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

Osteoporosis and osteonecrosis are serious skeletal side effects during or following treatment of childhood acute lymphoblastic leukemia (ALL).[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.[2] This condition mainly affects the weight-bearing joints and can result in chronic pain, functional limitations, and articular collapse.[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.[2] Osteoporosis is induced by the leukemia itself as well as its treatment, and is co-determined by genetic susceptibility.[1,4] In addition, it is associated with the occurrence of vertebral and non-vertebral fractures in ALL patients.[1]

Bisphosphonates, potent antiresorptive agents, are widely used to treat osteoporosis in postmenopausal women and older men, and are increasingly being used used to treat bone fragility due to primary or secondary osteoporosis in children (including those with ALL).[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.[6] Furthermore, intermittent administration of recombinant human parathyroid hormone (rhPTH), an anabolic agent, has been shown to increase bone mineral density in postmenopausal women and in children with steroid-treated Duchenne muscular dystrophy.[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.[7]

The influence of bisphosphonates or rhPTH administration on sensitivity to chemotherapy has not been elucidated, since the use of these agents 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.[8] Therefore, we assessed whether *in vitro* administration of the bone-modifying agents ZA, pamidronic acid (PA), and rhPTH has an impact on the 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-dimethylthiazoyl-2yl]-2, 5-diphenyltetrazolium bromide; Life Technologies Europe BV, Bleiswijk, the Netherlands) assays were performed to assess leukemia cell viability *in vitro*. The T-ALL cell lines LOUCY, Jurkat, HBP-ALL, and 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, daunorubicin, dexamethasone, 6-mercaptopurine, pegylated asparaginase, and prednisone were included in the experiments.

Experiments were performed to determine potential effects of the single bone-modifying agents on leukemia cell viability. 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. Subsequently, 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-bottomed plates with a serial dilution of the chemotherapeutic agents. These fixed concentrations were 1.25 μg/mL (5-fold peak plasma concentration), 10 μg/mL (5-fold), and 0.002 μg/mL (15-fold) for ZA, PA, and rhPTH, respectively. In addition, experiments with 1-, 3-, or 5-fold peak plasma concentrations of ZA or PA were performed for dexamethasone as well as prednisone in a subset of the leukemia cell lines (SupT1, SUP-B15, RS4;11, and NALM-6).

The 50th 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 chemotherapeutic agents and bone-modifying agents on the chemotherapeutic agent-induced cytotoxicity. We considered a median combination index of <0.90 as synergism and >1.10 as antagonism. Calculations were

