Background: Using immunomodulatory methods to address the challenging issue of craniofacial bone repair may be a potentially effective approach. The protease inhibitor saquinavir has been shown to inhibit the inflammatory response by targeting the toll-like receptor 4/myeloid differentiation primary response complex. Independently, inhibition of toll-like receptor 4 or myeloid differentiation primary response led to enhanced skull bone repair. Therefore, the authors aimed to investigate the effects of saquinavir on skull bone healing.

Methods: The effects of saquinavir on skull bone healing were assessed by means of gene expression, histology, immunohistochemistry, and tomography in a mouse calvarial defect model. Subsequently, the role of saquinavir in cell viability, migration, and osteogenic and osteoclastogenic differentiation was also evaluated in vitro.

Results: One-week saquinavir administration improved skull bone healing based on micro–computed tomographic and histomorphometric analyses. Compared to the vehicle control, 1-week saquinavir treatment (1) enhanced osteoclast infiltration (tartrate-resistant acid phosphatase staining) at day 7, but not at days 14 and 28; (2) induced more CD206+ M2 macrophage infiltration, but not F4/80+ M0 macrophages at days 7, 14, and 28; and (3) elevated osteoclastogenic gene RANKL (quantitative polymerase chain reaction) expression and other osteogenic and cytokine expression. Furthermore, in vitro data showed that saquinavir administration did not influence MC3T3-E1 cell migration or mineralization, whereas higher concentrations of saquinavir inhibited cell viability. Saquinavir treatment also enhanced the osteoclastic differentiation of bone marrow–derived precursors, and partially reversed high-mobility group box 1–driven osteoclastogenesis inhibition and elevated proinflammatory cytokine expression.

Conclusion: The improved skull bone repair following short-term saquinavir treatment may involve enhanced osteoclastogenesis and modulated inflammatory response following skull injury. (Plast Reconstr Surg. 150: 1264e, 2022.)

Clinical Relevance Statement: The authors’ work demonstrates improved skull bone healing by short-term application of saquinavir, a drug traditionally used in the treatment of acquired immunodeficiency syndrome. As such, saquinavir may be repurposed for skeletal repair.

Disclosure: The authors have no competing financial interests to report.
management, diverse natural and/or synthetic biomaterials used for cranioplasty, and tissue engineering/regenerative medicine-based approaches have achieved promising outcomes to improve craniofacial bone healing.\textsuperscript{3,6} Despite these advances, undesirable clinical outcomes exist, especially in cases of patients younger than 2 years or severely infected craniofacial fractures.\textsuperscript{6,8} In light of this, there is a great need for highly efficacious, interdisciplinary approaches that can enhance calvarial healing.

One novel approach to address these challenges involves immunomodulation of the postfracture inflammatory response to enhance bone regeneration. The acute inflammatory response within the first 7 days after injury seems to be effective, because acute inflammation initiates the regenerative cascades that directly contribute to the bone healing outcome.\textsuperscript{3,13} Recent research also includes engineering new biomaterials that harness the inflammatory response toward efficacious bone healing.\textsuperscript{11–13} Despite its potential, one recurrent obstacle in identifying the useful immunomodulators is how to achieve temporally or spatially precise control of the host immune response for functional bone repair.

Toll-like receptor 4 (TLR4), a member of the toll-like receptor family, plays a unique role in sensing tissue damage.\textsuperscript{14} Our previous work demonstrated accelerated bone repair in a TLR4 gene knockout mouse model, potentially by means of enhanced osteoclast differentiation after fracture.\textsuperscript{15} This corroborated existing literature that showed TLR4 and other TLR family members and their signaling mediators play important roles in regulating bone inflammation, regeneration, and metabolism under various conditions.\textsuperscript{16–18} Saquinavir, a first-generation antiretroviral agent originally developed as a protease inhibitor of human immunodeficiency virus protease, was shown to target the mammalian protease cathepsin V. Cathepsin V is essential for the formation of 700 similarly as described in our prior work.\textsuperscript{20} It was administered as an analgesic immediately after surgery. Mice were divided randomly into control and various saquinavir treatment groups. The mice in the saquinavir group were injected intraperitoneally with 5 mg/kg saquinavir (0.5 mg/ml) daily for 3, 7, and 28 days; and the control group mice were injected with an equal volume of saline. All mice were euthanized by carbon dioxide overdose followed by cervical dislocation at designated time points. The animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee Guide of Ten People’s Hospital of Tongji University, Shanghai, People’s Republic of China.

