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Preliminary report

Differential action of 3-hydroxyanthranilic acid on viability and activation of stimulated lymphocytes

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ABSTRACT

Lymphocytes proliferation after antigen-driven activation leads to an increase in cell count, which could last some week, until apoptosis mechanisms allow the homeostatic control of the system. During the first days of this stimulation, activated lymphocytes display high resistance to apoptosis and to most immunosuppressive drugs. According to the literature, few compounds have been described to kill recently activated cells, by inhibiting metabolic processes fundamental to proliferation.

The aim of our work was to evaluate comparatively these different compounds, in order to identify the best strategy to kill cells that have undergone proliferation, while sparing the repertoire of resting cells.

After preliminary experiments, 3-HAA and bortezomib were selected as the most suitable compounds for our purposes. The possible synergic effect of 3-HAA with bortezomib or with manganese ions was also assessed. 3-HAA was confirmed to be the most reliable pharmacologic approach to inhibit proliferation with acceptable toxicity on resting cells. While in the case of PHA stimulation 3-HAA led to death of most lymphocytes, only a minor percentage of cells were killed after allo-stimulation, suggesting that the effect is proportional to the percentage of stimulated lymphocytes. Manganese ions further enhanced this effect, while results with bortezomib seemed to be less consistent.

These results deserve further investigations to develop new procedures for targeting activated cells with pharmacological approaches.

1. Introduction

Antigen driven lymphocyte activation is a key process in the development of adaptive immunity, both in physiologic and in pathologic conditions. In a normal immune response, antigen-driven proliferation leads to the expansion of multiple T cell clones and to an increase in lymphocyte count, which usually lasts some weeks until activation-induced cell apoptosis allows the homeostatic control of the system. Indeed, in the first days after stimulation, activated lymphocytes display high resistance to apoptosis and are poorly susceptible to the action of most immunosuppressive drugs [1,2]. The selective action on recently activated lymphocytes compared to resting cells can be clinically relevant; thus, the effect of the drugs on disease-causing cells should be dissociated from their immunosuppressive side effects.

Different strategies have been developed to identify and deplete allo-reactive lymphocytes, by incubating them *ex vivo* with stimulator cells (usually allo-geneic B cells or dendritic cells) in mixed lymphocyte reaction (MLR). In these settings, activated cells can be identified because of the expression of activation-markers, such as CD25, and eliminated by immunomagnetic depletion or photodynamic purging. However, this strategy bears intrinsic problems; indeed, the targeting of CD25 can lead to deplete even regulatory T cells, which are also characterized by the expression of high levels of CD25.

Allo-depletion with drugs with a selective action on proliferating cells could offer a more affordable and simple alternative, but it has not been evaluated deeply in the clinic.

Treatment with methotrexate before or after the stimulus has been reported to be selective in killing activated T cells, by inhibiting several key enzymes in folate metabolism (dihydrofolate reductase and thymidylate synthase), preventing de-novo purine biosynthesis [3,4]. Another approach exploited the inhibition of membrane transport of electrons by the drug phenoxodiol. This drug causes cell apoptosis via both intrinsic and extrinsic pathways. By blocking plasma membrane electron transport it interferes with the redox balance and thus impairs

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cell survival and proliferation [5]. 3-hydroxyanthranilic acid (3-HAA) has been shown to play a role in the antiproliferative effect of the enzyme IDO (indoleamine 2,3-dioxygenase) [6]. By depleting intracellular GSH, this drug exposes cells to proliferation related oxidative burst and apoptosis, an effect that could be enhanced by the addition of manganese ions, which further increase oxidation processes [7,8]. Other strategies to inhibit recently stimulated lymphocytes include the block of JAK3 signaling pathway by tasocitinib [9] and the inhibition of proteasome (bortezomib) which impairs NF-κB signaling, causing death in activated cells [10]. Tasocitinib is a small molecule inhibitor of JAK3, a cytoplasmic tyrosine kinase involved in receptor signaling for cytokines, lymphocyte development and homeostasis. At least part of the efficacy observed in these models is likely the result of the inhibition of JAK3-mediated IL-2 signaling. In addition, JAK3 inhibition may affect the function of a number of other cytokines including IL-4 and IL-9 [11]. Bortezomib, contains a boronate moiety linked to a dipeptide with a high affinity, specificity and selectivity for catalytic activity of the proteasome. Inhibition of IκB degradation by proteasome keeps NF-κB in the cytoplasm, hereby preventing its effect on the transcription of genes related to survival and proliferation [12].

Unfortunately, most of these reports quote few studies performed in different experimental settings and never compared each other.

The aim of our work was to evaluate comparatively these different compounds, in order to identify the best strategy to kill activated and proliferating cells while sparing the repertoire of resting cells, which is the necessary condition for developing a possible clinical use of these compounds.

