Introduction
Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently administered after orthopedic surgery for their anti-inflammatory, antipyretic, and analgesic capabilities. Their anti-inflammatory effects result from inhibition of prostaglandin (PG) biosynthesis via action on the cyclooxygenase (COX) involved in catabolism of arachidonic acid, which is present in the phospholipid membrane of cells[1–3].

Acetaminophen (paracetamol) is a widely used NSAID for its analgesic and mild anti-inflammatory actions. Although its pharmacologic action has not been attributed to a significant inhibition of peripheral prostanoids, Hinz et al[4] recently demonstrated that acetaminophen inhibited COX-2 to a degree comparable to the effect of other NSAIDs and selective COX-2 inhibitors.

Several studies demonstrated that indomethacin, ketorolac, diclofenac, and celecoxib, among other NSAIDs, suppress bone growth, remodeling, and repair[5–7], although the mechanism of this action is not fully understood. Research has focused on the activity of osteoblasts, which play an important role in bone formation and regeneration and are reported to have both bone-forming and immunological functions. We draw attention to: the high expression of antigens involved in antigen presentation in bone tissue sections[8], primary cultures, and human osteosarcoma cell line MG-63[9–12]; the elevated expression of various cytokines[13, 14]; and their phagocytic capacity of osteoblasts against targets of different size and nature[11, 12].

Unlike other NSAIDs, the effect of paracetamol on bone tissue has not yet been investigated, despite its very wide therapeutic utilization, especially as an analgesic. We therefore designed a study to explore its effects on molecular, cellular, and functional parameters of osteoblasts treated with a dose of 5 and/or 25 µmol/L, which are within the therapeutic range[15]. We selected the human osteosarcoma cell line MG63, which is frequently used as model to study the effect of pharmaceuticals on these osteoblasts[12, 16, 17].

Materials and methods
Cell line
We used the human osteosarcoma cell line MG63, purchased from American Type Cultures Collection (ATCC, Manassas, VA).
Drugs
Paracetamol (Sigma Chem Comp, St Louis, MO, USA).

Cell Culture
The human osteosarcoma cell line MG63 was maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 µg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 µg/mL amphotericin B (Sigma, St Louis, MO, USA), 1% glutamine (Sigma, St Louis, MO, USA), 2% HEPES (Sigma, St Louis, MO, USA), and supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were detached from the culture flask with a solution of 0.05% Trypsin (Sigma, St Louis, MO, USA) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, MO, USA) and then washed and suspended in complete culture medium with 10% FBS.

Cell proliferation assay
Osteoblasts were seeded at 1×10⁴ cells/mL per well into a Cell proliferation assay (Sigma, St Louis, MO, USA) and then washed and suspended USA) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, MO, USA) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, MO, USA) and then washed and suspended in a 24-well plate (Falcon, Becton Dickinson Labware, NJ, USA) without FBS and cultured for 24 h with the different concentrations of paracetamol (5–25 µmol/L) at 37 °C. Cells were detached, washed, and suspended in PBS and then immediately analyzed in a flow cytometer Ortho Absolute (Ortho Diagnostic System, Raritan, USA). All experiments included an internal control, i.e., cells incubated under the same conditions but without paracetamol.

Antigenic phenotype by flow cytometry
Cultured osteoblastic cells were previously treated with 25 µmol/L of paracetamol in FBS-free medium for 24 h at 37 °C. They were then detached from the culture flask by treatment with 0.4% EDTA solution, washed, and suspended in phosphate-buffered saline (PBS) at 2×10⁵ cells/mL. Cells were labeled by direct staining with labeled latex beads. One hundred microliters of cell suspension were incubated with 10 µL of the appropriate MAB for 30 min at 4 °C in the dark. Cells were washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with an argon laser (Facs Vantage Becton Dickinson, Palo Alto, California, USA) at a wavelength of 488 nm to determine fluorescent cell percentage. As controls, untreated cells were stained with the MABs. The percentage of antibody-positive cells was calculated from counts of 2000–3000 cells. At least four experiments were run for each antigen.

