(50) Title: USE OF N-ACETYL-CYSTEINE IN THE MANUFACTURE OF TOPICAL PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF ALLERGIC PATHOLOGIES OF THE RESPIRATORY TRACT

(57) Abstract: The use of NAC in the manufacture of topical pharmaceutical compositions for the treatment of allergic pathologies of the respiratory tract is described.
"Use of N-acetyl-cysteine in the manufacture of topical pharmaceutical compositions for the treatment of allergic pathologies of the respiratory tract"

The present invention relates to the use of N-acetyl-cysteine for the preparation of topical pharmaceutical compositions for the treatment of allergic pathologies of the respiratory tract, more particularly, for the treatment of allergic pathologies of the respiratory tract with symptomatology mainly caused by the eosinophil activation.

The role of eosinophils in the onset of inflammation and in the obstruction of the airways is known. In fact, eosinophils release a wide array of pro-inflammatory and cytotoxic materials such as superoxide anion (O$_2^-$), eosinophil-cationic protein (ECP) and eosinophil peroxidase (EPO). EPO-dependent mechanisms lead to the formation of ipohalous acids and of hydroxy radical (OH$^-$) which contribute to the eosinophil-mediated cytotoxicity and are considered strong activators of macrophages and of epithelial cells.

Superoxide anion and EPO represent oxidant inflammatory activity while the release of ECP is a marker of non-oxidant inflammatory activity of the cells.

It is known that the allergic pathologies of the respiratory tract, such as asthma and allergic rhinitis, are characterised by a chronic inflammatory disease of the airways where excess reactive-oxygen species (ROS) production and defective endogenous anti-oxidant defence mechanism are present.

N-acetyl-cysteine (NAC) is a known drug used for the treatment of respiratory pathologies (The Merck Index, XII ed., no. 89, page 16). By topical route, NAC is used as mucolytic in inhalatory form, for the treatment of respiratory diseases characterised by thick and viscous hypersecretion, acute and chronic bronchitis, pulmonary emphysema, mucoviscidosis and bronchiectasia, as well as in the form of rhinologic drops, in association with a nasal decongestant, for the treatment of acute and subacute rhinitis, with mucopurulent and slowly healing exudant, chronic and mucocrostosis rhinitis, vasomotory rhinitis and sinusitis.

However, notwithstanding the well-consolidated use in therapy, as far as we know, the effect of NAC on the eosinophil function, and then its use in the treatment of allergic pathologies of the respiratory tract, has never been described.

On the contrary, there are some papers in the literature which could suggest a lack of effect
of NAC in the treatment of these allergic pathologies. For example, in WO 98/48839 (Warner-Lambert Co.) topical nasal anti-inflammatory compositions for the treatment of allergic rhinitis containing anti-inflammatory corticosteroids optionally in association with NAC as mucolytic are described.

We have now found that NAC is able to inhibit the inflammatory response of the eosinophils and it is then useful, by topical administration, in the treatment of allergic pathologies of the respiratory tract.

Therefore, object of the present invention is the use of NAC for the preparation of topical pharmaceutical compositions for the treatment of allergic pathologies of the respiratory tract.

For the use object of the present invention, NAC is generally used in the form of aqueous solution for inhalation or for nasal instillation, optionally in admixture with usual excipients such as preserving agents, buffering agents, complexing agents, salving agents, and so on.

A preferred example of pharmaceutical composition of NAC for the use object of the present invention is the following.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC</td>
<td>1.000 g</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>0.0125 g</td>
</tr>
<tr>
<td>Hydroxypropylmethylcellulose</td>
<td>0.750 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.020 g</td>
</tr>
<tr>
<td>Monosodium phosphate</td>
<td>0.300 g</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>0.300 g</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.100 g</td>
</tr>
<tr>
<td>Sorbitol 70%</td>
<td>2.000 g</td>
</tr>
<tr>
<td>Mint flavour</td>
<td>0.0188 g</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.310 g</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>0.360 g</td>
</tr>
<tr>
<td>Purified water</td>
<td>q.s. 100 ml</td>
</tr>
</tbody>
</table>

The efficacy of NAC was determined by evaluating its capacity to inhibit the production of superoxide anion, EPC and EPO.

As already reported these three parameters are characteristic evidences of the oxidant and
non-oxidant inflammatory activity of the eosinophils.
NAC showed to significantly inhibit all the three parameters.
Furthermore, the effect of NAC on the eosinophil survival was evaluated.

This is a further important parameter of the NAC efficacy in the treatment of allergic pathologies of the respiratory tract. In fact, in these pathologies an up-regulation of cytokine expression can be observed which, by inhibiting the natural apoptosis, prolongs eosinophil survival. NAC showed to be effective in reducing the survival of eosinophils cultured in the presence of GM-CSF.

