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Human β -defensin-2 induces extracellular accumulation of adenosine in *Escherichia coli*.

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ABSTRACT

Human β -defensins are host-defense peptides performing antimicrobial as well as immunomodulatory functions. The present study investigated whether the effect of human β -defensin-2 treatment on *Escherichia coli* could generate extracellular molecules of relevance for immune regulation. Mass spectrometry analysis of bacterial supernatants detected accumulation of purine nucleosides triggered by β -defensin-2 treatment. Other cationic antimicrobial peptides tested presented variable outcomes regarding extracellular adenosine accumulation, being human β -defensin-2 the most efficient in inducing this response. Structural and biochemical evidence indicated that a mechanism other than plain lysis was involved in the observed phenomenon. Using isotope (^{13}C) labeling, extracellular adenosine was found to be derived from pre-existent RNA, and a direct interaction between the peptide and bacterial nucleic acid was documented for the first time for β -defensin-2. Taken together, the data suggest that defensin activity upon bacterial target may alter local levels of adenosine, a well-known immunomodulator influencing inflammatory processes.

Keywords: defensin/adenosine/ immunomodulation/ inflammation

INTRODUCTION

Human β -defensins (hBD) are cationic antimicrobial peptides produced predominantly by epithelial cells (1). Notably in the intestine, an environment populated by a dense and rich microbial community, epithelial defensins reinforce the host innate defense (2). These peptides are normally expressed at low basal levels, but hBD-2 and -3 can be upregulated by a variety of microbial and inflammatory stimuli (3, 4). In particular, altered hBD-2 expression has been reported to correlate with the development of intestinal inflammation (5, 6). On the other hand, intestinal inflammation has been associated with alterations in the microbiota, for example, shifts in the relative abundance of Proteobacteria such as *Escherichia coli* (7, 8). Elucidating how the interaction between hBD and its bacterial targets impact inflammatory processes can aid our understanding of the host-microbial networking in health and disease.

The most studied mechanism of action of cationic antimicrobial peptides involves membrane permeabilization with eventual cell lysis; however, other mechanisms and cellular targets are described for many peptides including human defensins (9-12). Moreover, alongside their antimicrobial activity, manifold immunomodulatory properties of defensins are reported, many of which describe direct effects upon immune cells and cytokine expression (13-15). In some cases, the interaction of peptides with bacterial components is implicated in their immunomodulatory effects (16, 17). In this context, the present study hypothesized if hBD-2 activity upon *E. coli* could result in the generation of extracellular mediators with relevance for immunomodulation.

MATERIALS AND METHODS

Chemicals. Lyophilized synthetic human beta-defensin-2, human beta-defensin-3 and human alpha-defensin-5 were purchased from Peptide Institute Inc., Sheep Myeloid Antimicrobial Peptide 29 (SMAP29) from AnaSpec Inc. and magainin I from Sigma-Aldrich. Adenosine (Ado), guanosine (Guo), cytidine (Ctd) and uridine (Urd), as well as their corresponding monophosphates and nitrogen bases were purchased from Sigma-Aldrich. Trimethylsilylation reagent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), 1 % trimethylchlorosilane (TMCS) was purchased from Supelco Analytical.

Culture conditions. *Escherichia coli* strain W (DSM 1116) was cultivated in mineral medium, herein referred as M4, composed of MgSO₄ 0.02 g/l, citric acid 0.2 g/l, K₂HPO₄ 1 g/l, NaNH₄HPO₄ 0.32 g/l and supplemented with 0.2 % glucose. Cultures were inoculated (160 μ l; six to eight replicates for each condition in all experiments) in 100-wells plates in

BioscreenC growth analysis system (Oy Growth Curves) and incubated at 37 °C using medium-amplitude shaking. O.D. measurements were taken every 15 min. At logarithmic phase (4 h) cultures were treated with 40 µl of aqueous solution of antimicrobial peptide. Untreated control cultures were handled in parallel in all experiments, by the addition of 40 µl of sterile water instead. Supernatants were filtered through 0.2 µm pore membrane for further analyses 2 h after treatment, except when indicated otherwise. Three replicate cultures from each condition were monitored for further 20 to 24 h to generate the corresponding growth curves.