Osteocalcin synthesis
Osteocalcin was quantified in culture supernatants by using the N-tact® osteo SP kit for IRMA osteocalcin (Diasorin-Stillwater, Minnesota, USA). For this purpose, cells were cultured for 24 h in FBS-free medium in the presence of 25 µmol/L of paracetamol, and the supernatant was obtained by centrifuge at 1600 r/min. Samples were analyzed in duplicate using the N-tact® osteo SP kit. Results were expressed in picograms of osteocalcin/mL×10⁴ cells. Supernatants of cultures kept under the same conditions but without treatment served as controls.

Phagocytic activity of MG63 lines
Phagocytic activity was studied by flow cytometry. Cultured human MG63 osteosarcoma cells treated with 25 µmol/L of paracetamol and untreated control cells were cultured without FBS and detached from the culture flask by treatment with 0.04% EDTA solution, washed, and then suspended in complete culture medium with 10% FBS at 2×10³ cells/mL. Cells were labeled by direct staining with labeled latex beads. One hundred microliters of cell suspension were incubated with 200 µL carboxylated FITC-labeled latex beads of 2 µm diameter (Sigma Adrich, St Louis, USA) for 30 min at 37 °C in darkness. Cells were washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer (Facs Vantage Becton Dickinson, Palo Alto, California, USA). Results were expressed as percentage of cells positive for phagocytosis and mean channel fluorescence, which correlates with the number of particles phagocytosed.

Table 1. Monoclonal antibodies (MABs) used to study antigenic phenotype on cultured human osteoblastic-like cells, with their specificity, the fluorochrome used to label the antibody, and the supplier. FITC: Fluorescein-isothiocyanate. PE: Phycoerythrin.

| MABs  | CD/specificity | Fluorochrome | Supplier                |
|-------|----------------|--------------|-------------------------|
| Control PE | -              | PE           | Caltag (Burlingame, CA) |
| Control FITC | -             | FITC         | Caltag (Burlingame, CA) |
| OKM13 | CD13           | FITC         | Caltag (Burlingame, CA) |
| CD21  | CD21           | PE           | Caltag (Burlingame, CA) |
| CD44  | CD44           | FITC         | Caltag (Burlingame, CA) |
| IOL1b | CD54           | FITC         | Caltag (Burlingame, CA) |
| CD80  | CD80           | FITC         | Caltag (Burlingame, CA) |
| CD86  | CD86           | FITC         | Caltag (Burlingame, CA) |
| OKDR  | HLA-DR         | FITC         | Caltag (Burlingame, CA) |

Statistical analysis
Statistical comparisons were determined by using the Student’s t-test, and P<0.05 was considered significant.

Results
Effect of paracetamol treatment on proliferation of MG63 cell line
The effects of 24-h treatment with each paracetamol dose (5 or 25 µmol/L) on cell growth of the MG63 cell line were determined by cell count, finding a statistically significant reduction in MG63 cell proliferation with both doses (P<0.01 for both dose). After 24 h culture of 10⁵ cells in each case, the mean viable cell count was 39.53×10⁴ cells/mL in the control cultures versus 28.37×10⁴ cells/mL in the 5 µmol/L-treated cultures and 26.18×10⁴ cells/mL in the 25 µmol/L-treated cultures (Figure 1). Because a greater effect on proliferation was observed with
the higher dose (25 µmol/L), it was selected as the dose to be used in analyses of the effects on the remaining study parameters.

**Effect of paracetamol treatment on antigenic phenotype of MG63 cell line**

Using flow cytometry, we studied the effect of treatment with 25 µmol/L paracetamol on the expression of different surface markers of the MG63 cell line in order to determine its antigenic profile. Results obtained showed that treatment with 25 µmol/L of paracetamol for 24 h significantly increased the expression of CD21, CD44, CD80, CD86, and HLA-DR antigens ($P<0.01$ for CD21, $P<0.05$ for CD44 and $P<0.01$ for CD80, CD86, and HLA-DR), but had no effect on CD13 and CD54 antigens (Figure 2, Table 2).

**Effect of paracetamol treatment on osteocalcin synthesis in MG-63 cell line**

The effect of paracetamol on cell differentiation/maturation was studied by using osteocalcin synthesis as a marker. According to the osteocalcin levels in culture supernatants, treatment with 25 µmol/L paracetamol for 24 h significantly decreased osteocalcin synthesis ($P<0.05$), finding mean values of 0.50 pg/mL×10^4 cells in the treated cultures versus 2.60 pg/mL×10^4 cells in the untreated (control) cultures (Figure 3).