In order to better illustrate the present invention the following example is now given.

Example 1

Materials and methods

Human eosinophils isolated from peripheral blood obtained from healthy volunteers were used.

NAC was dissolved in deionized water and then diluted in a buffer.

Cells were activated by particulate stimuli (serum opsonized zymosan - SOZ) and soluble mediators such as fMLP (N-formylmethionyl-leucyl-phenylalanine) which acts through specific receptors and PMA (phorbol 12-myristate 13-acetate), a phorbol ester which directly activates protein kinase C.

SOZ was prepared immediately before the use by boiling in saline (10 mg/ml) zymosan A particles for 10 minutes, washing twice in HBSS and then resuspending in HBSS at 5 mg/ml; zymosan (0.5 mg/ml) was then incubated with 10% autologous serum. NAC concentrations were expressed as molar concentration.

Experimental procedure

Isolation of human eosinophils

Fresh anticoagulated human blood from healthy volunteers was diluted with PBS and aliquots were layered onto Percoll solution as described by Hansel et al. (J. Immunol. Methods, 145, 105-110, 1991) for the subsequent purification of eosinophils. The magnetic cell separation system was applied as described by Hansel et al. and by Hatzelmann et al: (Br. J. Pharmacol., 114, 821-831, 1995) obtaining eosinophils with >95% purity (determined
Measurement of superoxide anion release

The release of superoxide from human eosinophils was measured essentially as described by Sedgwick et al. (J. Allergy Clin. Immunol., 81, 876-83, 1988) with the modifications described by Cortijo et al., (Br. J. Pharmacol., 119, 99-106, 1996). In brief, the generation of superoxide was quantified as the superoxide dismutase-inhibitable reduction of ferricytochrome c with a modified microassay. With 96-well plates and a 200 µl reaction volume, 8 x 10⁴ cells were added to 100 µmoles/l of cytochrome c in HBSS/gel and 5 µg/ml of cytochalasin B. To initiate the reaction, the cells were incubated with fMLP (30 nM), PMA (10 ng/ml) or SOZ (0.5 mg/ml). Immediately after the addition of the activator, the reaction wells were measured by absorbance at 550 nm in a Microplate AutoReader (EL309, Bio-Tek Instruments) followed by repeated readings for 30 minutes (fMLP) and 120 minutes (PMA, SOZ). Each reaction was performed in duplicate and against an identical control reaction containing 20 µg/ml of SOD. The results were adjusted to represent a 1 ml reaction volume and superoxide generation was calculated with an extinction coefficient of 21.1 x 10⁵ moles/l/cm as nmoles of cytochrome c reduced per 5 x 10⁵ cells per minute (minute) minus SOD control.

Treated cells were pre-incubated with NAC at a concentration of 0.1, 0.5 and 1 mM by first testing 0.5 mM for 5 minutes at 37°C before cell activation and the exposure was continued up to the end of the experiment. In further experiments the incubation time with NAC was 30 minutes. Control experiments were carried out in parallel with drug vehicle. The drug effects were expressed as percent inhibition from control values.

In order to determine the ability of NAC to directly react with the oxygen metabolites generated by activated cells, the formation of superoxide anion in a comparable magnitude was triggered in a cell-free system (the other conditions being the same) with a xanthine/xanthine oxidase system as described by Gillissen et al. (Respiration, 64, 16-22, 1997).

Determination of EPO release

EPO release was assessed by the method of Munoz et al. (J. Leukocyte Biol., 58, 667-674,
Eosinophils were resuspended in HBSS-FCS and 10^5 cells were loaded onto microplate wells. NAC in HBSS-FCS was added to each well and the plate was incubated for 30 minutes at 37°C. Then, cells were activated with fMLP (1 μM) + Cyto B (5 μg/ml), incubated for 30 minutes and then centrifugated at 350 g for 5 minutes. This concentration of fMLP+Cyto B was selected according to what reported by Munoz et al. Duplicate aliquots of supernatant (100 μl) were transferred onto a new plate. Kinetic assay for EPO was carried out for supernatant of treated and untreated cells. The EPO release was expressed in ng/10^6 cells and the drug effect was expressed as percent inhibition from control values.

**ECP production**

All assays were carried out in duplicate at a cell concentration of 10^6 cells/ml in supplemented Dulbecco’s PBS (Hatzelmann et al.). The cell suspensions (1 ml) were pre-incubated with NAC for 15 minutes at 37°C before stimulation; then the cells were activated with fMLP (100 nM) for 30 minutes and the experiments terminated by centrifugation (Hatzelmann et al.). Supernatants and cell pellets were prepared for storage (-20°C). Aliquots of the samples were taken for ECP measurement by a RIA method according to the manufacturer instructions. The effects of the drug were expressed as percent inhibition from control values.