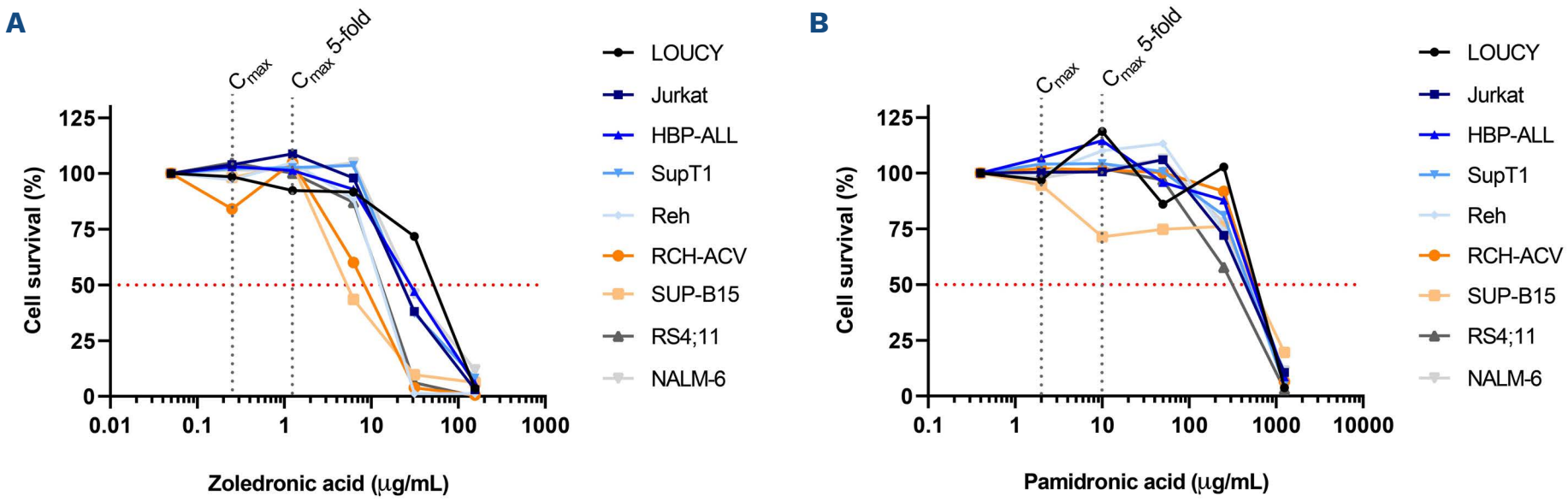

Figure 1. Leukemia cell viability (%) after treatment with bone-modifying agents. (A) Dose-response curves for all T-cell acute lymphoblastic leukemia (ALL) and B-precursor ALL cell lines after 4 days of exposure to 0.050-156.3 μg/mL zoledronic acid. (B) Dose-response curves for all T-ALL or B-precursor 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. The leukemia cell lines included were: 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). C_{max}: peak plasma concentration (as achieved in patients during clinical application); rhPTH: recombinant human parathyroid hormone.

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 to 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 achieved in patients during clinical application, nor at the intended fixed concentrations (Figure 1). In the dexamethasone-resistant leukemia cell lines (i.e., LOUCY, Jurkat, HPB-ALL, Reh, and RCH-ACV) as well as in the 6-mercaptopurine-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 to dexamethasone and 6-mercaptopurine could not be determined in these cell lines. Administration of ZA, PA, or rhPTH at the intended fixed concentrations in combination with daunorubicin, 6-mercaptopurine, or pegylated asparaginase showed median combination index values between 0.90-1.10, indicating no synergistic or antagonistic effect. However, dexamethasone in combination with ZA or PA at a 5-fold peak plasma concentration resulted in median combination index values of 1.153 and 1.343, which may point towards a slight antagonistic and moderate antagonistic effect, respectively. For rhPTH the effect in combination with dexamethasone was 0.9610 (Figure 2, *Online Supplementary Table S1*). Despite the fact that these fixed concentrations will not be readily attained in the plasma of patients, we performed additional experiments with 1-, 3-, and 5-fold peak plasma concentrations of ZA or PA in leukemia cells exposed to dexamethasone or prednisone to investigate whether this was a general effect of corticosteroids or a dexamethasone-specific effect. ZA and PA at 1- and 3-fold peak plasma concentrations did not seem to negatively influence either dexamethasone- or prednisone-induced cell death, with median combination index values between 0.90-1.10. However, dexamethasone-exposed leukemia cells in combination with a 5-fold peak plasma concentration of ZA or PA repeatedly showed median combination index values above 1.10 (1.150 and 1.336, respectively) (Figure 3, *Online Supplementary Table S2*). In addition, our results indicate that ZA, PA, and rhPTH in combination with vincristine treatment act antagonistically rather than synergistically, with median combination index values of 1.192, 1.926, and 2.719, respectively. However, due to pronounced

variability across three independent experiments, it was not possible to obtain reproducible effects of the bone-modifying agents on sensitivity to vincristine (*Online Supplementary Table S1, Online Supplementary Figure S1*).

Our results support the concerns raised by Janke *et al.*, who observed that ZA may reduce the antileukemic efficacy of dexamethasone and pegylated asparaginase in immunocompetent murine ALL models,[8] although they were unable to identify the exact mechanism behind this effect. 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 in which 1-, 3-, or 5-fold peak plasma concentrations were used. 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.[9,10] This suggests that alternate administration of dexamethasone and bisphosphonates (ZA or PA) may be safe and that administration does not have adverse effects on the sensitivity of leukemia cells to chemotherapy. However, there is currently no definitive evidence