**Micro–Computed Tomographic Analysis**

Mice from both control and saquinavir treatment groups were euthanized at 7, 14, or 28 days after surgery. The skulls were dissected and fixed in 4% paraformaldehyde overnight. Bone healing was assessed using a high-resolution microcomputed tomography system (eXplore Locus; GE Healthcare, Chicago, Ill.) at a fixed isotropic voxel size of 45 μm. Three-dimensional images obtained from micro–computed tomographic imaging were reconstructed using software OsiriX Dicom viewer (OsiriX, Bernex, Switzerland), and a region of interest (4.0 mm$^2 \times 2.09$ mm) was defined. Qualitative and quantitative data were analyzed by OsiriX with a global fixed threshold of 700 similarly as described in our prior work.\textsuperscript{20}

**Histologic and Immunohistochemistry Analyses**

Calvarial samples from parietal defects were obtained from each group and fixed in 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid, and dehydrated in graded ethanol solutions. The samples were embedded in paraffin wax, sectioned at a thickness of 5 μm, addition, the role of saquinavir in cell viability, migration, and osteogenic and osteoclastogenic differentiation in vitro was investigated.
and stained with Harris hematoxylin and eosin reagent (Jiancheng, People’s Republic of China). Histomorphometric analysis was performed to quantify the two-dimensional area of new bone formation as described in our prior work. Tartrate-resistant acid phosphatase (TRAP) staining was conducted on paraffin-embedded samples and incubated in TRAP buffer (Lushen, People’s Republic of China) for 1 hour at 37°C. Immunohistochemical staining was performed as described previously. Briefly, sections were incubated in primary antibodies [i.e., rabbit polyclonal anti-CD206 (1:300 dilution; Abcam, Cambridge, United Kingdom), rabbit monoclonal anti-F4/80 (1:1000 dilution; Cell Signaling Technology, Danvers, Mass.), rabbit monoclonal anti-CD31 (1:50 dilution; Cell Signaling Technology), and mouse monoclonal anti-VEGFA (1:1000 dilution; Abcam) overnight at 4°C. Color was developed by application of the 3,3′-diaminobenzidine kit (BD5001; Bioworld Technology, Louis Park, Minn.) as described in our prior work. All images were visualized and captured using bright-field microscopy (D-35578; Leica, Wetzlar, Germany).

Cell Viability Assay

Mouse osteoblastic MC3T3-E1 cells (Cell Bank, Chinese Academy of Sciences, Shanghai, People’s Republic of China) were seeded at a density of 2 × 10³ cells/cm² in 96-well culture plates in Dulbecco’s Modified Eagle Medium (keyGEN BioTECH, Nanjing, People’s Republic of China), 20% fetal bovine serum (HyClone Laboratories, Logan, Utah), and 1% penicillin-streptomycin (Gibco, Grand Island, N.Y.). Cell viability was measured using the Cell Counting Kit-8 assay (Dojindo Laboratories, Japan) at 12, 24, and 48 hours after seeding, in accordance with the manufacturer’s protocol. Briefly, 10 μl of Cell Counting Kit-8 reagent was added to each well and incubated for 2 hours at 37°C and 5% carbon dioxide. Optical density value was recorded by a microplate reader (BioTek, Winooski, Vt.) with 450 nm set as the excitation wavelength. Cell viability was determined by comparing the obtained absorbance value to a standard calibration curve.

In Vitro Scratch Assay

MC3T3-E1 cells were seeded at a density of 5 × 10⁴ cells/well in 12-well culture plates with Dulbecco’s modified Eagle medium, 20% fetal bovine serum, and 1% penicillin-streptomycin to observe chemotaxis through creation of an artificial wound. On reaching 90% confluence, the scratches were created in each well with micropipette tips. Detached cells were removed with phosphate-buffered saline washing. At 0, 24, and 48 hours after wound creation, the magnitude of cell migration was observed and captured through light microscopy (D-35578). This assay was conducted five times and representative images were presented.

Osteogenic Differentiation Assay

MC3T3-E1 cells were seeded in 24-well plates at 5 × 10⁵ cells/cm² and cultivated in osteogenic medium composed of α-Minimum Essential Medium (HyClone), 10% fetal bovine serum, 1% penicillin-streptomycin, 10 mM β-glycerophosphate (Sigma, St. Louis, Mo.), 10 mM Dexamethasone (Sigma) and 50 μg/ml ascorbic acid (Sigma), with media change every 3 days. Alizarin red S staining (Electron Microscopy Sciences, Hatfield, Pa.) was used to determine osteogenic mineralization in cultivated cells. Briefly, the MC3T3-E1 cells were cultured for 14 days, fixed with 4% paraformaldehyde and washed with double-distilled water three times. Alizarin red S reagent (200 μl) was added to each well and incubated for 1 hour. Stained images were captured under bright-field microscopy (D-35578) and representative images are recorded.