2. Materials and methods

2.1. Experimental plan

First of all, preliminary experiments were performed to choose the compounds with the best therapeutic potential, based on the difference between killing of PHA-stimulated and -unstimulated cells. After that, the selected agents were evaluated in MLR, as a more physiologic stimulus, measuring viability and proliferation. Possible synergic effects of different compounds have been evaluated as well.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll gradient from blood of healthy donors. For each condition, 200,000 cells were plated in 96-well plates in 200 μL X-VIVO 15 medium (Lonza, Belgium) containing 10% Human Serum AB (Sigma Aldrich, Germany).

Compounds were added to cell cultures: 3-hydroxyanthranilic acid was used at increasing concentrations from 0.3 to 0.7 mM (Sigma Aldrich, Germany) bortezomib from 0.125 to 2 μM (Selleck Chemical LLC, TX, USA), phenoxodiol from 0.0625 to 1 mM (A160 dehydroequol, Synchem OHG, Germany), methotrexate from 0.25 to 4 mM (Wyeth Lederle, Italy), tasocitinib from 4 to 64 μM (CP-690550, Selleck Chemical LLC, TX, USA), manganese chloride from 1 to 30 μM ($MnCl_2$, Sigma Aldrich, Germany). 3-HAA was dissolved in water; bortezomib, tasocitinib, phenoxodiol were dissolved in dimethylsulfoxide (DMSO) so that the final concentration of DMSO would not exceed 1%. Methotrexate was formulated for injection (25 mg/mL saline solution containing NaOH) and was diluted in the medium. Medium pH was measured at the higher concentration of drug and no variations were found.

To induce proliferation, 1 μg/mL phytohemagglutinin (PHA, Biochrom AG, Germany) was simultaneously added to the cell culture. MLR was performed using EBV-transformed B lymphocytes at a ratio of 1:2 to responder PBMCs [13].

After 60 hours of culture, cell proliferation and viability were evaluated.

2.3. Proliferation assay

The effect of the selected substances on the proliferation of PBMCs was determined by labeling metabolically active cells with [methyl-^{3}H] thymidine (3H-thy) (PerkinElmer).

Two hundred thousand cells were plated in ninety-six-well plates in 200 μL X-VIVO 15 + 10% Human Serum AB and treated with the compounds as described above. 3H-thy (2.5 μCi/mL/well) was added for the final 22 hours of the incubation period. Cells were then transferred into a 96-well filter microplate (Millipore), attached to a vacuum pump and washed twice. Twenty five microliters of scintillation cocktail (Optiphase SuperMix Filter Count, PerkinElmer Life Science) were added to each filter and the amount of incorporated radioactivity was determined as cpm by a liquid scintillation analyzer (Wallac 1450 Microbeta, Liquid Scintillation Counter).

2.4. Viability evaluation

The effect of the compounds on cell viability was assessed by cytometric 7-aminoactinomycin D (7AAD) staining. Briefly, cells were harvested and washed with saline. 7AAD dye (eBioscience) was added to the cell suspension according to the customer indications. After 5 minute incubation, cells were acquired with BD FACScan cytometer (Becton Dickinson), and data were analyzed with FlowJo software (TreeStar, v 7.6).

3. Results

3.1. Selection of the compounds

Based on the scientific literature, the following five substances were chosen: methotrexate, phenoxodiol, tasocitinib, 3-HAA and bortezomib. Different concentrations of each compound were tested on PBMCs in order to identify the setting associated with the higher difference between toxic effects on PHA-stimulated and -unstimulated cells. After 60-hour incubation, 7AAD cytometric analysis of viability indicates that 0.5 mM 3-HAA and 0.5 μM bortezomib have the greatest preferential action on stimulated cells, while methotrexate, phenoxodiol and tasocitinib seem to have little or no effect on cell viability both in PHA-stimulated and -unstimulated cells (data not shown).

3.2. 3-HAA in association with manganese ions

The effect of the addition of manganese ions, that further increase 3-HAA induced oxidation processes, on the killing of stimulated cells was assessed. After PHA stimulation, almost all the cells were alive (10.8% 7AAD positive cells, Fig. 1A) and activated, but the addition of $MnCl_2$ allowed to reach the highest difference between the death of stimulated and unstimulated cells in particular at 1 μM $MnCl_2$ (see double arrow in the Fig. 1A). Proliferation was also strongly affected by manganese ions (Fig. 1B). Using strong stimuli, such as PHA, the addition of concentration of ≥1 μM $MnCl_2$ allowed to reduce the proliferation to levels comparable to unstimulated cells (difference not significant). For milder stimuli, such as allo-geneic EBV-transformed cells, a reduction to baseline proliferation levels was reached by means of 3-HAA alone.

3.3. 3-HAA and manganese ions in association with bortezomib

The possible synergic effect of 3-HAA and $MnCl_2$ with bortezomib was assessed. While preliminary data suggested the use of bortezomib as a promising approach for our purposes, in these experiments the addition of this agent did not show any advantage compared to