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Differential activities of thalidomide and isoprenoid biosynthetic pathway inhibitors in multiple myeloma cells

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ABSTRACT

Thalidomide has emerged as an effective agent for treating multiple myeloma, however the precise mechanism of action remains unknown. Agents known to target the isoprenoid biosynthetic pathway (IBP) can have cytotoxic effects in myeloma cells. The interactions between thalidomide and IBP inhibitors in human multiple myeloma cells were evaluated. Enhanced cytotoxicity and induction of apoptosis were observed in RPMI-8226 cells. Examination of intracellular levels of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) revealed a wide variance in basal levels and response to IBP inhibitors. These findings provide a mechanism for the differential sensitivity of myeloma cells to pharmacologic manipulation of the IBP.

1. Introduction

Multiple myeloma, a disorder of malignant plasma cells, accounts for approximately 10% of hematological malignancies and leads to 11,000 deaths per year in the United States. Thalidomide has been the standard of care for newly diagnosed multiple myeloma. Despite its wide-spread use, the precise mechanisms of action underlying thalidomide's therapeutic effects in myeloma remain unknown. Thalidomide appears to have both direct and indirect effects on myeloma cells and in general, is thought to have immunomodulatory and antiangiogenic properties [1–4]. Zoledronic acid (ZA), an aminobisphosphonate, is widely used in the management of myeloma-induced bony disease. This agent has a very high affinity for bone mineral and causes inhibition of osteoclast function by inducing changes in the cytoskeleton, loss of the ruffled border, and apoptosis [5,6]. ZA also exerts direct effects on multiple myeloma cells, with prior studies showing that ZA can induce apoptosis in myeloma cell lines [7]. The mechanism of action for these effects is believed to be due to the ability of ZA and other aminobisphosphonates to inhibit farnesyl pyrophosphate synthase (FDPS), an enzyme which catalyzes a step in the isoprenoid biosynthetic pathway (IBP) (Fig. 1) [8–10].

The IBP is responsible for the production of a variety of sterol and non-sterol moieties [11]. The rate limiting step in this pathway is catalyzed by the enzyme HMG-CoA reductase, leading to the production of mevalonate. FDPS catalyzes the conversion of the five-carbon units isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) to the 10-carbon unit geranyl pyrophosphate (GPP), as well as the conversion of geranyl pyrophosphate to the 15-carbon unit farnesyl pyrophosphate (FPP). Geranylgeranyl pryophosphate (GGPP) synthase (GGDPS) catalyzes the addition of IPP to FPP, yielding the 20-carbon GGPP. Both FPP and GGPP serve as substrates for isoprenyltransferases, specifically farnesyl transferase (FTase) and geranylgeranyl transferases (GGTase) I and II, respectively. Prenylated proteins, including Ras and Rab family members, play central roles in diverse cellular processes, including cell survival, proliferation, differentiation, cytoskeletal organization, and membrane trafficking [12]. The function of these proteins is dependent on proper membrane localization, which is achieved in part through prenylation [13].

Preclinical studies have demonstrated that inhibitors of the IBP, including statins (HMG-CoA reductase inhibitors), aminobisphosphonates, and isoprenyltransferase inhibitors, can induce myeloma cell death [14–21]. The anti-myeloma effects of IBP inhibitors appear to be a consequence of disruption of geranylgeranylation, however the specific targets have not been delineated [21–24].

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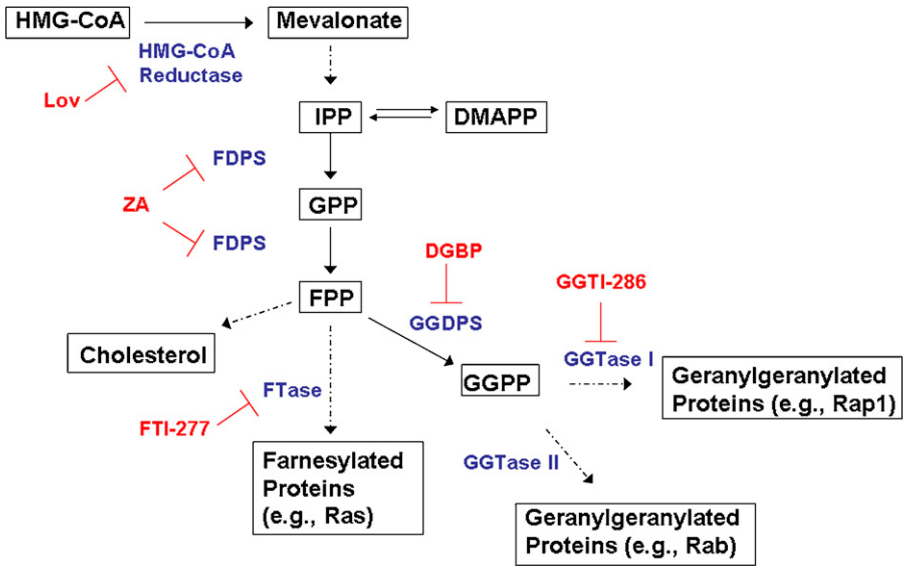

Fig. 1. The isoprenoid biosynthetic pathway (IBP) and pharmacological inhibitors. Substrates and products of the pathway are shown in black, enzymes are shown in blue, and specific inhibitors are shown in red.

