic, suggesting the long-term effectiveness of the impregnated bone cement. The cell lines showed different sensitivity depending on their rate of proliferation. This finding supports the mechanism of MTX action as an inhibitor of DNA synthesis. Consistent with this, confluent osteoblast cultures were shown in our study to be unaffected by concentrations of MTX up to 0.1 mM.

Solvent NMP had a diffusion kinetic very similar to that of MTX and was released at concentrations of 0.5 mM after day 1 and of 0.27 mM after day 56. In dose-response experiments all five cell lines of osteosarcoma showed a similar sensitivity to NMP: cytotoxic effects were observed at concentrations higher than 10 mM. Concentration-effect curves for NMP and experiments with MTX/NMP-mixtures clearly showed that the eluted concentrations of solvent were not toxic and did not influence the effects of MTX. Thus, the unexpected high cytotoxic effect of eluates from day 1 on the fast proliferating cell lines MG-63 and MNNG-HOS, but not on the slow-growing cell lines, must be due to unidentified components eluted from the bone cement. The components methylmethacrylate (MMA, monomer) and N,N-dimethyl-p-toluidine (DMPT, cocatalyst) have been reported to be cytotoxic. In preliminary studies increased concentrations of MMA (50 to 70 µg/ml) were detected in eluates from NMP-containing cements compared with NMP-free control cement eluates. In MMA dose-response curves, only 10 mg/ml were found to be cytotoxic on osteosarcoma cells. These findings agree with those of studies reporting the cytotoxicity of MMA at concentrations between 1 mg/ml and 10 mg/ml on various cell types. Therefore MMA is probably not responsible for the additional cytotoxic effect. The cocatalyst DMPT was shown to be released from Palacos-R at low concentrations over several months and higher concentrations were found at day 1 of elution. Stea et al reported that DMPT eluted from bone cements was cytotoxic to MG-63 osteosarcoma cells and that the induced delay at the beginning of the cell-replication cycle (S-phase) was found to be reversible. Our findings suggest therefore that DMPT may be a possible candidate responsible for the additional cytotoxicity observed in our study. Since this effect was restricted to day-1 eluates, it is doubtful if it plays a role under conditions in vivo.

In normal bone cells, as examined for osteoblasts by dose-response curves, NMP had the same effect as in osteosarcoma cells and was cytotoxic at concentrations higher than 10 mM. In vitro, NMP was released from bone cements at a concentration lower than this threshold and therefore NMP-containing 10-day eluates were found to have no cytotoxic effects on osteoblasts.

Studies using MTX powder for cement preparation showed that the drug is released at effective concentrations both in vitro and in vivo. Kirchen et al studied in vitro the effect of MTX released from Palacos bone cement on...
giant-cell tumour of bone. Concentrations of MTX from 9.9 µM to 39.6 µM were tested and shown to be biologically active. Wasserlauff et al\textsuperscript{4} examined both in vitro and in vivo the diffusion of cis-platinum, 5-fluourouracil and MTX from various types of bone cement including Palacos-R with gentamicin, Palacos-R without gentamicin, CMP, Simplex P and Zimmer low-viscosity. They found that Palacos cements released the highest amount of the three cytotoxic drugs, whereas MTX had the best liberation properties. Released MTX was shown to be cytotoxic on mouse fibrosarcoma cells in tissue culture. Furthermore, using a rabbit model, high levels of MTX were found in drainage fluid.\textsuperscript{5} Circulating blood levels of MTX decreased from 1.76 µM on day 1 to 0.31 µM on day 14 after surgery and were undetectable on day 21. Langendorff et al\textsuperscript{6} studied the cytoxicity of the released MTX in a mouse model in vivo. They found that growth of transplanted osteosarcoma and mammary carcinoma could be reduced by inserting plugs of MTX cement into the tumours. They observed a wide zone of necrotic tissue around the implant, but found vital tumour cells in the periphery of the tumour. Hernigou et al\textsuperscript{8} studied the effect of MTX-containing bone cement in two animal models. In rats, growth of the induced osteosarcoma was temporarily reduced and survival increased. MTX-loaded implants of bone cement induced necrosis of the tumour but some persistence was observed in the periphery. In dogs, they measured the blood levels of MTX for five days after surgery and described a decrease in concentration of MTX from 0.05 µM to 0.016 µM which was not always detectable after the third day.\textsuperscript{6} Cement blocks replaced between two and three months after implantation continued to release MTX in vitro, however, suggesting that there would be continued diffusion in vivo until recovery of the cement. Despite the low serum level of MTX in dogs, local administration appears to reduce local recurrence of the tumour. Hernigou et al\textsuperscript{8} also presented pharmacological data from 14 patients with primary or metastatic tumours. They measured very high local concentrations of MTX in drainage fluid. On the first day after surgery concentration in the drain fluid was 10\textsuperscript{4} times that in the blood and continued to be over 100 times more for the five days of drainage. MTX was still detected in the blood at day 10 (0.02 µM) and in the urine up to at least the third week. The release and diffusion from cement were about the same at all sites and shapes of implant. Preliminary clinical investigations on MTX-loaded bone cement were reported by Hernigou et al.\textsuperscript{18} They selected 30 patients with primary or metastatic bone tumours for local chemotherapy, since general chemotherapy was inappropriate because of the age of the patient or because the excision of the tumour was too marginal. Local chemotherapy was well tolerated and no general MTX toxicity occurred. There was only one recurrence during four years of follow-up.

Application of MTX-impregnated cement may also reduce the risk of metastasis. Although dissemination of tumour was not totally prevented, Wang et al\textsuperscript{4} found a dose-dependent reduction of metastases and delayed development of pulmonary metastases using a VX\textsubscript{2} rabbit model. In the same animal model they observed that the local administration of MTX markedly decreased the amount of local destruction of bone and proliferation of osteoclasts.\textsuperscript{19} These data suggest that proliferation of normal bone cells also including osteoblasts may be affected by local release of MTX from bone cement. In our study we found that proliferation of osteoblasts was influenced by concentrations of MTX higher than 1 µM, but that of osteosarcoma cells was much more affected (≥0.1 µM MTX). Using a rabbit model Degreif et al\textsuperscript{20} found no evidence of an inhibitory effect of MTX on the rate of regeneration of bone. Since the effectiveness of MTX is related to the proliferation rate of cells, they suggest that the regenerative process in bone may be too slow for MTX to display any significant effect.

Our results indicate that bone cement containing MTX dissolved in NMP will release the drug in a suitable and effective way. The released MTX potently inhibits proliferation of osteosarcoma cells and has little effect on the growth of osteoblasts. NMP appeared not to be toxic at the released concentrations in osteosarcoma cells and osteoblasts. MTX-impregnated cement may therefore be of value in the treatment of bone tumours.

We thank V. Eckervogt, M. Hartig, B. Truckenbrod and W. Franz for technical assistance and Dr. H.-P. Wiesmann for generously providing osteoblasts. We are grateful to H.-J. Pfefferle for his critical reading of the manuscript. Although none of the authors have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article, benefits have been or will be received but are directed solely to a research fund, foundation, educational institution, or other non-profit institution with which one or more of the authors is associated.

References


THE JOURNAL OF BONE AND JOINT SURGERY