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Influence of bisphosphonates or recombinant human parathyroid hormone on \textit{in vitro} chemotherapy sensitivity of acute lymphoblastic leukemia cells

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DTCdW, JEvA, JGCAMB-G, SJCMNN, JPPM, and MMvdH-E contributed to the design of the study. DTCdW and JGCAMB-G contributed to data acquisition. DTCdW, JEvA, JGCAMB-G, SJCMNN, JPPM, and MMvdH-E contributed to data analysis and interpretation. DTCdW, JEvA, JGCAMB-G, SJCMNN, JPPM, and MMvdH-E drafted the manuscript. DTCdW, JEvA, JGCAMB-G, RP, SJCMNN, JPPM, and MMvdH-E reviewed the manuscript and were involved in critical revision of the manuscript for important intellectual content.

\textbf{Data-sharing statement}
Original data are available from the corresponding author on reasonable request.
Osteoporosis and osteonecrosis are serious skeletal side effects during or following treatment of childhood acute lymphoblastic leukemia (ALL).\textsuperscript{1,2} Osteonecrosis results from impaired blood supply to the bone, which may be caused by intravascular emboli, increased marrow pressure and/or direct blood vessel injury.\textsuperscript{2} This mainly affects the weight-bearing joints and can result in chronic pain, functional limitations, and articular collapse.\textsuperscript{3} The exact pathophysiology is not completely understood, but hypercoagulability following exposure to corticosteroids (especially concomitantly with asparaginase) has been shown to be related to the occurrence of osteonecrosis.\textsuperscript{2} Osteoporosis is induced by the leukemia itself as well as its treatment, and is co-determined by genetic susceptibility.\textsuperscript{1,4} In addition, it is associated with the occurrence of vertebral and non-vertebral fractures in ALL patients.\textsuperscript{1}

Bisphosphonates, potent antiresorptive agents, are widely used to treat osteoporosis in postmenopausal women and older men, and are increasingly used to treat bone fragility due to primary or secondary osteoporosis in children (including those with ALL).\textsuperscript{5} Although the working mechanism is not completely understood, small (case) studies have reported that bisphosphonates can also ameliorate pain symptoms, enhance musculoskeletal function, and consequently improve mobility in ALL patients with osteonecrosis.\textsuperscript{6} Furthermore, intermittent administration of recombinant human parathyroid hormone (rhPTH), an anabolic agent, has been shown to increase BMD in postmenopausal women and in children with steroid-treated Duchenne muscular dystrophy.\textsuperscript{7} Nevertheless, rhPTH has only rarely been used in children and there are currently no studies of rhPTH being used in the pediatric cancer setting due to concerns regarding possible oncogenicity (osteosarcoma) in patients with open epiphyses.\textsuperscript{7}

The influence of bisphosphonates or rhPTH administration on chemotherapy sensitivity has not been elucidated, since their use has only been described in small (case) studies, in which no strong evidence for oncological safety has been reported. A recent preclinical study on the effect of zoledronic acid (ZA) on ALL treatment efficacy raises concerns about potential adverse effects of ZA on leukemic drug sensitivity.\textsuperscript{8} Therefore, we assessed whether \textit{in vitro} administration of the bone-modifying agents ZA, pamidronic acid (PA), and rhPTH impacts cytotoxic effects of several chemotherapeutic agents that are commonly used during ALL treatment.

In various T-cell and B-cell leukemia cell lines, methyl-thiazol-tetrazolium (MTT; 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide; Life Technologies Europe BV, Bleiswijk, the Netherlands) assays were performed. The T-ALL cell lines LOUCY, Jurkat, HBP-ALL, SupT1 as well as the B-precursor ALL cell lines Reh, RCH-ACV, SUP-B15, RS4;11, and NALM-6 were used (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). We obtained ZA and PA from Sigma-Aldrich (Schnelldorf, Germany) and Teriparatide (rhPTH (1-34)) from Forsteo®, Eli Lilly Nederland
B.V. (Utrecht, the Netherlands). The chemotherapeutic agents vincristine (VCR), daunorubicin (DNR), dexamethasone (DEXA), 6-mercaptopurine (6-MP), pegylated asparaginase (PEG-ASP), and prednisone (PRED) were included in the experiments.