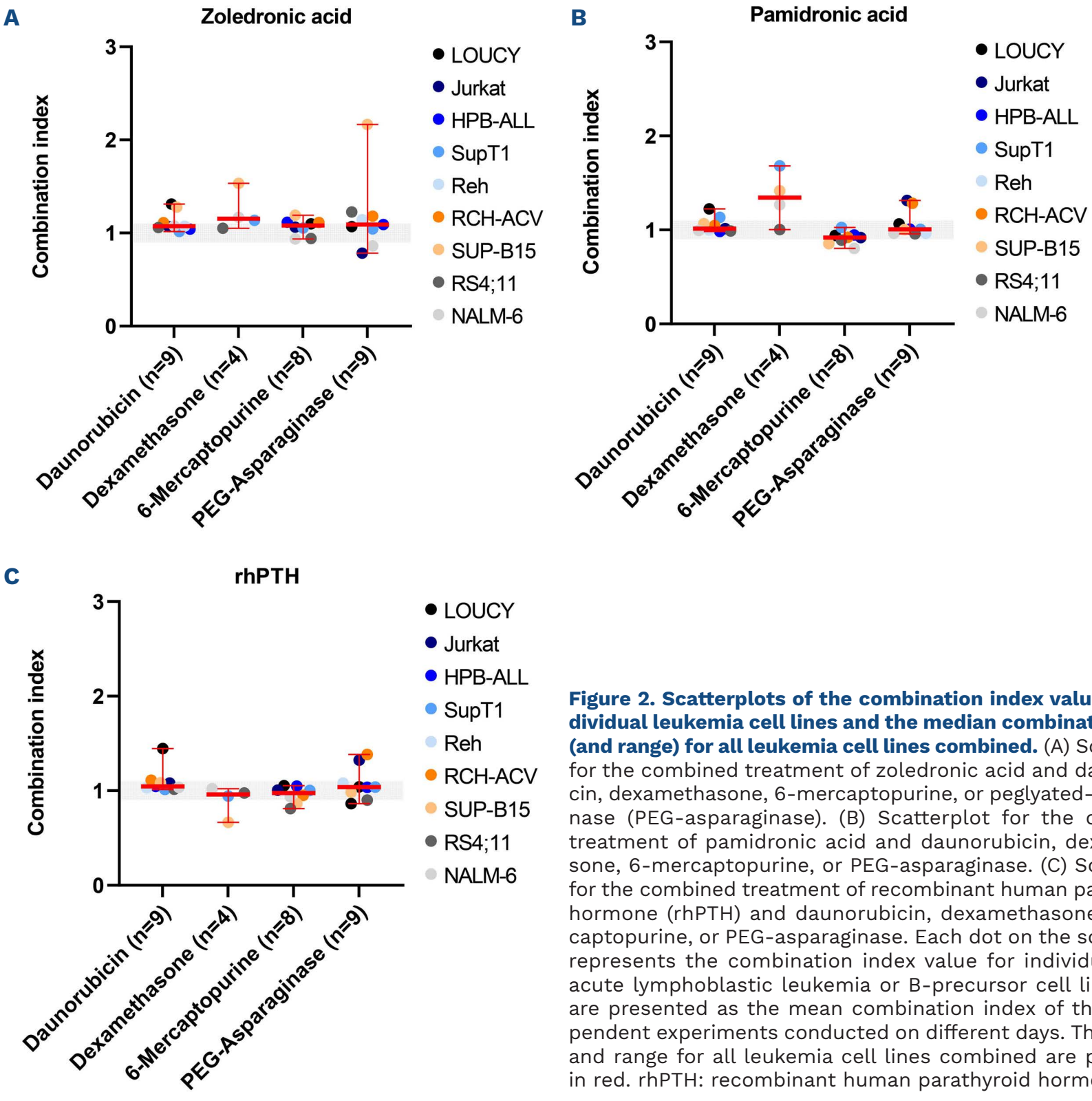

Figure 2. Scatterplots of the combination index values for individual leukemia cell lines and the median combination index (and range) for all leukemia cell lines combined. (A) Scatterplot for the combined treatment of zoledronic acid and daunorubicin, dexamethasone, 6-mercaptopurine, or peglyated-asparaginase (PEG-asparaginase). (B) Scatterplot for the combined treatment of pamidronic acid and daunorubicin, dexamethasone, 6-mercaptopurine, or PEG-asparaginase. (C) Scatterplot for the combined treatment of recombinant human parathyroid hormone (rhPTH) and daunorubicin, dexamethasone, 6-mercaptopurine, or PEG-asparaginase. Each dot on the scatterplot represents the combination index value for individual T-cell acute lymphoblastic leukemia or B-precursor cell lines. Data are presented as the mean combination index of three independent experiments conducted on different days. The median and range for all leukemia cell lines combined are presented in red. rhPTH: recombinant human parathyroid hormone.

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 who received bisphosphonates are necessary to provide further insight.

In conclusion, we showed that ZA, PA, and rhPTH, as single agents, did not have 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 daunorubicin, 6-mercaptopurine, and pegylated asparaginase. However, when using 5-

