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Research paper

NB 06: From a simple lysosomotropic aSMase inhibitor to tools for elucidating the role of lysosomes in signaling apoptosis and LPS-induced inflammation

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ABSTRACT

Ceramide generation is involved in signal transduction of cellular stress response, in particular during stress-induced apoptosis in response to stimuli such as minimally modified Low-density lipoproteins, TNFalpha and exogenous C_6-ceramide. In this paper we describe 48 diverse synthetic products and evaluate their lysosomotropic and acid sphingomyelinase inhibiting activities in macrophages. A stimuli-induced increase of C_{16}-ceramide in macrophages can be almost completely suppressed by representative compound NB 06 providing an effective protection of macrophages against apoptosis. Compounds like NB 06 thus offer highly interesting fields of application besides prevention of apoptosis of macrophages in atherosclerotic plaques in vessel walls. Most importantly, they can be used for blocking pH-dependent lysosomal processes and enzymes in general as well as for analyzing lysosomal dependent cellular signaling. Modulation of gene expression of several prominent inflammatory messengers IL1B, IL6, IL23A, CCL4 and CCL20 further indicate potentially beneficial effects in the field of (systemic) infections involving bacterial endotoxins like LPS or infections with influenza A virus.

1. Introduction

Apoptosis is a mandatory process in development and control of biological systems and organisms. It takes place under physiological conditions, is accompanying cell growth, cell division and differentiation and is essential for tissue homeostasis and immune response of organisms [1]. Macrophages and smooth muscle cells in the vessel wall are able to internalize cholesterol and modified LDL (mLDL) via scavenger receptors (scavenger receptor class A (SRA I/II/III)) [2,3]. Once macrophages are saturated with (minimally) modified (oxidatively/enzymatically) Low-Density Lipoproteins (mmLDL), they transform into foam cells. Foam cells, however, located in larger gatherings in the vessel wall, form fatty streaks and significantly contribute to plaque formation. Minor injuries result in fibrous plaque containing foam cells or smooth muscle cells, respectively fragments from apoptosis or necrosis. In further steps, a complex injury to the vessel wall with a necrotic core is emerging, finally leading as unstable plaque, to thrombosis or infarction [2]. Macrophages are particularly interesting as a test system for acid sphingomyelinase (aSMase) inhibitors, as they play a crucial role in the development of atherosclerosis and represent a potential therapeutic target for compounds under investigation. In cell culture experiments apoptotic rates of human macrophages correlate with the mmLDL concentration applied [4]. Oxidized phospholipids in mmLDL induce apoptotic signaling in arterial smooth muscle cells via activation of aSMase [5]. In addition to mmLDL, TNFalpha is a prototype activator of aSMase, increasing

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concentration of ceramide in cells and inducing apoptosis [6,7]. Unlike mmLDL-stimulated acid sphingomyelinase, neutral sphingomyelinase (nSMase) plays an important role in increasing ceramide levels in lipid extracts of cells. Ceramides with fatty acids of varying chain lengths bound as amides, are components of the sphingomyelin cycle and are well established signaling molecules. Activation of SMases and subsequent ceramide generation is involved in signal transduction of cellular stress response, in particular during stress-induced apoptosis. Sphingomyelin as a substrate of these pathways is a physiologically inert phosphosphingolipid abundant in all eukaryotic cell types [8–10].

(C_{16}-) ceramide with its raft- and transport-vesicles forming characteristics links both pathways [11]. A lysosomotropic compound lacks a direct effect on free enzymes, it is rather an inhibitor of all lysosome-dependent signaling pathways as well as lysosomal luminal enzymes, transporters and structures depending on an acidic pH value. It thus can be used as an enzyme cross-inhibitor of the lysosome for blocking pH-dependent lysosomal processes. Based on this concept, further conclusions on pro-apoptotic stimuli such as mmLDL, TNFalpha, exogenous C_6-ceramide and synthetic ceramide analogues (1R)-(E)-(2-methyl-oxazol-4-yl)-hexadec-2-ene-1-ol (HPL-1R36N) and 4-[(1R)-(E)-1-Hydroxy-3-phenyl-allyl]-(2RS,4R)-2-phenyl-thiazolidin-3-carbonic acid-*t*-butyl ester (HPL-39N) [12] as well as from inhibitor treatment (NB 06 (**23**)) [4] could be derived; in fact they all require an intact lysosome. An active compound not only engages with classic metabolic processes such as proteolysis and degradation of membrane lipids, but also alters presentation of lipid antigens since lysosomal lipid-binding proteins are necessary to present lipid or glycolipid antigens, such as e.g. the four MHC-I-like glycoproteins CD1a-d [13].