In Vitro Bone Marrow–Derived Osteoclast Differentiation Assay

Mouse bone marrow–derived cells were extracted from the femora and tibiae of 4- to 6-week-old female C57BL/6 mice in accordance with the Institutional Animal Care and Use Committee Guide (Tenth People’s Hospital of Tongji University, Shanghai, People’s Republic of China). Briefly, bone marrow was flushed from the medullary cavities and seeded onto a 10-cm-diameter cell culture dish overnight. Unattached cells were collected and seeded in medium containing α minimum essential medium, 10% fetal bovine serum, and 1% penicillin-streptomycin at 1 × 10⁴ cells/well on a 96-well plate (Osteo Assay Surface; Corning, Corning, N.Y.) for TRAP staining and 1 × 10⁵ cells/well on a six-well plate to collect RNA samples. After 24 hours, the media were changed to basal medium containing 30 ng/ml macrophage colony-stimulating factor (Novoprotein Scientific, Shanghai, People’s Republic of China) 30 ng/ml for 3 days. Subsequently, cells were cultured in 30 ng/ml macrophage colony-stimulating factor, 100 ng/ml receptor activator of nuclear factor kappa-B ligand (Novoprotein Scientific), with/without supplementation of various concentrations of saquinavir (0.05, 0.25, 0.5, and 2.5 μg/ml) or 100 ng/ml HMGB1 (Sino Biological, Inc.,
Beijing, People’s Republic of China) for up to 8 days for TRAP staining and 7 days for quantitative polymerase chain reaction analysis as indicated.

Quantitative Polymerase Chain Reaction for In Vitro and In Vivo Studies

Cells from osteoclast assays and tissue samples surrounding the initial bone defects (at 3 and 7 days after surgery) were harvested and extracted using TRIzol and chloroform (Invitrogen, Waltham, Mass.). Prime Script RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) was used to synthesize cDNA from collected RNA. The resultant cDNA was amplified using SYBR Premix Ex Taq II (Takara Bio) in an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, Mass.). Primers used are listed in Table 1. The expression levels of each gene were normalized to the selected housekeeping gene (GAPDH), and the relative expression level was measured by the 2-ΔΔCT method as described previously.15

Statistical Analyses

Measurements were analyzed using GraphPad Prism Software 6.0 (GraphPad Software, Inc., San Diego, Calif.) and IBM SPSS Version 22.0 (IBM Corp., Armonk, N.Y.). Data were analyzed using t test or analysis of variance. For t test, if the data violated the normality assumption, a nonparametric test (Mann-Whitney U) was applied and data were presented using box plots instead of bar charts.21 For the one-way analysis of variance test, if equality of variance was violated, analysis of variance with Welch test was applied and followed by post hoc Turkey test. Statistically significant differences are indicated in figures and legends. A value of p < 0.05 was considered statistically significant.

RESULTS

One- and 4-Week Saquinavir Administrations Have Opposing Effects on Skull Bone Healing

To understand the effects of saquinavir on bone healing, 5 mg/kg saquinavir per mouse daily over three durations (3 days, 1 week, and 4 weeks) was administered and the mice were euthanized at 28 days for bone healing assessment by means of micro–computed tomography and histology. Micro–computed tomographic results indicated differential effects of saquinavir on bone formation based on its administration duration. Micro–computed tomographic analyses revealed larger areas of mineralized, opaque tissues in the 1-week saquinavir treatment group compared to the other three groups, although no group showed complete bone healing over the course of the 28-day study. Statistical analysis showed significantly higher new bone formation (bone volume fraction) in the 1-week saquinavir group compared to the control group. Significantly lower bone formation was observed in the 4-week saquinavir group compared to the 3-day and 1-week saquinavir treatment groups (Fig. 1).

Histologic observations were consistent with micro–computed tomographic analysis. At day 7 after surgery, saquinavir-treated and control tissues appeared histologically similar. Both groups exhibited high cellularity, disorganized connective tissue, and small islands of new bone matrix at the defect edges, with typical large, cuboid, osteoblasts on the surface of the newly formed bone matrix. At day 14 and day 28, both groups showed evidence of woven bone and bone marrow, whereas the 1-week saquinavir-treated group appeared to have larger regions of woven bone