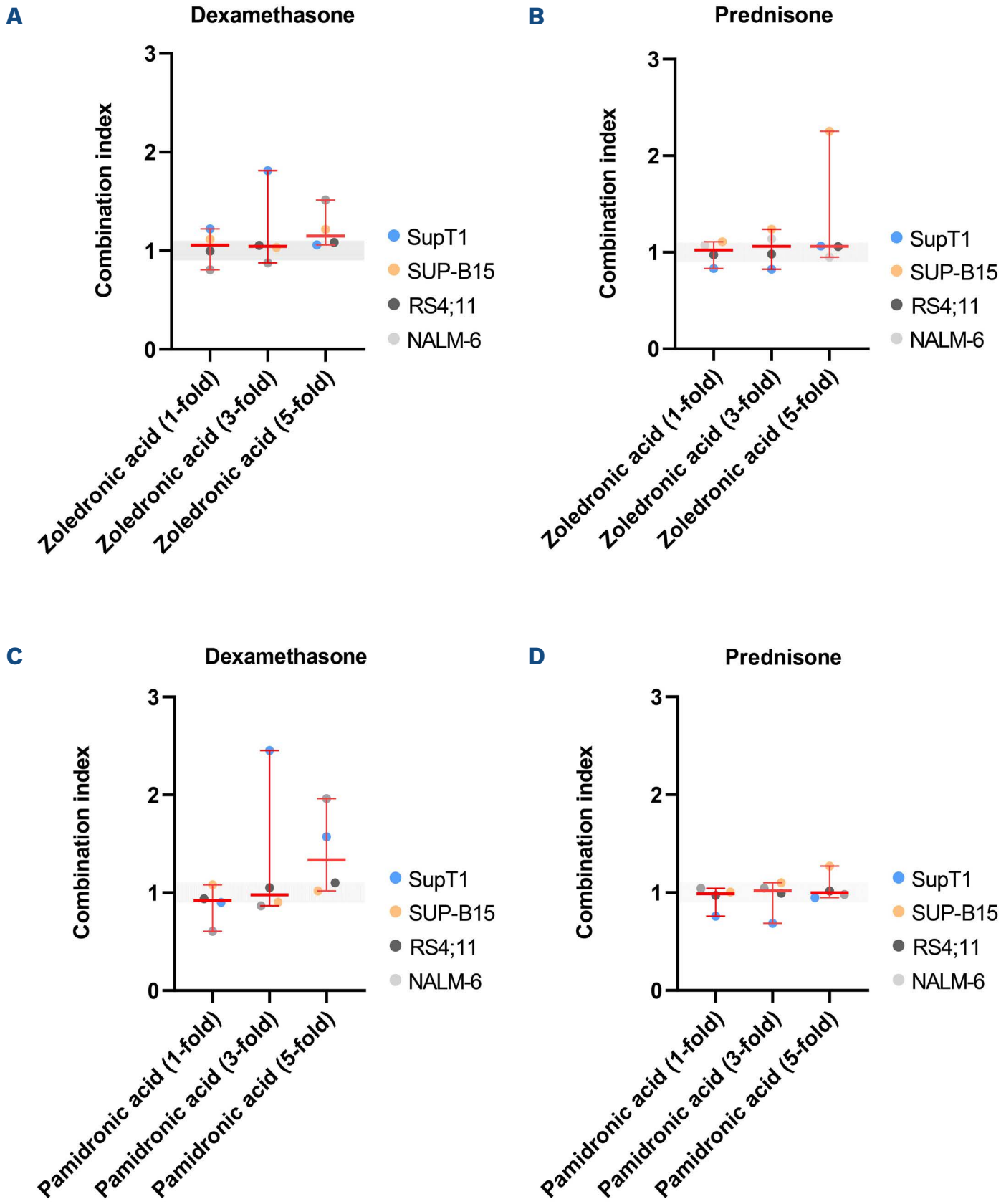

Figure 3. Scatterplots of the combination index values for individual leukemia cell lines and the median combination index (and range) for all leukemia cell lines combined. (A) Scatterplot for the combined treatment of zoledronic acid (at 1-, 3-, or 5-fold peak plasma concentration) and dexamethasone. (B) Scatterplot for the combined treatment of zoledronic acid (at 1-, 3-, or 5-fold peak plasma concentration) and prednisone. (C) Scatterplot for the combined treatment of pamidronic acid (at 1-, 3-, or 5-fold peak plasma concentration) and dexamethasone. (D) Scatterplot for the combined treatment of pamidronic acid (at 1-, 3-, or 5-fold peak plasma concentration) and prednisone. Each dot on the scatterplot represents the combination index value for individual T-cell acute lymphoblastic leukemia or B-precursor cell lines. Data are presented as the mean combination index of three independent experiments conducted on different days. The median and range for all leukemia cell lines combined are presented in red.

fold peak plasma concentrations, we observed that ZA and PA had a slight and moderate antagonistic effect, respectively, on dexamethasone-induced cell death. Our results underscore the caution required when using these bone-modifying agents in children with ALL (especially for dexamethasone in combination with ZA or PA), and support the current clinical practice of administering them only 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.

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Disclosures

No conflicts of interest to disclose.

Contributions

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

Data-sharing statement

Original data are available from the corresponding author on reasonable request.

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