We performed MTT assays to determine potential cytotoxic effects of single bone-modifying agents on leukemia cell viability in vitro. The applied concentration ranges of the bone-modifying agents were based on previous pharmacokinetics studies in adult patients\(^9\)-\(^{11}\) (as these studies were not available in children), and we used the measured peak plasma concentrations after typically-prescribed doses as a reference. To test potential effects of the bone-modifying agents on chemotherapeutic agent-induced cytotoxicity, leukemia cell suspensions with or without a fixed concentration of ZA, PA, or rhPTH were added to 96-well u-bottom plates with a serial dilution of the chemotherapeutic agents. These fixed concentrations were 1.25 µg/mL (five-fold peak plasma concentration), 10 µg/mL (five-fold), and 0.002 µg/mL (fifteen-fold) for ZA, PA, and rhPTH respectively. In addition, experiments with one-, three-, or five-fold peak plasma concentrations of ZA or PA were performed for DEXA as well as PRED in a subset of the leukemia cell lines (SupT1, SUP-B15, RS4;11, and NALM-6).

The 50\(^{th}\) percentile of the maximal inhibitory concentration (IC\(50\)) of the chemotherapeutic agents, the bone-modifying agents, and the chemotherapeutic agents in combination with the bone-modifying agents were determined for each leukemia cell line. The combination index method as described by Chou\(^{12}\) was used to quantify the combined effects of the bone-modifying agents on chemotherapeutic agent-induced cytotoxicity. We considered a median combination index (CI) of <0.90 as synergism and >1.10 as antagonism. Calculations were conducted in R (Vienna, Austria).

We investigated potential effects of the bone-modifying agents ZA, PA, and rhPTH on leukemia cell viability as well as on cytotoxic responses of the chemotherapeutic agents in vitro. ZA, PA, and rhPTH, as single agents, showed no direct cytotoxic effects on leukemia cell viability in all T-ALL and B-precursor ALL cell lines within ranges of plasma concentrations as achieved in patients during clinical application, nor at the intended fixed concentrations (Figure 1). In the DEXA-resistant leukemia cell lines (i.e. LOUCY, Jurkat, HPB-ALL, Reh, and RCH-ACV) as well as in the 6-MP-resistant leukemia cell line (i.e. Reh) IC\(50\) values were not reached. Therefore, potential synergistic or antagonistic effects of ZA, PA, or rhPTH on the cytotoxic responses of DEXA and 6-MP could not be determined in these cell lines. Administration of ZA, PA, or rhPTH at the intended fixed concentrations in combination with DNR, 6-MP, or PEG-ASP showed median CI values between 0.90-1.10, indicating no synergistic or antagonistic effect. However, DEXA in combination with ZA or PA at a five-fold peak plasma concentration resulted in median CI values of 1.153 and 1.343, that may point towards a slight antagonistic and moderate antagonistic effect, respectively.
For rhPTH the effect in combination with DEXA was 0.9610 (Table S1, Figure 2). Despite the fact that these fixed concentrations will not be readily attained in plasma of patients, we performed additional experiments with one-, three-, and five-fold peak plasma concentrations in DEXA as well as in PRED-exposed leukemia cells to investigate whether this was a general corticosteroid or a DEXA specific effect. The results showed that ZA and PA at one- and three-fold peak plasma concentration did not seem to negatively influence DEXA- nor PRED-induced cell death, with median CI values between 0.90-1.10. However, DEXA-exposed leukemia cells in combination with a five-fold peak plasma concentration of ZA or PA repeatedly showed median CI values above 1.10 (1.150 and 1.336, respectively) (Table S2, Figure 3). In addition, our results indicate that ZA, PA, and rhPTH in combined treatment with VCR act antagonistically rather than synergistically, with median CI values of 1.192, 1.926, and 2.719, respectively. However, due to high variability across three independent experiments, it was not possible to obtain reproducible effects of the bone-modifying agents on VCR-sensitivity (Table S1, Figure S1).