Chlorpromazine (**15**), imipramine (**1**) and desipramine (**2**) are used therapeutically as neuroleptics (**15**) or anti-depressants (**1**, **2**) in panic disorder, bulimia and agoraphobia with panic attacks [14]. High concentrations of weakly basic compounds promote detachment of aSMase from intralysosomal membranes and thus, allow proteolytic degradation of this enzyme [15]. The release of the active enzyme from the inner lysosomal membrane and an enhanced proteolytic degradation was suggested as a cause for inactivation. At a concentration of 20 μM, **2** initiates dissociation of 50% of aSMase from the lysosomal membrane-bound substrate sphingomyelin. Complete dissociation is achieved at 50 μM [16]. Since the mid-eighties, compounds like **2** have been known as irreversible inhibitors of aSMase (EC 3.1.4.12) [17,18]. Compound **2** reduces or suppresses enzyme activity, thus completely mimicking a Niemann-Pick syndrome in intact cells when administered at non-cytotoxic concentrations. Neither **15** nor **2** show a similar peeling effect on other acidic lysosomal lipid hydrolases [18,19]. Starting from known lysosomotropic aSMase inhibiting compounds **2** and **15**, we designed and prepared a group of 48 congeners introducing novel structures into potent inhibitors. Synthetic products were characterized in macrophages with regard to anti-apoptotic effects, lysosomotropic activities and aSMase inhibition, using well-known aSMase inhibitors like **1**, **2**, **15** and amitriptyline (**88**) as references. Based on methylamine - a common prerequisite for effective inhibitors (weak base, pKa >6, lipophilic) [15,20], our structural variations comprise: a) introduction of ring systems with varying lengths of carbon chains linking the aliphatic *N*,*N*-dimethylamine and the ring nitrogen, b) 4-methoxy or 3,4-dimethoxy substitution of the phenylethyl moiety, c) replacement of the endocyclic nitrogen by carbon and introduction of an adjacent exocyclic nitrogen ring, d) replacement of the hetero atoms in the ring system (sulfur by oxygen, switch from 10*H*-phenothiazines to 4*H*-phenoxazines) and e) steric restriction of free rotational elements by fusion to additional ring systems or by introduction of carbon double bonds. Unlike previous studies on approved pharmaceutical compounds and their classification as “Functional inhibitors of aSMase” [15,20] based on a structure-activity relationship model (SARM) using three connection-specific values (pKa, log P, steric hindrance factor of the most basic nitrogen atom), the focus here was on a rational design and a targeted variation of distinct structural features for investigating biological activities related to lysosomotropic effects. Due to common prerequisites for the effectiveness towards aSMase (i.e. weak base, pKa >6, lipophilic) parallels to structure-activity relationships found by Kornhuber are inevitable [15,20]. Our results, however, provide accurate statements about partial structures responsible for the (in) efficacy of a compound as lysosomotropic as well as an aSMase inhibitor. Special attention is paid also to the symmetry of compounds (**132** (NB 45)), blockade of basic nitrogen (e.g. **53** (NB 22)) as well as strong increase in lipophilicity due to e.g. fluorine substitution (**113** (NB 32)). Hybridization experiments elucidating time-dependent gene expression effects in human Mono-Mac 6 cells stimulated with the bacterial endotoxin LPS, also in presence of the active lysosomotropic compound **23** (NB 06), have been performed. The results provide further insights into modulating properties of lysosomotropic compounds on gene expression of various inflammatory messengers (interleukins and cytokines).

2. Results and discussion

2.1. Chemistry

Compounds **1**, **2** and **15** are already known as lysosomotropic compounds active as aSMase inhibitors [17,18]. Therefore, in initial syntheses tricyclic compounds **1**, **2** and **15** are modified by incorporating additional *N*-methyl amine moieties like 4-methoxy- or 3,4-dimethoxy-phenylethyl residues. To investigate structure-activity-relationships, compounds with varying ring systems and a shorter aliphatic carbon chain (carbazole (**19**), diphenylamine (**31**), 10*H*-phenothiazine (**41**) and 10*H*-phenoxazine (**36**)), were prepared. Depending on the ring structure, final compounds IVa/b were synthesized using Scheme 1A (6,11-dihydro-5*H*-benzo[b][1] benzazepine (7-ring)), 10*H*-phenothiazines, 10*H*-phenoxazines or Scheme 1B (carbazoles). Each synthesis consists of three steps: spacer attachment to a compound containing a heterocycle (step A), *N*-Alkyl spacer demethylation with 1-chloroethyl chloroformate (ACE-Cl), a reagent for selective *N*-dealkylation of tertiary amines [21] (step B) and finally an *N*-Alkyl spacer substitution (step C). Final compounds based on the same intermediates III are designated as compounds IVa/IVb in Scheme 1/Table 2 in Material and Methods. An overview of inserted residues is given in Scheme 1C. More synthetic details with regard to each subgroup of compounds presented here are provided in the Experimental section.

2.2. aSMase enzyme inhibition and anti apoptotic potential in vital cells (PBMC)

Starting from known aSMase inhibitors **2** and **15** [17,18] a total number of 48 compounds were prepared and tested on PBMCs subjected to aSMase stimulation and mmLDL-induced apoptosis, **2** and **15** were used as reference compounds. Based on results obtained in these experiments, molecules to be synthesized next were designed and a compound library generated. Selected active compounds were further investigated as for biological effects in cell lysates, on TNFalpha-induced apoptosis, ceramide pattern and levels as determined in the lipid extracts of cells, as well as in transcription and gene expression experiments.

Apoptosis assay: PBMC were pre-incubated with 2 μM of each compound for 30 min, then mmLDL (27 μg/mL) or TNFalpha (3 ng/mL) was added, cells incubated 4 h and stained with YO-PRO®-1

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