Table 1. Primer Sequences for Quantitative Polymerase Chain Reaction

| Gene Name                  | Abbreviation | Gene Symbol | Forward Primer Sequence | Reverse Primer Sequence |
|----------------------------|--------------|-------------|-------------------------|-------------------------|
| Interleukin 1β             | IL-1β        | Il1b        | GCAACTGTTTCTGAACCTAACT  | ATCTTTTGCGGCTCCTAAGCT   |
| Interleukin 4              | IL-4         | Il4         | GGCTCTAACCCTCAGTCAGTG   | GGGATGATCTCTTCAGTCTAG    |
| Interleukin 6              | IL-6         | Il6         | ACAACACGGCCCTTCCTACT    | CGACATTCCAGAAAGATGAG    |
| Interleukin 10             | IL-10        | Il10        | GCTTCTACTGACCTGGCATAG   | CGAGGCCTTAGACGCTGAG    |
| Tumor necrosis factor-alpha| TNF-α        | Tnf         | AAGGCTCTAGGCCCCACGTGTA  | CGGACACACTGTTGTTGTTTT    |
| Toll-like receptor 4       | TLR4         | Tlr4        | ATGGCAATGCTCTACAACACC   | CGAGGCCTACTCTACACACA    |
| Toll-like receptor 2       | TLR2         | Tlr2        | GGAAAGCTGTGTCCTGATG     | AGGGCTGCTCCCCCTATGGTATT |
| Receptor for advanced glycan end products | RAGE     | Age         | CTTGCTCTATGCGGGCGACGCT    | GGAGATTTGAGCCACGCT     |
| Osteoprotegerin            | OPG          | Tnfrsf11B   | ACCCAAACTGCTACACTAGC    | CTGCAGAACAACACTCAGACTCAG |
| Receptor activator of nuclear factor kappa-β | RANKL    | Tnfrsf1        | GCTGTCGGTTACATGGCTTCCGCC   | CATTTGCCACACTCTGACCAATAT |
| High-mobility group protein 1 | Hmgbl   | Hmgbl       | GCCGGAGCTCTCCGCTGTCATCC | GGGTGCTGTTGCTGTTGCG    |
| Alkaline phosphatase       | ALP          | TACCATTTTTTGCCAGAGA | GCCGATTTGGTGTTGAGCCCTTT    | CTTGCGTGGGAATTGTTGTT    |
| Runt-related transcription factor 2 | Runx2   | Runx2       | AAGGAGCTCTGAGATTGTTGTTGAG   | CATTGGGAGATTTGTTGTT    |
| Osterix                    | Oss          | Sp7         | ATGGGCTCTGCTGTGCTGTT    | TGAAGGCTACGGCTAATGCT    |
| Glyceraldehyde-3-phosphate | GAPDH        | Gapdh       | ACAAGCAACGGGTGTGCAGC    | TTTAGGAGGCTCAGCAGCACTTT |

1267e
compared to controls. In addition, histomorphometric analyses showed significantly larger areas of newly formed bone matrix and woven bones were observed (on the endocortical side of the skull bone lateral to the defect perimeters) in the saquinavir (SQV) treatment groups compared to control groups on day 14 and day 28, respectively, http://links.lww.com/PRS/F472.

**Enhanced Osteoclast Differentiation and M2 Macrophage Infiltration after 1-Week Saquinavir Administration In Vivo**

To determine the effect of 1-week saquinavir treatment on osteoclast differentiation during bone healing, TRAP staining was performed at postsurgery days 7, 14, and 28. Osteoclast infiltration was visualized by means of TRAP staining of tissue samples surrounding the mice parietal bone defects and noninjured bone areas (i.e., sagittal suture region). As illustrated in Figure 2 and Figure, Supplemental Digital Content 2, more intense TRAP staining, indicating increased infiltration of osteoclasts, was observed after 1-week of saquinavir treatment at postsurgery day 7, but not at days 14 and 28, compared to the control group (Fig. 2). [See Figure, Supplemental Digital Content 2, which shows TRAP staining and quantitative polymerase chain reaction analysis. (Above) Comparable TRAP-positive staining at the sagittal suture areas was observed in the saquinavir-treated and control groups. SQV, saquinavir. (Center) Representative TRAP staining images of mice after 1-week of saquinavir administration at postoperative days 14 and 28. Comparable TRAP-positive staining at the defect areas was observed in the saquinavir-treated and control groups. Arrows indicate TRAP-positive regions. (Below) Tissue
samples surrounding the initial bone defects were harvested at postsurgery day 3 for quantitative polymerase chain reaction analysis and the statistical results were indicated (mean ± SEM; *p < 0.05; n = 4 per group), http://links.lww.com/PRS/F473.] Comparable TRAP staining was observed in both groups at noninjured bone areas (i.e., sagittal suture areas). Compared to controls, a significant increase in expression of RANKL, Osx, and RUNX2, was observed in 1-week saquinavir-treated samples, whereas OPG expression was not significantly changed after 1-week saquinavir administration. These data suggest that 1-week saquinavir administration enhanced osteoclast differentiation after bone injury.