Our results support the concerns raised by Janke et al., who observed that ZA may reduce the antileukemic efficacy of DEXA and PEG-ASP in immunocompetent murine ALL models,8 but they were unable to identify the exact mechanism of the effect of ZA on antileukemic efficacy. Bisphosphonates accumulate in bone, due to extensive uptake shortly after intravenous infusion, and once embedded, due to slow release (>120 days).13 We hypothesize that leukemia cells and chemotherapeutic agents may be in close contact with (high concentrations of) bisphosphonates in bone tissue, as the osteoblastic bone marrow niches, which are localized near the inner bone surface, are notorious for harboring leukemia cells as well as chemotherapeutic agents.14 This could potentially be the interphase where leukemia cells are exposed to higher levels of bisphosphonates, thereby influencing the drug sensitivity to a greater extent than measured in our experiments containing one-, three-, or five-fold peak plasma concentrations. On the other hand, newly formed bone in the interface between bisphosphonate infusions is bisphosphonate-naïve during growth in the juvenile skeleton,15 because bisphosphonates that are not rapidly taken up by bone will be excreted by the kidneys rapidly after administration.9 This may suggest that alternate administration of DEXA and bisphosphonates (ZA or PA) may be safe and that administration has no adverse effects on leukemia cell sensitivity. However, there is currently no definitive evidence to support the hypothesis that interference of high concentrations of accumulated bisphosphonates in bone tissue with leukemia therapy can be avoided. Hence, preclinical experiments that study the interactions in the bone-microenvironment and clinical follow-up studies that assess the frequency of relapse in children with ALL that received bisphosphonates are necessary to provide further insight.
In conclusion, we showed that ZA, PA, and rhPTH, as single agents, had no direct cytotoxic effects on leukemia cell viability at any dosage. Furthermore, *in vitro* administration of ZA, PA, and rhPTH did not seem to affect the leukemic drug sensitivity of DNR, 6-MP, and PEG-ASP. However, when using a five-fold peak plasma concentration, we observed a slight and moderate antagonistic effect of ZA and PA on DEXA-induced cell death, respectively. Our results underscore the caution to use these bone-modifying agents in children with ALL (especially for DEXA in combination with ZA or PA), and support the current clinical practice to only administer them in highly selected cases (preferably in clinical trial settings). Moreover, it is still questionable how effective these bone-modifying agents are in ALL patients with (severe) osteonecrosis, as no large studies with high quality evidence are available.
References


**Figure legends**

**Figure 1. Leukemia cell viability (%) after treatment with bone-modifying agents.**

A. Dose-response curves for all T-ALL and B-precursor ALL cell lines after 4 days of exposure to 0.050-156.3 µg/mL zoledronic acid. B. Dose-response curves for all T-ALL or B-precursor ALL cell lines after 4 days of exposure to 0.400-1250 µg/mL pamidronic acid. C. Dose-response curves for all T-ALL or B-precursor ALL cell lines after 4 days of exposure to 0.0004-1.3 µg/mL recombinant human parathyroid hormone (rhPTH). The experimental conditions in this experiment were performed in duplicate. Data are presented as the mean of these duplicate conditions. The dashed red line represents the IC_{50} value. C_{max}: peak plasma concentration (as achieved in patients during clinical application). Included leukemia cell lines: LOUCY (T-ALL), Jurkat (T-ALL), HBP-ALL (T-ALL with HOX11L2/TLX3-BCL11B), SupT1 (T-ALL), Reh (ETV6-RUNX1, BCP-ALL), RCH-ACV (E2A-PBX), SUP-B15 (BCR-ABL1), RS4;11 (MLL-AF4), and NALM-6 (B-precursor ALL).

**Figure 2. Scatterplots (A-C) visualizing the combination index (CI) values per individual leukemia cell line and the median CI (and range) for all leukemia cell lines combined.**

A. Scatterplot for the combined treatment of zoledronic acid and daunorubicin (DNR), dexamethasone (DEXA), 6-mercaptopurine (6-MP), or PEG-asparaginase (PEG-ASP). B. Scatterplot for the combined treatment of pamidronic acid and DNR, DEXA, 6-MP, or PEG-ASP. C. Scatterplot for the combined treatment of recombinant human parathyroid hormone (rhPTH) and DNR, DEXA, 6-MP, or PEG-ASP. Each dot on the scatterplot represents the CI value per individual T-ALL or B-precursor cell line. Data are presented as the mean CI of three independent experiments conducted at different days. The median and range for all leukemia cell lines combined is presented in red.