**Eosinophil survival**

Freshly isolated eosinophils were resuspended at a concentration of 2 X 10^6 cells/ml in supplemented RPMI (Hallsworth et al., Br. J. Pharmacol. 117, 79-86. 1996). 25 μl (~50,000 cells) of the cell suspension were cultured in a 96-well plate containing 75 μl of supplemented RPMI with 1 ng/ml of recombinant human GM-CSF and various NAC concentrations, simultaneously added with GM-CSF. The cells were cultured for a period of 4 days after which viability was assessed in duplicate by trypan blue exclusion. In each experiment, untreated eosinophils were cultured also in the presence (positive control) and in the absence (negative control) of GM-CSF (1 ng/ml). Preliminary experiments showed that the eosinophil viability when cultured in the presence of GM-CSF was >50% on day 4 while in the absence of GM-CSF the eosinophil survival began to decrease after day 1 in culture, dropping to ~5% by day 4 (Hallsworth et al.).
Analysis of results

Results were expressed as means ± s.e. of mean. Statistical analysis was carried out by Student’s t test or by analysis of variance with appropriate post-hoc tests. Significance was accepted when P<0.05.

Results

Measurement of superoxide anion release

Stimulation of eosinophils with fMLP (30 nM), PMA (10 ng/ml) or SOZ (0.5 mg/ml) produced superoxide anion generation. The control values (that is values measured in cells activated but not treated, and expressed as nmoles superoxide generated by 5 x 10⁵ cells) in the different groups of experiments were 7.7±1.4 (n=4), 7.5±1.6 (n=4), 8.3±1.9 (n=4) and 7.1±1.6 (n=4) for fMLP groups, 25.2±3.2 (n=5), 35.3±1.7 (n=3), 26.3±0.3 (n=3) and 28.4±1.7 (n=4) for PMA groups and 11.7±1.6 (n=7), 9.3±0.9 (n=3), 12.0±1.9 (n=5) and 12.2±1.7 (n=4) for SOZ groups.

NAC decreased the superoxide generation by fMLP, PMA and SOZ with similar potencies (-log IC₅₀ = 3.66±0.08, 3.22±0.19 and 3.28±0.16, respectively).

In further experiments it was found that by prolonging the incubation time up to 30 minutes no significant changes of the NAC inhibitory effects were observed.

Determination of EPO release

Activation of eosinophils with fMLP (1 μM) caused an augmented release of EPO into the supernatants. NAC produced a concentration-related inhibition of EPO release with -log IC₅₀ value of 4.72±0.09.

ECP production

The activation of eosinophils with fMLP (100 nM) resulted in ECP release. The values detected in the supernatants of unstimulated cells and the increase (2.7-fold) of ECP in the supernatant of the fMLP-stimulated cells are consistent with the values reported by Hatzelmann et al. NAC (0.5 mM) inhibited ECP release as reported in the following table 1.
Table 1

Effect of NAC on ECP release in fMLP-stimulated cells with (100 nM) in the supernatant of human eosinophils. The values are expressed as ng/10^6 cells.

<table>
<thead>
<tr>
<th></th>
<th>unstimulated cells</th>
<th>fMLP 100 nM</th>
<th>fMLP + NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. #1</td>
<td>130.7</td>
<td>302.3</td>
<td>317.4</td>
</tr>
<tr>
<td>Expt. #2</td>
<td>193.1</td>
<td>264.8</td>
<td>157.2</td>
</tr>
<tr>
<td>Expt. #3</td>
<td>196.8</td>
<td>306.3</td>
<td>118.5</td>
</tr>
<tr>
<td>Expt. #4</td>
<td>123.7</td>
<td>353.4</td>
<td>225.2</td>
</tr>
<tr>
<td>means</td>
<td>168.0</td>
<td>447.5</td>
<td>204.6</td>
</tr>
<tr>
<td>±sem</td>
<td>16.7</td>
<td>141.5</td>
<td>43.6</td>
</tr>
</tbody>
</table>

Eosinophil survival

In the absence of GM-CSF, the eosinophil survival in culture by day 4 was ~10-20%. By contrast, in the presence of 1 ng/ml of GM-CSF, the viability of cultured eosinophils increased up to >50%. NAC (0.5-1 mM) showed to increase the viability of eosinophils cultured without GM-CSF but to decrease the survival of eosinophils cultured with GM-CSF.
Claims

1) Use of N-acetyl-cysteine for the manufacture of topical pharmaceutical compositions for the treatment of allergic pathologies of the respiratory tract.

2) Use according to claim 1 wherein the topical pharmaceutical composition is an aqueous solution for inhalatory use or for nasal instillation.

3) Use according to claim 1 for the treatment of asthma and allergic rhinitis.