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Bortezomib and ixazomib protect firefly luciferase from degradation and can flaw respective reporter gene assays

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ABSTRACT

Firefly luciferase-based reporter gene assays are the most commonly used assays to investigate the transcriptional regulation of gene expression. However, direct interaction of tested compounds with the firefly luciferase leading to altered enzymatic activity may lead to misinterpretation of experimental data. When investigating the proteasome inhibitors bortezomib, carfilzomib, and ixazomib, we observed increased luminescence for bortezomib and ixazomib, but not for carfilzomib, in a pregnane-X-receptor (PXR) reporter gene assay, which was inconsistent with the mRNA expression levels of the main PXR target gene *CYP3A4*. To further scrutinize this phenomenon, we performed experiments with constitutively expressed firefly luciferase and demonstrated that the increase in cellular firefly luciferase activity is independent from PXR activation or CYP3A4 promoter. Using cell-free assays with recombinant firefly luciferase enzyme, we made the counterintuitive observation that firefly luciferase activity is inhibited by bortezomib and ixazomib in a reversible and competitive manner. This inhibition stabilizes the firefly luciferase enzyme against proteolytic degradation (e.g., toward trypsin), thereby increasing its half-life with subsequent enhancement of total cellular luminescence that eventually mimicked PXR-driven luciferase induction. These data show that particular compounds can strikingly interfere with firefly luciferase and once more illustrate the importance of careful interpretation of data obtained from luciferase-based assays.

Luciferase-based reporter gene assays are widely used to investigate the regulation of gene expression as well as to identify new potential drugs in high-throughput screenings. The reporter gene construct contains the regulatory elements (e.g., promoter) of a gene of interest and the sequence for the luciferase (reporter), which enzymatic activity acts as a surrogate for the expression of the analyzed gene and/or the activation/inhibition of the regulatory elements. The firefly luciferase, encoded by the *luc* gene from the American firefly *Photonius pyralis,* catalyzes the reaction from firefly luciferin, ATP, and O_2 with Mg^{2+} as a cofactor to AMP, oxyluciferin, and pyrophosphate, thereby emitting yellow–green light with a spectral maximum of 560 nm [1,2]. The main advantages of bioluminescence-based reporter gene assays are their sensitive signal with a wide dynamic range due to their relatively short half-life (3 h compared with, e.g., 26 h for green fluorescent protein) [3,4] and their low background signal [1,2]. Small increases of the short half-life can, however, have significant impact on the readout of luciferase-based assays. Using a model by Hargrove and Schmidt [5], Auld and coworkers calculated that an increase of only approximately 30% in protein half-life can lead to a 150% increase in the cellular luciferase concentration and, thus, luciferase signal [3]. In addition, a substrate-competitive mechanism for luciferase inhibitors was proposed because decreased or absent inhibition was demonstrated in the presence of excess luciferase substrates [6]. More recently, great effort has been put into the construction of comprehensive luciferase inhibitor databases [6–10] in order to support the interpretation of high-throughput screening results and to avoid the further pursuit of false-positive hits.

When evaluating the potential for drug–drug interactions of the proteasome inhibitors bortezomib, ixazomib, and carfilzomib, we observed that bortezomib and ixazomib in fact increased the luciferase signal in a pregnane-X-receptor (PXR) reporter gene

Abbreviations used: PXR, pregnane-X-receptor; RT–qPCR, reverse transcription–quantitative polymerase chain reaction; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin; Tris, tris(hydroxymethyl)aminomethane; DMEM, Dulbecco's modified Eagle's medium; AhR, aryl hydrocarbon receptor; G6PDH, glucose-6-phosphate dehydrogenase; ANOVA, analysis of variance.

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assay. Being one of the major targets for this transcription factor, the mRNA expression of *CYP3A4,* however, was not induced, as demonstrated by reverse transcription–quantitative polymerase chain reaction (RT–qPCR). In contrast, the proteasome inhibitor carfilzomib neither induced mRNA expression of *CYP3A4* nor increased the luciferase signal in the PXR reporter gene assay. Thus, the mechanism of proteasome inhibition leading to luciferase accumulation and enhanced luminescence could be excluded as the underlying reason. Instead, we hypothesized that bortezomib and ixazomib stabilize the luciferase enzyme, whereas carfilzomib does not. Interestingly, luciferase is known to be stabilized by inhibition, a mechanism already demonstrated for other compounds, which leads to very counterintuitive observations; whereas cellular assays demonstrate an increased luciferase signal, assays with recombinant or purified luciferase protein reveal an inhibition of the luciferase activity [11,12].

In this study, therefore, we scrutinized the underlying reason for the bortezomib- and ixazomib-mediated increase in the luciferase signal in cellular reporter gene assays that cannot be attributed to PXR activation.