**Figure 3. Scatterplots (A-D) visualizing the combination index (CI) values per individual leukemia cell line and the median CI (and range) for all leukemia cell lines combined.**

A. Scatterplot for the combined treatment of zoledronic acid (with an one-, three-, or five-fold peak plasma concentration) and dexamethasone. B. Scatterplot for the combined treatment of zoledronic acid (with an one-, three-, or five-fold peak plasma concentration) and prednisone. C. Scatterplot for the combined treatment of pamidronic acid (with an one-, three-, or five-fold peak plasma concentration) and dexamethasone. D. Scatterplot for the combined treatment of pamidronic acid (with an one-, three-, or five-fold peak plasma concentration) and dexamethasone.
concentration) and prednisone. Each dot on the scatterplot represents the CI value per individual T-ALL or B-precursor cell line. Data are presented as the mean CI of three independent experiments conducted at different days. The median and range for all leukemia cell lines combined is presented in red.
Supplementary Material

Influence of bisphosphonates or recombinant human parathyroid hormone on *in vitro* chemotherapy sensitivity of acute lymphoblastic leukemia cells

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Table S1. Median combination index (CI) values for the effects of the bone-modifying agents (zoledronic acid, pamidronic acid, and recombinant human parathyroid hormone (rhPTH) with a concentration up to five-fold peak plasma concentration) on the chemotherapeutic agent-induced cytotoxicity of vincristine, daunorubicin, dexamethasone, 6-mercaptopurine and PEG-asparaginase.

N = total of evaluable leukemia cell lines. Ranges of CI values: 0.90-1.10 = (near) additive (±), 0.30-0.70 = synergism (+++), 0.70-0.85 = moderate synergism (++), 0.85–0.90 = slight synergism (+), 1.10-1.20 = slight antagonism (-), 1.20-1.45 = moderate antagonism (--), and 1.45-3.30 = antagonism (---).

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<th>Pamidronic acid (5fold)</th>
<th>rhPTH (15fold)</th>
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<td>Median (range) Effect</td>
<td>Median (range) Effect</td>
<td>Median (range) Effect</td>
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<tr>
<td>Vincristine (n=9)</td>
<td>1.192 (0.849-2.291) -</td>
<td>1.926 (0.625-3.584) -</td>
<td>2.719 (0.706-6.294) ---</td>
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<td>Daunorubicin (n=9)</td>
<td>1.074 (1.017-1.312) ±</td>
<td>1.014 (0.983-1.224) ±</td>
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<td>Dexamethasone (n=4)</td>
<td>1.153 (1.051-1.535) -</td>
<td>1.343 (1.003-1.682) --</td>
<td>0.9610 (0.666-1.022) ±</td>
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<tr>
<td>6-Mercaptopurine (n=8)</td>
<td>1.082 (0.937-1.191) ±</td>
<td>0.9194 (0.804-1.027) ±</td>
<td>0.9764 (0.812-1.054) ±</td>
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<td>PEG-Asparaginase (n=9)</td>
<td>1.092 (0.784-2.167) ±</td>
<td>1.007 (0.959-1.313) ±</td>
<td>1.039 (0.863-1.384) ±</td>
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Table S2. Median combination index (CI) values for the effects of the bone-modifying agents (zoledronic acid and pamidronic acid with an one-, three-, or five-fold psychological peak plasma concentration) on the chemotherapeutic agent-induced cytotoxicity of dexamethasone and prednisone.

N = total of evaluable leukemia cell lines. Ranges of CI values: 0.90-1.10 = (near) additive (±), 0.30-0.70 = synergism (+++), 0.70-0.85 = moderate synergism (++), 0.85-0.90 = slight synergism (+), 1.10-1.20 = slight antagonism (-), 1.20-1.45 = moderate antagonism (--), and 1.45-3.30 = antagonism (---).

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<td>1.057 (0.806-1.221) ±</td>
<td>1.044 (0.877-1.812) ±</td>
<td>1.150 (1.058-1.515) -</td>
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<td>Prednisone (n=4)</td>
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<tr>
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<td>1.024 (0.831-1.110) ±</td>
<td>1.061 (0.824-1.239) ±</td>
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<td>Dexamethasone (n=4)</td>
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<td>0.9202 (0.606-1.082) ±</td>
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<td>1.336 (1.019-1.961) --</td>
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<td>0.9883 (0.759-1.045) ±</td>
<td>1.020 (0.687-1.100) ±</td>
<td>0.9989 (0.948-1.272) ±</td>
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Figure S1. Scatterplots (A-C) visualizing the combination index (CI) values per individual leukemia cell line and the median CI (and range) for all leukemia cell lines combined.

A Scatterplot for the combined treatment of zoledronic acid and vincristine. B Scatterplot for the combined treatment of pamidronic acid and vincristine. C Scatterplot for the combined treatment of recombinant human parathyroid hormone and vincristine. Each dot on the scatterplot represents the CI value per individual T-ALL or B-precursor cell line. Data are presented as the mean CI of three independent experiments. The median and range for all leukemia cell lines combined is presented in red.