To determine angiogenesis and immunomodulatory effects of 1-week saquinavir treatment during skull bone healing, immunohistochemistry staining of VEGF, CD31, F4/80 (M0 macrophage marker), and CD206 (M2 macrophage marker) and expression of inflammatory cytokines were characterized. Immunohistochemistry results demonstrated notable increases in vascularization and inflammatory response during bone healing, although no obvious difference in the staining intensity of VEGF, CD31, and F4/80 was observed between the two groups. Interestingly, more intense CD206 staining and significantly increased expression of IL-4, TNF-α, TLR4, and RAGE were observed in the 1-week saquinavir treatment group compared to the control group (Fig. 3). [See Figure, Supplemental Digital Content 3, which shows immunohistochemistry staining images of F4/80 and CD206 and quantitative polymerase chain reaction analysis. (Above) Representative immunohistochemistry staining images of mice after 1-week saquinavir (SQV) administration at postoperative days 14 and 28. (Below) Tissue samples surrounding the initial bone defects were harvested at postsurgery day 3 for quantitative polymerase chain reaction analysis and the statistical results were indicated (mean ± SEM; *p < 0.05; n = 3 or 4 per group), http://links.lww.com/PRS/F474. See Figure, Supplemental
Digital Content 4, which shows representative immunohistochemistry staining images of VEGF (left) and CD31 (right) after 1-week saquinavir (SQV) administration at postoperative day 7. Comparable F4/80 staining intensity, indicating M0 macrophage infiltration, was observed between 1-week saquinavir administration and control groups. More intense CD206 staining, indicating increased M2 macrophage infiltration, was observed in 1-week saquinavir administration compared to the control group. (Right) Tissue samples surrounding the initial bone defects were harvested at postsurgery day 7 for quantitative polymerase chain reaction analysis and the statistical results were indicated (mean ± SEM; *p < 0.05; n = 3 or 4 per group).

Effects of Saquinavir on Cell Migration, Viability, Osteogenic Differentiation, and Osteoclastogenic Differentiation of MC3T3-E1 Cells In Vitro

To determine the direct effects of saquinavir on migration, viability, and osteogenic differentiation, MC3T3-E1 cells were treated with concentrations of saquinavir ranging from 0 to 5 µg/ml saquinavir supplementation in MC3T3-E1 culture. These concentrations did not grossly influence cell migration or cell mineralization, whereas higher concentrations of saquinavir treatment inhibited cell viability as demonstrated by Cell Counting Kit-8 cytotoxicity assays. Alizarin red S staining conducted at day 14 indicated no significant mineralization across all groups. Saquinavir supplementation in osteoclast differentiation medium (0.05 to 2.5 µg/ml) enhanced osteoclast differentiation of bone marrow–derived precursors. [See Figure, Supplemental Digital Content 5, which shows the effects of saquinavir on cell migration, viability, and osteogenic differentiation. MC3T3-E1 cells were treated with various concentrations of saquinavir and assessed for migration, metabolism, and osteogenic differentiation. (Above, left) Cell migration assay showed that comparable cell migration distances were observed at different time points among different groups; only a slight increase in cell migration was observed after 48 hours of saquinavir treatment at the concentration of 2.5 µg/ml. (Above, right) Cell viability assay showed that, compared to the control group, 2.5 µg/ml and 0.5 µg/ml saquinavir treatment inhibited cell proliferation at 24 hours, and 2.5 µg/ml saquinavir treatment inhibited cell proliferation at 48 hours. No statistically significant difference in cell viability was observed in the 0.25-µg/ml saquinavir treatment group compared to the control group at all time points (*p < 0.05; mean ± SEM). CCK8, Cell Counting Kit-8; OD, optical density. (Center, right) Alizarin red S staining conducted at day 14 indicated no significant mineralization across all groups. Saquinavir supplementation in osteoclast differentiation medium (0.05 to 2.5 µg/ml) enhanced osteoclast differentiation of bone marrow–derived precursors. (Below) Tissue samples surrounding the initial bone defects were harvested at postsurgery day 7 for quantitative polymerase chain reaction analysis and the statistical results were indicated (mean ± SEM; *p < 0.05; n = 3 or 4 per group).
indicated that saquinavir treatment did not obviously influence cell mineralization. (Below) TRAP staining showed that enhanced osteoclastogenic activity was observed in cells treated with saquinavir \( (n = 5) \), [http://links.lww.com/PRS/F476](http://links.lww.com/PRS/F476).

