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A cell-based test system for the assessment of pharmacokinetics of NOD1 and NOD2 receptor agonists

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ABSTRACT

Agonists of nucleotide oligomerization domain (NOD) 1 and NOD2 receptors represent a promising class of immunostimulants and immunological adjuvants. Here, we describe a cell-based test system to assess their pharmacokinetics. In this system, NOD1 and NOD2 agonist concentrations in sera are determined using a reporter cell line, 293Luc, which contains an NF-κB-inducible luciferase reporter construct and naturally expresses NOD1 and NOD2. The 293Luc cells dose-dependently respond to different NOD1 and NOD2 agonists in the nanomolar to low-micromolar concentration range. To verify that the NF-κB-inducing activity of serum samples is due to the administered agonist and not to secondarily induced endogenous molecules, a 293Luc-derived NOD1/NOD2 double-knockout clone is used. Within-run and between-run precisions of the system are < 15% and < 20%, respectively. Applicability of the novel assay is illustrated by studying pharmacokinetics of two specific NOD2 agonists (*N*-acetyl-D-glucosaminyl-*N*-acetyl-D-muramyl-L-alanyl-D-isoglutamine and *N*-glycolyl-D-muramyl-L-alanyl-D-isoglutamine) and a specific NOD1 agonist (*N*-acetyl-D-glucosaminyl-*N*-acetyl-D-sorbitolamine-D-lactoyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid). In summary, the test system described here can potentially be used to assess pharmacokinetics of NOD1 and NOD2 agonists in different animal species.

1. Introduction

Muropeptides are monomeric fragments of bacterial peptidoglycan that trigger innate immune responses via NOD1 and NOD2 receptors [1,2]. Muropeptides are viewed as a promising class of immunostimulants and immunological adjuvants. A number of natural agonists of NOD1 and/or NOD2 have been characterized [3], many synthetic and semisynthetic muropeptide analogs have been generated [4–7], and some of them have been approved for clinical use [8–10].

An important issue in the clinical development of NOD1 and NOD2 agonists is the analysis of their pharmacokinetics. However, no standard method exists. Different studies have utilized radioactively labelled muropeptide analogs coupled with scintillation counting of either unfractionated samples [11] or high-performance liquid chromatography (HPLC)-fractionated samples [12]; HPLC coupled with mass-spectrometry (HPLC-MS) [13]; gas chromatography – mass spectrometry (GC–MS) to detect free muramic acid after acidic hydrolysis [14]; and chemiluminescence immunoassay [15]. All of these assays have advantages and disadvantages, as detailed in Discussion. Their common disadvantage is that they do not measure biological activity of NOD1 and NOD2 agonists detected in the circulation.

Recently, we generated a panel of reporter cell lines and clones designed to measure agonism towards NOD1 and NOD2 receptors [16]. The panel is based on a 293Luc reporter cell line, which was derived from the HEK293T cell line by stable transfection with a reporter construct (a luciferase gene under an NF-κB-inducible promoter). The 293Luc cells express natural NOD1 and NOD2 and robustly respond to agonists of either receptor and to tumor necrosis factor (TNF), the latter serving as a positive control. Using CRISPR/Cas9 technology, we generated three 293Luc derivative clones with knockouts of NOD1, NOD2 or both genes. These clones are used to assess specificity of compounds towards NOD1 and NOD2. Here, we describe the use of this cell panel as a biological test system to study pharmacokinetics of NOD1 and NOD2 agonists. Concentrations of agonists in sera are measured using luciferase induction in the parental 293Luc cell line. The double-knockout clone (293Luc-ΔNOD1/2) is used as a specificity control, in order to verify that luciferase induction in the 293Luc cells is caused by the tested agonist and not by secondarily induced endogenous molecules.

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2. Materials and methods

2.1. Chemicals

N-acetyl-D-glucosaminyl-*N*-acetyl-D-muramyl-L-alanyl-D-isoglutamine (GMDP), a specific NOD2 agonist, was synthesized as described [17] and kindly provided by Evgeny A. Makarov (RAM ltd, Moscow). Another specific NOD2 agonist, *N*-glycolylmuramyl-L-alanyl-D-isoglutamine (GlycMDP) [2,18], was purchased from Invivogen (San Diego, CA). *N*-acetyl-D-glucosaminyl-*N*-acetyl-D-sorbitolamine-D-lactoyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid (GS-triDAP), a specific NOD1 agonist, was prepared from the natural muropeptide *N*-acetyl-D-glucosaminyl-*N*-acetyl-D-muramyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid (GM-triDAP) by sodium borohydride treatment as described [16] and kindly provided by Vyacheslav L. L'vov and Nikolai P. Arbatsky (Institute of Immunology, Moscow). GS-triDAP was termed 'reduced GM-triDAP' in our earlier publications [16]. Exact molecular masses of GMDP, GlycMDP and GS-triDAP are 695.29, 508.2 and 870.37, respectively. Recombinant

