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Original article

Racemic Etodolac is cytotoxic and cytostatic for B-cell precursor acute lymphoblastic leukemia cells☆

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Abstract

Several epidemiological studies have provided evidence that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) could have a prophylactic effect against some cancers such as sporadic colorectal cancer and leukemia. Indeed, various NSAIDs have been shown to induce apoptosis in malignant cells. We evaluated the effect of racemic Etodolac on proliferation and cell survival in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. Etodolac decreased survival of Nalm-16 and Nalm-6 BCP-ALL cell lines and also decreased cell proliferation in Nalm-16 cell line. Ours findings indicate, for the first time to our knowledge, that Etodolac is cytotoxic and cytostatic for BCP-ALL cells.

Keywords: B-cell precursor acute lymphoblastic leukemia; Etodolac; Apoptosis; Tumor growth; Treatment

1. Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) leads to death within a few months, but nowadays 80% of newly diagnosed pediatric patients become long-term survivors after treatment with multi-agent chemotherapy [1].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a large group of compounds with analgesic, antipyretic and anti-inflammatory properties [2]. Several epidemiological studies have provided evidence that administration of NSAIDs could have a prophylactic effect against some cancers such as sporadic colorectal cancer [3,4], lung cancer [5] and leukemia [6]. Etodolac is a safe NSAID used for osteoarthritis, rheumatoid arthritis and for management of acute pain for more than 20 years. Etodolac is a racemic mixture of two enantiomers, R and S, each one with different pharmacological properties. The major molecular targets of NSAIDs are the two forms of cyclooxygenase (COX) required for prostaglandin synthesis [7]. NSAIDs may also exert their effects through COX-independent mechanisms [8], such as the peroxisome proliferators-activated receptor (PPAR) family of nuclear receptors that function as ligand-dependent transcription factors. The PPARγ was found to be expressed in human BCP-ALL and treatment with PPARγ ligands induced growth inhibition and apoptosis [9].

2. Materials and methods

2.1. Leukemic cell lines

Nalm-16 and Nalm-6 were kindly provided by Dr. Maria Isabel Doria Rossi, Federal University of Rio de Janeiro,

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Brazil. They were originally established from peripheral blood of BCP-ALL relapsed patients. According to EGIL criteria, Nalm-16 and Nalm-6 were characterized as Common BCP-ALL ($CALLA^+$) and Pré-B ALL, respectively [10]. The cell lines were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 100 U/ml G sodium penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mg/ml sodium pyruvate, 40 μM essential amino acids, and 40 μM non-essential amino acids, all from Gibco–BRL, Gaithersburg, MD, USA. The pH was adjusted to 7.2 with 1.5 g/l sodium bicarbonate and cells were maintained at 37 °C in humidified atmosphere with 5% CO_2.

2.2. Etodolac treatment

Racemic Etodolac was purchased from Sigma–Aldrich. The drug was dissolved in dimethylsulfoxide (DMSO, Sigma–Aldrich) and diluted in culture medium immediately before use. Nalm-16 or Nalm-6 cells were plated at 3×10^5 cells/ml in 24-well plates. They were treated with 250 or 500 μM Etodolac and compared to cells maintained in DMSO.

The range of Etodolac's concentration was chosen based on a phase II clinical trial for treatment of B-cell chronic lymphocytic leukemia (B-CLL) that has observed reduction in average lymphocyte count in patients administered 1000–2400 mg of R-Etodolac twice daily, at steady-state blood levels of approximately 300–600 μM [11].

2.3. Cell cycle and cell death analysis

After 24, 48 and 72 h culture cells were collected and their viability was measured by Trypan dye exclusion assay. Double staining with Annexin V–FITC (1:10 dilution, Molecular Probes, Eugene, OR, USA) for apoptotic cells and propidium iodide (PI; Sigma–Aldrich) for dead cells, done according to manufacturer's instructions, was followed by data acquisition using a FACSCalibur flow cytometer (BDB, San Jose, CA, USA). A total of 30,000 events were acquired. DNA content was monitored by flow cytometry after PI staining using the method of Vindelov and colleagues [12] at 24, 48 and 72 h. Cells with sub-diploid DNA content (sub-G0) were excluded from cell cycle analysis. A total of 10,000 events were acquired within live cells-gate. All FACS data were analyzed using Infinicyt software (Cytognos, Salamanca, Spain).

2.4. Statistical methods

Statistical significance was determined using the non-parametric Mann–Whitney *U*-test, using Prism 2.01 software (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Racemic Etodolac induces cell death in BCP-ALL cell lines

We found that the assayed Etodolac doses decreased viable cell expansion in both cell lines tested at 250 and 500 μM (Fig. 1A and C). No effect was observed with 100 μM (data not shown). Etodolac treatment also reduced total cell number expansion (Fig. 1B and D), suggesting a potentially relevant impact on blocking tumor growth. In order to better understand the kinetics of Etodolac-induced cell death, we used Annexin-V/PI double staining to identify all cell death stages. FACS analysis revealed that Etodolac reduced the percentage and the absolute cell number of viable cells, confirming the cytotoxic activity of Etodolac (Fig. 1E, F).

Immunophenotyping showed that Etodolac induced early apoptosis (Annexin-V^+/PI^- staining) at 24 h (data not shown). During cell culture progression, the treated leukemic cells were found in the late apoptosis (Annexin-V^+/PI^+) and the necrosis stage (Annexin-V^-/PI^+), as observed at 72 h (Fig. 1G). Our results clearly showed that Etodolac is cytotoxic to the BCP-ALL cell lines tested.

3.2. Racemic Etodolac decreases cell proliferation in Nalm-16 BCP-ALL cell line

No difference was found at 24 h, but at 48 h we found a marked difference in the S-cell cycle fraction when comparing cells treated with 500 μM Etodolac to its respective control (Fig. 2A–C). No differences were found for 100 μM (data not shown). As depicted in Fig. 2B, Etodolac at the highest concentration reduced the proliferation compartment (S-fraction) by around 60% at 48 h.

4. Discussion

The design of new therapeutic strategies in BCP-ALL is pivotal to improve survival of patients with high risk of relapse, and to reduce the therapy side-effects in patients with low risk of relapse.

Here, we described for the first time to our knowledge the use of Etodolac, a classical NSAIDs, against BCP-ALL. Etodolac induced decrease of cell survival. This cytotoxic effect induced by Etodolac may be of potential clinical interest, although further pre-clinical and clinical studies are necessary. We also showed that Etodolac decreased cell proliferation, as found by low percentage of cells in S-cell cycle phase after treatment. This cytostatic effect induced by Etodolac is in accordance to the prophylactic effect of NSAIDs against leukemia observed by epidemiological studies.

These data provide evidence that racemic Etodolac decreases leukemic lymphocyte survival and proliferation and thus, it could be useful as an anti-leukemic agent. Further works should verify whether these effects are also observed in patient samples and also clarify the molecular mechanisms