**Saquinavir Enhanced Osteoclast Differentiation of Bone Marrow–Derived Osteoclast Precursors In Vitro**

Because enhanced osteoclastogenesis was suggested after 1-week saquinavir administration during the bone healing process, we further explored the effects and potential mechanisms of saquinavir on osteoclastogenesis in vitro. Therefore, the effects of HMGB1/saquinavir signaling on osteoclastogenesis was assessed in bone marrow–derived osteoclast precursors. As shown in Figure 4, in the control group, precursors differentiated into multinucleated, TRAP+ osteoclasts. Saquinavir treatment (1 µg/ml) enhanced, whereas HMGB1 treatment (100 ng/ml) markedly reduced, the formation of mature osteoclasts from their precursors. The addition of 1 µg/ml saquinavir into the cell culture partially reversed the inhibitory effects of HMGB1 on osteoclastogenesis.

We further examined the expression of HMGB1 receptors (TLR4, RAGE, and TLR2) and its triggered inflammatory cytokines (IL-6, TNF-α) under this culture condition. HMGB1 induced significantly higher expression of its receptors TLR4, TLR2, and RAGE, and higher expression of proinflammatory cytokines IL-6 and TNF-α. A significant decrease in the expression of TLR4, TLR2, and RAGE was observed after the addition of 1 µg/ml saquinavir treatment.

**DISCUSSION**

Saquinavir is a protease inhibitor, which is used in combination with nucleoside/nucleotide reverse transcriptase inhibitors or a nonnucleoside reverse transcriptase inhibitor as a therapeutic approach (i.e., highly active antiretroviral therapy) for patients with human immunodeficiency virus infection.22 Patients with long-term highly active antiretroviral therapy treatments have experienced some common complications, including bone disorders (e.g., osteonecrosis and osteoporosis).23,24 Although there have been conflicting reports of the effects of highly active...
antiretroviral therapy treatment (including saquinavir) on bone metabolism, a more established theory is that these bone metabolism disorders share a similar phenotype of decreased bone formation and increased bone resorption, which is highly associated with osteoclast differentiation and function.\textsuperscript{23,24} Using a mouse skull defect model in the current study, we have discovered a positive effect on bone formation by short-term (1-week) saquinavir treatment, contrary to existing literature.\textsuperscript{23,24} Interestingly, our results suggest a time-dependent effect of saquinavir on bone healing where long-term saquinavir treatment inhibited bone healing, seemingly similar to long-term highly active antiretroviral therapy. Regardless, it is possible that short-term, controlled saquinavir treatment may prove beneficial to enhance bone regeneration.

Previous research has shown that saquinavir can inhibit HMGB1-driven inflammation by targeting the TLR4/MyD88 signaling pathway.\textsuperscript{10} HMGB1 can be released by cells or extracellular matrix in response to tissue damage,\textsuperscript{29} where it acts like a cytokine mediator to activate receptor families including RAGE, TLR2, TLR4, and TLR9.\textsuperscript{26,27} There are numerous studies investigating the roles of HMGB1 and its receptors on bone metabolism, bone growth, or bone healing, with contradictory results. For example, TLR2, TLR4, and RAGE have been shown to mediate the chronic inflammatory response and osteoclast-mediated bone resorption in periodontitis or other chronic bone destructive diseases.\textsuperscript{28,29} Contrarily, TLR4 enables innate inflammatory response from osteoblasts with the up-regulation of inducible nitric oxide synthase activity and inhibition of the differentiation of precursor cells toward mature osteoclasts.\textsuperscript{37} In addition, our prior work demonstrated enhanced bone healing in TLR4 knockout mice,\textsuperscript{15} and TLR4 is also essential for allograft remodeling and its mediated bone repair.\textsuperscript{30} Thus, in the current study, we tested whether saquinavir administration influenced bone healing outcomes and its potential mechanism.

Our study prioritized investigating the effects of 1-week saquinavir treatment in bone healing and its potential mechanisms, primarily because of the observed enhanced bone healing relative to other saquinavir treatment groups (i.e., 3-day and 4-week treatment groups). Nevertheless, characterization of the inhibitory effects of long-term saquinavir on bone healing and its role in modulating osteoclastogenesis during the bone remodeling phase would also be important to further characterize the underlying mechanism. Also, our study selected 28 days as the endpoint of bone healing observation. This rationale was based on our prior publications on bone healing.\textsuperscript{15,20} According to our previous observations, we found that in this mouse skull defect model, no significant healing occurred after 21 days, based on pentachrome staining.\textsuperscript{15}