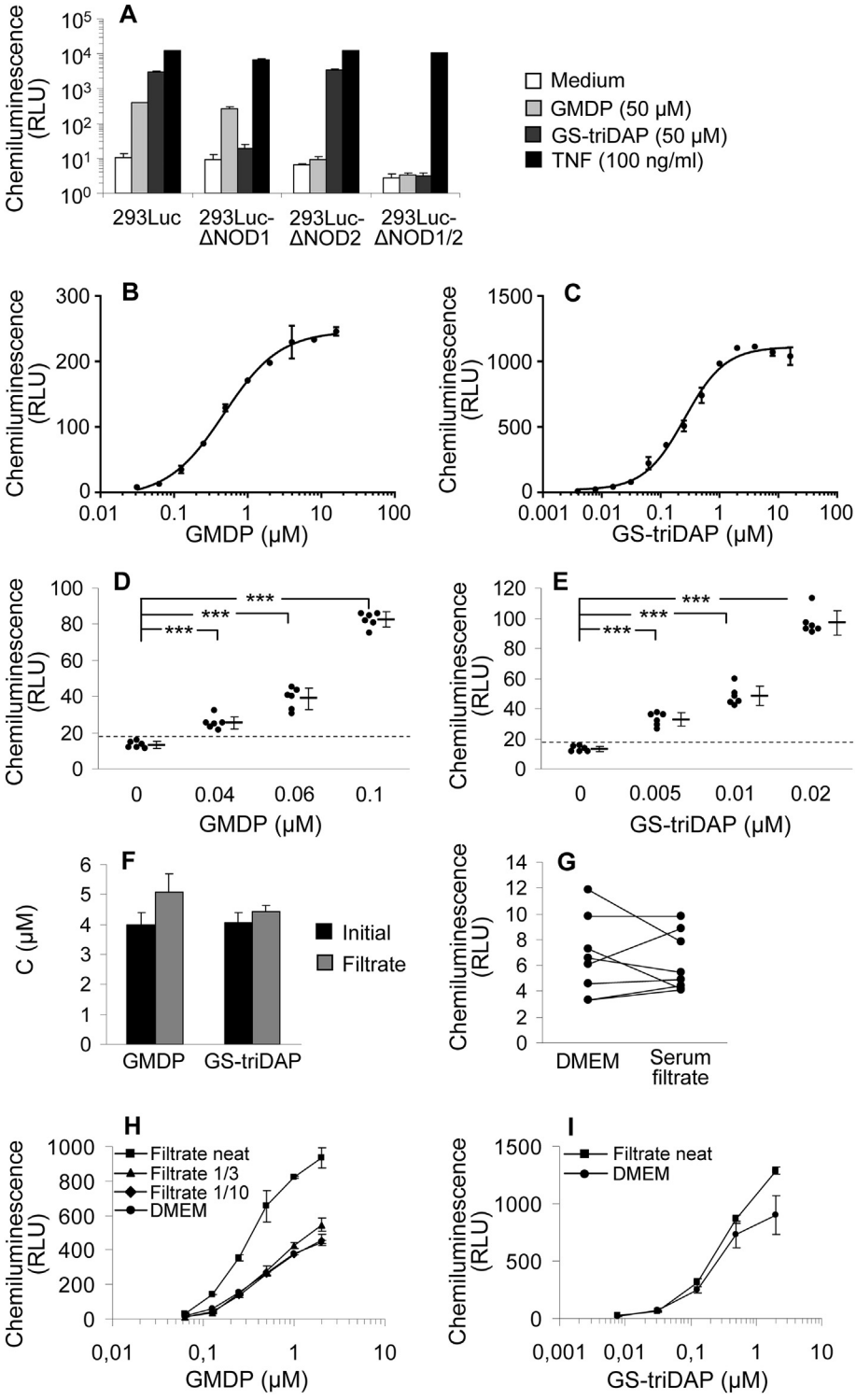

Fig. 1. Validation of the 293Luc cell-based assay to measure NOD1 and NOD2 agonist concentrations. A, responses of the parental 293Luc cell line and its daughter NOD1- and/or NOD2-knockout clones to GMDP, GS-triDAP and TNF. A representative experiment, mean ± SD of triplicates. B and C, representative dose-response curves of 293Luc cells to GMDP (B) and GS-triDAP (C). D and E, establishment of the limit of quantitation (LoQ) for GMDP (D) and GS-triDAP (E). For each agonist concentration, 6 individual measurements as well as mean and SD are shown. *** $p < 0.001$ by Student's *t*-test. Dashed lines, specific signal cut-off (limit of blank) corresponding to mean + 1.645 SD of the background signal [20]. F, concentrations of GMDP and GS-triDAP diluted at 4 μM in DMEM before and after passing through a 3-kD centrifuge ultrafilter. The initial solutions and the filtrates were added to 293Luc cells at 25 μl per 75 μl culture medium (1/4). Mean ± SD of three experiments. G, responses of 293Luc cells to 3-kD filtrates of normal rabbit serum. Each dot represents a mean of triplicate from an individual experiment, with a total of 8 experiments. H and I, potentiation of responses of 293Luc cells to GMDP (H) and GS-triDAP (I) by a 3-kD filtrate of normal rabbit serum. Neat or pre-diluted (1/3 or 1/10) filtrates were spiked with GMDP or GS-triDAP to 4× final concentrations. The spiked filtrates were added to culture medium at a 1/4 ratio. X-axis indicates final concentrations of GMDP and GS-triDAP in the culture wells.