There has been a report evaluating the combination of thalidomide and lovastatin in *ex vivo* studies involving bone marrow mononuclear cells from patients with multiple myeloma [25]. In this study there appeared to be an increase in apoptosis with the combination of drugs. Increased cytotoxicity with the combination of ZA and thalidomide in RPMI-8226 cells, but not ARH-77 cells, has been demonstrated [26]. Finally, an interaction between simvastatin and lenalidomide, a second-generation immunomodulatory agent, has been observed in myeloma cells [27]. The mechanisms underlying these observations have yet to be defined.

In the studies presented here, the effects of combining thalidomide with inhibitors of the IBP in human myeloma cells are examined. Agents which specifically inhibit discrete steps in the IBP (lovastatin as an HMG-CoA reductase inhibitor, ZA as a FDPS inhibitor, and digeranylbisphosphonate (DGBP) as a GGDPS inhibitor) or directly inhibit the prenyltransferases (FTI-277 as a FTase inhibitor and GGTI-286 as a GGTase I inhibitor) are utilized. These studies reveal differential sensitivity of myeloma cell lines not only to inhibitors of the IBP, but also to the combination of thalidomide with IBP inhibitors. FPP and GGPP levels, both basal and in response to IBP inhibitors, were found to vary amongst cell lines, providing a mechanism for the differential sensitivity.

2. Materials and methods

2.1. Materials

Lovastatin, DL-mevalonic acid lactone (converted to mevalonate prior to use), farnesyl pyrophosphate, geranylgeranyl pyrophosphate, and thalidomide were obtained from Sigma (St. Louis, MO). Zoledronate was purchased from Novartis (East Hanover, NJ). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), FTI-277, and GGI-286 were obtained from Calbiochem (San Diego, CA). Digeranyl bisphosphonate [28] was supplied by Terpenoid Therapeutics, Inc. (Coralville, IA). Anti-pan-Ras was obtained from InterBiotechnology (Tokyo, Japan). Anti-PARP, anti-β-tubulin, anti-Rap1a, anti-Rab6, and anti-goat IgG horseradish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-rabbit HRP-linked antibodies were obtained from Amersham (GE Healthcare, Piscataway, NJ). Annexin V-FITC was obtained from BD Pharmingen (BD Biosciences, San Jose, CA). D*-GCVLS and D*-GCVLL (dansyl-labeled peptides) were obtained from Bio-Synthesis (Lewisville, TX). Rat recombinant FTase and GGTase I were purchased from Jena Biosciences (Jena, Germany). HPLC-grade water was prepared with a Milli-Q system (Millipore, Bedford, MA). All solvents were of optima or HPLC grade.

2.2. Cell cultures

Human multiple myeloma cell lines (RPMI-8226, H929, and U266) were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 media with 10% (RPMI-8226 and H929) or 15% (U266) heat-inactivated fetal calf serum (per ATCC suggestion) supplemented with glutamine and penicillin–streptomycin at 37 °C and 5% CO_2.

2.3. MTT assay

Cells were seeded (5×10^4 cells/150 μL per well) in 96-well flat-bottom plates. Cells were incubated with drugs for 24–48 h at 37 °C and 5% CO_2. The MTT assay was performed as previously described [29]. The absorbance for control cells treated with solvent only was defined as an MTT activity of 100%.

2.4. Annexin V staining and flow cytometry

Following incubation with drugs, cells ($0.5–0.75 \times 10^6$ cells/sample) were washed with PBS, pelleted, and then resuspended in HEPES buffer solution (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, and 1.8 mM $CaCl_2$). Annexin V-FITC (2.5 μg/mL) was added and a 10–15 min incubation at room temperature was performed. Propidium iodide solution (1 μg/mL) was then added. Flow cytometry was performed with a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cellquest software (Becton Dickinson) was used for acquisition (Cellquest V3.3) and analysis (Cellquest Pro V4.0) of data. Forward scatter (FSC) and orthogonal scatter (SSC) were collected using linear amplification. Annexin V-FITC and propidium iodide fluorescence were collected using log amplification. 10,000 events were collected in listmode. A bitmap gate was placed around the cell population on the basis of forward and orthogonal light scatter to eliminate small debris and aggregates. The bitmap was large enough so that apoptotic cells were not eliminated. Cells satisfying the bitmap gate were analyzed using quadrant statistics in an Annexin V-FITC versus propidium iodide dual parameter histogram.

2.5. Western blot analysis

Cells (5×10^6/5 mL) were incubated drugs. At the conclusion of the incubations, cells were collected, washed with PBS, and lysed in RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, and 0.05 M Tris–HCl) containing protease and phosphatase inhibitors. Protein content was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Equivalent amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with the appropriate primary antibodies, and detected using HRP-linked secondary antibodies and Amersham Pharmacia Biotech ECL Western blotting reagents per manufacturer's protocols.

2.6. Intracellular FPP and GGPP measurements

Intracellular FPP and GGPP levels were measured using the previously reported reversed phase HPLC methodology [30]. Briefly, following incubation with drugs, cells were collected and counted using Trypan blue staining and a hemocytometer. Cells were then washed with PBS. Isoprenoid pyrophosphates were extracted from cell pellets ($3–5 \times 10^6$ cells/sample) with 2× 1.2 mL of extraction solvent (butanol/75 mM ammonium hydroxide/ethanol 1:1.25:2.75). After drying down by nitrogen gas, the FPP and GGPP in the residue were incorporated into fluorescent GCVLS or GCVLL peptides by FTase or GGTase I. The prenylated fluorescent pep-