Our data showed that short-term saquinavir treatment enhanced the number of TRAP\textsuperscript{+} cells and increased RANKL expression. Although enhanced osteoclast activity has been reported to be a main driving force for many bone destructive diseases,\textsuperscript{16,28} it also plays an important role for initiating the bone healing response. Similarly, our prior work showed that TLR4 knockout mice (both global knockout or conditional knockout in myeloid cells) showed improved bone repair with the enhanced osteoclast differentiation.\textsuperscript{15,20} Specifically, the increased expression of RANKL in TLR4 knockout mice was shown at early time points (3 hours and days 1, 2, and 4), but not at late time points (days 14, 21, and 28) compared to the control group.\textsuperscript{15} This phenotype is indeed similar to what we have observed in the 1-week saquinavir treatment group. Enhanced osteoclast infiltration (TRAP staining) (Fig. 2; see Figure, Supplemental Digital Content 2, http://links.lww.com/PRS/F473) was observed at day 7, but not at days 14 and 28. Furthermore, 1-week saquinavir treatment also showed elevated M2 macrophage infiltration indicated by intense CD206 staining. Contradictorily, elevated expression of both proregenerative and proinflammatory cytokines was observed during bone healing after 1-week saquinavir treatment. To explore how saquinavir influences postinjury inflammatory response, further experiments will be needed, such as including expression of more M1 or M2 macrophage-associated cytokines, or exploring whether the presence of CD206\textsuperscript{+} M2 macrophages is attributable to macrophage local activation or infiltration/migration. Our present study revealed enhanced bone formation after 1-week saquinavir treatment, which is likely to be associated with enhanced osteoclastogenesis and modulated inflammatory response.

The immune and skeletal systems are functionally coupled by overlapping signaling networks and shared cellular components. Such functional coupling may contribute to the causes of immune-associated bone abnormalities and the bone healing response.\textsuperscript{30} When designing an immunomodulatory approach for efficacious bone healing, a crucial element will be to identify an effective, controllable immunomodulator. Within immune and skeletal signaling
crosstalk, osteoclasts and their related signaling molecules are a key link between these two systems. Therefore, targeting osteoclastogenesis may be a potentially effective approach. It is also worth noting that subtle differences between skull and long bones—including their development, structure, and healing responses—may also influence how immunomodulators can be used for the ultimate healing outcomes.

CONCLUSIONS

In this study, we evaluated the influence of saquinavir on skull bone healing and elucidated potential signaling mechanisms. Our key findings include the following: (1) the duration of saquinavir administration influenced skull bone healing outcomes (bone repair was enhanced with short-term saquinavir application but inhibited with long-term saquinavir treatment); and (2) the improved bone healing after short-term saquinavir treatment was potentially mediated by enhanced osteoclastogenesis after injury.

Dan Wang, Ph.D.
422A, Lo Kwee-See Integrated Biomedical Sciences Building
Area 39, Institute for Tissue Engineering and Regenerative Medicine
Chinese University of Hong Kong
Shatin, New Territories
Hong Kong SAR, People’s Republic of China
wangmd@cuhk.edu.hk

Yuanzhi Xu, Ph.D.
Department of Stomatology
Tenth People’s Hospital of Tongji University
299 Yan’An Road, Jingan District
Shanghai, People’s Republic of China
18917683819@163.com

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China No. 8102141 (principal investigator, Dan Wang). Funding sources were not involved in the study design, data collection, data analysis, manuscript preparation, or submission.