**Effect of paracetamol treatment on phagocytic activity of MG63 line**

Table 3 and Figure 4 show the effects of paracetamol on the phagocytic capacity of the MG63 line (analyzed by flow cytometry) which was significantly decreased after a 24-h
tissues showed that NSAIDs produce cell cycle arrest and cell death; they reported that the cytotoxic and apoptotic effects of NSAIDs on osteoblasts might not be prostaglandin-mediated, but alterations during early wound-healing may delay bone regeneration or, in implants, osseointegration. These results are in agreement with the effects of other NSAIDs on osteoblast growth reported in in vitro and in vivo studies[6, 7, 19, 20]. For some time, this adverse effect on bone tissue has largely been attributed to the role of PGE2 in bone formation[6, 7, 19]. However, Chang et al[21] showed that NSAIDs significantly arrest the cell cycle in G0/G1 and induce cytotoxicity and osteoblast cell death; they reported that the cytotoxic and apoptotic effects of NSAIDs on osteoblasts might not be prostaglandin-related. Hence, it has been proposed that the induction by NSAIDs of cell cycle arrest and cell death in osteoblasts may be an important mechanism contributing to their suppressive effect on bone formation. Studies on cell lines from other tissues showed that NSAIDs produce cell cycle arrest and induce apoptosis[22-25]. However, the underlying mechanism is thought to be complex and various factors have been implicated, including PG synthesis inhibition, apoptosis induction by different pathways, cell cycle alteration or arrest, and the dose of the NSAID in question[26-28].

The present study showed that paracetamol affects osteocalcin synthesis, antigenic profile, and phagocytic activity. Type I collagen, alkaline phosphatase, and osteocalcin can all be used as markers of osteoblast maturation. Osteocalcin was selected for this study because it is the most abundant non-collagenous protein of bone, and its synthesis increases during final stages of osteoblast differentiation and maturation[29]. Osteocalcin synthesis was lower during cell differentiation, suggesting that this pharmaceutical not only decreases proliferative capacity but may also arrest osteoblast differentiation.

A similar effect on the antigenic profile of paracetamol treatment was previously reported in osteoblasts in inflammatory situations in vitro and in vivo[13, 30]. This effect is explained as the response of osteoblasts to proinflammatory situations, in which there appears to be an activation of their immune function to the detriment of their bone forming function and their differentiation/maturation. This is supported by our findings of no change in the percentage expression of CD54 molecule and a decrease in osteocalcin synthesis.

Osteoblasts and dendritic cells have characteristics in common, including cytokine synthesis, phagocytic capacity, antigenic presentation to T lymphocytes, and the expression of certain antigens. It has been reported that dendritic cells increase expression of class II and costimulatory molecules in some situations, eg, infections, finding that the expression of chemokine receptors and adhesion molecules was modified in the presence of microbial products[31]. This effect is usually found in parallel with a decrease in the phagocytic capacity of the cell, interpreted as a process of cell activation aimed at responding to specific situations, with an increase in antigen presentation to T lymphocytes and a reduction in phagocytic capacity. Paracetamol may act in a similar manner, activating the osteoblast and producing an increase in the expression of certain membrane antigens and a decrease in its phagocytic capacity. These results reflect a common situation observed in many cell types, which transform their function under treatment, as reported for macrophages, decidual cells and fibroblasts[32-35].

These experimental data indicate that paracetamol may have an effect on bone tissue, modifying various physiological parameters of osteoblasts. It should be taken account that the MG63 cell line was used as osteoblast model, with the limitations that this implies, although it is one of the most widely used lines in the study of the effect of drugs on osteoblasts[12, 16, 17]. Further research is warranted to evaluate the use of this drug in patients requiring bone regeneration.

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### Author contribution

Lourdes DÍAZ-RODRÍGUEZ: performed research; analyzed data and wrote the paper; Olga GARCÍA-MARTÍNEZ: per-
formed research; Manuel ARROYO-MORALES: collaboration in development of performed research; Belén RUBIO-RUIZ: collaboration in development of performed research; Concepción RUIZ: designed research, analyzed data, and wrote the paper.

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