1. Materials and methods

1.1. Materials

Culture media, medium supplements such as antibiotics (penicillin/streptomycin) and glutamine, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), bovine serum albumin (BSA), and the GenElute Mammalian Total RNA Miniprep Kit were purchased from Sigma–Aldrich (Taufkirchen, Germany). Rifampicin and bovine trypsin were purchased from AppliChem (Darmstadt, Germany). Fetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (TCDD, dioxin) was obtained from LGC Standards (Wesel, Germany). Microtiter plates (96 wells) were obtained from Nunc (Wiesbaden, Germany), opaque white luminometer plates (96 wells) were purchased from Greiner (Frickenhausen, Germany), and cell culturing bottles were obtained from Greiner. Absolute qPCR SYBR Green Mix was obtained from ABgene (Hamburg, Germany), and the RevertAid H Minus First Strand cDNA Synthesis Kit was obtained from Fermentas (St. Leon-Rot, Germany). The Steady-Glo Luciferase Assay System, the Dual-Glo Luciferase Assay System, the pGL4.21 vector, the pGL4.74 [hRluc/TK] renilla vector, the pGL4.50 [luc2/CMV/Hygro] vector, the Quantilum Recombinant Luciferase, the 1× Glo Lysis buffer, and FuGENE HD Transfection Reagent were obtained from Promega (Madison, WI, USA). The NR1I2 (NM_003889) Human cDNA TrueClone (pCMV6-XL4 vector containing the cDNA of the PXR gene NR1I2) was obtained from OriGene (Rockville, MD, USA). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Bortezomib was purchased from Absource Diagnostics (München, Germany), and ixazomib and carfilzomib were obtained from Sequoia Research Products (Pangbourne, UK). Tris(hydroxymethyl)aminomethane (Tris) and acetic acid were purchased from Carl Roth (Karlsruhe, Germany).

1.2. Cell lines

Human colon adenocarcinoma LS180 cells (available at American Type Culture Collection [ATCC], Manassas, VA, USA) were used for CYP1A2 and CYP3A4 induction and PXR reporter gene assays. Cells were cultured under standard cell culture conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

AZ-AhR cells (human hepatoma HepG2 cells stably transfected with a construct containing several AhR (aryl hydrocarbon receptor) binding sites upstream of a luciferase reporter gene) [13] were used for the AhR reporter gene assay. Cells were kindly provided by Zdenek Dvorak (Palacky University, Olomouc, Czech Republic). Cells were cultured under standard cell culture conditions in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.2 mg/ml hygromycin (every second passage).

1.3. PXR reporter gene assay

A PXR reporter gene assay using the reporter plasmid pGL4.21-CYP3A4-Luc, the PXR expression vector, and the pGL4.74 [hRluc/TK] renilla vector was conducted as described previously [14] to investigate whether the proteasome inhibitors bortezomib, carfilzomib, and ixazomib (0.5–50 nM) activate PXR after 24 h of exposure. Rifampicin (0.1–20 μM) served as a positive control. For detection of the luciferase activity, the Dual-Glo Luciferase Assay System was applied according to the manufacturer's instructions. Compound-induced increases of PXR activity were calculated by division of firefly luminescence by renilla luminescence (transfection efficiency control) and normalized to the PXR activity of non-drug-treated controls set to 1 (=100%). Each assay was conducted three or four times.

1.4. AhR reporter gene assay

For the AhR reporter gene assay, AZ-AhR cells were treated for 24 h with TCDD (5 nM, positive control), bortezomib (0.5–75 nM), ixazomib (0.5–75 nM), carfilzomib (0.5–75 nM), or vehicle control. The assay was performed as described previously [15] with the Steady-Glo Luciferase Assay System according to the manufacturer's instructions. Drug-induced increases of AhR activity were normalized to activity of non-drug-treated controls. Each assay was conducted three times.

1.5. CYP3A4 and CYP1A2 mRNA induction assay

For the induction experiments, LS180 cells were seeded in culturing flasks and incubated for 3 days. Cells were then treated with medium containing either bortezomib (0.1–5 μM), carfilzomib (0.5–10 μM), or ixazomib (0.5–10 μM) in quadruplicate for 4 consecutive days. Rifampicin (20 μM) served as a positive control for PXR-driven genes, and culture medium served as a negative control. All incubation solutions were adjusted to 0.02% DMSO. After harvesting, RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit. Quality and concentrations were measured spectrophotometrically, and isolated RNA was stored at −80 °C until analysis. cDNA was synthesized with the RevertAid H Minus First Strand cDNA Synthesis Kit according to the manufacturer's instructions. mRNA expressions of *CYP3A4* and *CYP1A2* were quantified by RT–qPCR with a LightCycler 480 (Roche Applied Science, Mannheim, Germany). PCR amplification was carried out in a 20-μl reaction volume with the Absolute QPCR SYBR Green Mix. Primers for CYP3A4 and CYP1A2 were published previously [16,17]. Data were evaluated by calibrator-normalized relative quantification with efficiency correction using the LightCycler 480 software version 1.5 (Roche Applied Science) as published previously [18]. The most suitable reference gene for normalization was identified using geNorm version 3.4 (Center for Medical Genetics, Ghent, Belgium), which determines most stable reference genes from a set of tested genes in a given cDNA sample panel [19]. Among a panel of seven reference genes tested, glucose-6-phosphate dehydrogenase (*G6PDH*) was the most stable reference gene in LS180 cells under the treatment with bortezomib and carfilzomib and glucuronidase