REFERENCES

1. Laloo R, Lucchesi LR, Bisignano C, et al. Epidemiology of facial fractures: Incidence, prevalence and years lived with disability estimates from the Global Burden of Disease 2017 study. Inj Prev. 2020;26(Suppl 4):1–16.
2. Taylor CA, Bell JM, Breiding MJ, Xu L. Traumatic brain injury-related emergency department visits, hospitalizations, and deaths—United States, 2007 and 2013. MMWR Surveill Summ. 2017;66:1–16.
3. Bykowski MR, Goldstein JA, Losee JE. Pediatric cranioplasty. Clin Plast Surg. 2019;46:173–183.
4. Fishero BA, Kohli N, Das A, Nover M. Current concepts of bone tissue engineering for craniofacial bone defect repair. Craniomaxillofac Trauma Reconstr. 2015;8:23–30.
5. Zuk PA. Tissue engineering craniofacial defects with adult stem cells? Are we ready yet? Pediatr Res. 2008;63:478–486.
6. Schutzman SA, Barnes P, Duhaime AC, et al. Evaluation and management of children younger than two years old with apparently minor head trauma: Proposed guidelines. Pediatrics. 2001;107:983–993.
7. Macsaac ZM, Shakir S, Naran S, et al. Repair of a complicated calvarial defect: Reconstruction of an infected wound with rhBMP-2. Ann Plast Surg. 2016;76:205–210.
8. Chao MT, Losee JE. Complications in pediatric facial fractures. Craniomaxillofac Trauma Reconstr. 2009;2:103–112.
9. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol. 2012;8:133–143.
10. Phillips AM. Overview of the fracture healing cascade. Injury 2005;36(Suppl 3):S5–S7.
11. Gao L, Li M, Yin L, et al. Dual-inflammatory cytokines on TiO2 nanotube-coated surfaces used for regulating macrophage polarization in bone implants. J Biomed Mater Res A 2018;106:1878–1886.
12. Shi M, Chen Z, Farnaggi S, et al. Copper-doped mesoporous silica nanospheres, a promising immunomodulatory agent for inducing osteogenesis. Acta Biomater. 2016;30:334–344.
13. Spiller KL, Nassiri S, Witherel CE, et al. Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. Biomaterials 2015;37:194–207.
14. Molteni M, Gemma S, Rossetti C. The role of toll-like receptor 4 in infectious and noninfectious inflammation. Mediators Inflamm. 2016;2016:978936.
15. Wang D, Gilbert JR, Gray JJ, Jr, et al. Accelerated calvarial healing in mice lacking Toll-like receptor 4. PLoS One 2012;7:e49945.
16. Vijayan V, Khandelwal M, Manglani K, Gupta S, Surolia A. Methionine down-regulates TLR4/MyD88/NF-κB and BMP/Smad signaling in osteoclast precursors to reduce bone loss during osteoporosis. Br J Pharmacol. 2014;171:107–121.
17. Huang RL, Yuan Y, Zou GM, Liu G, Tu J, Li Q. LPS-stimulated inflammatory environment inhibits BMP-2-induced osteoblastic differentiation through crosstalk between TLR4/MyD88/NF-κB and BMP/Smad signaling. Stem Cells Dev. 2014;23:277–289.
18. Kikuchi T, Matsuguchi T, Tsuboi N, et al. Methionine down-regulates TLR4/MyD88/NF-κB and BMP/Smad signaling in osteoclast precursors to reduce bone loss during osteoporosis. Br J Pharmacol. 2014;171:107–121.
19. Kikuchi T, Matsuguchi T, Tsuboi N, et al. Methionine down-regulates TLR4/MyD88/NF-κB and BMP/Smad signaling in osteoclast precursors to reduce bone loss during osteoporosis. Br J Pharmacol. 2014;171:107–121.
20. Wang D, Gilbert JR, Taylor GM, et al. TLR4 inactivation in myeloid cells accelerates bone healing of a calvarial defect model in mice. Plast Reconstr Surg. 2017;140:296e–306e.
21. Streit M, Gehlenborg N. Bar charts and box plots. Nat Methods. 2014;11:117.
22. Barbaro G, Scolozzi A, Mastrolorenzo A, Supuran CT. Highly active antiretroviral therapy: Current state of the art, new agents and their pharmacological interactions useful for improving therapeutic outcome. Curr Pharm Des. 2005;11:1805–1843.
23. Tebas P, Powderly WG, Claxton S, et al. Accelerated bone mineral loss in HIV-infected patients receiving potent antiretroviral therapy. *AIDS* 2000;14:F63–F67.

24. Aukrust P, Haug CJ, Ueland T, et al. Decreased bone formative and enhanced resorptive markers in human immunodeficiency virus infection: Indication of normalization of the bone-remodeling process during highly active antiretroviral therapy. *J Clin Endocrinol Metab.* 1999;84:145–150.

25. Harris HE, Andersson U. Mini-review: The nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol.* 2004;34:1503–1512.

26. Chen Q, Guan X, Zuo X, Wang J, Yin W. The role of high mobility group box 1 (HMGB1) in the pathogenesis of kidney diseases. *Acta Pharm Sin B* 2016;6:183–188.

27. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: Endogenous danger signaling. *Mol Med.* 2008;14:476–484.

28. Ilango P, Mahalingam A, Parthasarathy H, Katamreddy V, Subhareddy V. Evaluation of TLR2 and 4 in chronic periodontitis. *J Clin Diagn Res.* 2016;10:ZC86–ZC89.

29. Lalla E, Lamster IB, Feit M, et al. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. *J Clin Invest.* 2000;105:1117–1124.

30. Rho J, Takami M, Choi Y. Osteoimmunology: Interactions of the immune and skeletal systems. *Mol Cells* 2004;17:1–9.

31. Wang D, Gilbert JR, Zhang X, Zhao B, Ker DFE, Cooper GM. Calvarial versus long bone: Implications for tailoring skeletal tissue engineering. *Tissue Eng Part B Rev.* 2020;26:46–63.