Liquid chromatography-mass spectrometry (LC-MS). Bacterial supernatants were analyzed for the presence of defensin-induced compounds using a 6460 TripleQuad LC-MS system (Agilent Technologies, Santa Clara, CA, USA) with electro-spray ionization (ESI) coupled to a 1200 series LC. The samples were injected on reversed-phase C₁₈ column (50 mm x 2.1 mm ID; 1.8 µm particle size) and isocratically eluted with 95 % water, 5 % methanol, 0.1 % formic acid at a flow of 0.1 ml/min. Ionization was performed in the positive mode, with the following ion source parameters: N₂ flow 9 l/min and temperature 300 °C, nebulizer pressure 25 psi, sheath gas (N₂) flow 7 l/min and temperature 300 °C, capillary voltage 4000 V, charging voltage 1000 V and fragmentor voltage 135 V. Tandem-MS with collision-induced fragmentation was employed to determine compounds identity. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode, using commercial compounds as external standards. Specific parameters, expressed as [parental ion m/z (CE in V): quantifier fragment (m/z)/ qualifier fragment (m/z)] were as follows: Ado [268 (30): 136/119], Guo [152 (30): 135/110], Ctd [244 (30): 112/95], Urd [113 (30): 40/70], AMP [348 (30): 136/97], GMP [364 (30): 152/135], CMP [324 (50): 112/95], UMP [649 (10): 325/97], A [136 (30): 119/92], G [152 (30): 135/110], C [112 (30): 52/95], U [113 (30): 40/70]. In experiments where more than two compounds were simultaneously quantified, chromatographic separation was achieved using C₁₈ column (250 mm x 4.61 mm ID; 5 µm particle size) in 0.1% formic acid at 1 ml/min with water:methanol gradient as follows: 0 – 14 min 100:0, 20 °C; 14 – 28 min 90:10, 40 °C.

Viability tests and microscopy. In parallel to the analysis of cell-free supernatants, defensin-treated bacterial cultures were examined concerning viability and structural changes. For CFU counts cultures were serially diluted in sterile NaCl 0.9% solution and plated onto LB-agar plates. Determination of protein, DNA and ATP concentration was performed using the bicinchoninic acid (BCA) Protein Assay (Pierce Biotechnology), the Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen/Molecular Probes) and ATP BacTiter-Glo™

microbial cell viability assay (Promega), respectively. For fluorescence microscopy, cells were fixed 2 h after hBD-2 treatment with paraformaldehyde and glutaraldehyde (4% v/v), incubated in 10 mg/ml aqueous 4',6-diamidino-2-phenylindole (DAPI) for 5 min, washed twice with TE buffer and mounted onto glass slides for analysis under a Zeiss Photo microscope (365 nm excitation, 445/450 emission). Alternatively, fixed cells were processed for transmission electron microscopy (TEM), as described previously (18).

Isotopic labeling experiments. Isotopic labeling and determination of isotopic ratio were performed to elucidate the origin of defensin-induced extracellular compounds. Bacteria were labeled by the addition of 0.5 g/l [U-¹³C]-glucose (99 % ¹³C) (¹³C₆-Glu; Euriso-Top) to the culture medium. Total DNA and RNA were extracted and purified from cultures immediately before defensin treatment, using commercial kits (Qiagen) and subsequently degraded according to a standard enzymatic protocol (19). Products were lyophilized and incubated with trimethylsilylation reagent: pyridine (4:1) for 1 h at 100 °C for derivatization. Determination of ¹³C/¹²C ratios was performed by gas chromatography - isotopic ratio mass spectrometry (GC-IRMS) in GC IsoLink system connected via combustion interface to MAT 253 spectrometer (Thermo Scientific) as detailed elsewhere (20). Data were normalized following the calculations previously described (21).

Statistical analysis. Data obtained in three or more replicates were tested for statistically significant differences between means by one way analysis of variance (ANOVA), with $\alpha = 0.01$, using Holm-Sidak Test for pairwise comparison. The analyses were performed using SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL, USA).

RESULTS

Purine nucleosides accumulate extracellularly in E. coli after hBD-2 treatment. Supernatants sampled from hBD-2 treated *E. coli* cultures or untreated controls were comparatively analyzed by LC-MS, revealing two peaks present exclusively after hBD-2 treatment (Fig. 1A, #1 and #2). The first was composed of two main ions of m/z 136 and m/z 268, and the second of one major ion of m/z 152 (Fig. 1B). Fragmentation patterns generated by tandem-MS were used to identify the compounds in each peak. The ion of m/z 268 fragmented in a single ion of m/z 136. The fragment spectra for the ions m/z 136 and m/z 152 (Fig. 1C) were then compared to a web-based data repository (22) and to commercially available standards, and the compounds in peaks #1 and #2 were identified as the purine nucleosides adenosine (Ado) and guanosine (Guo), respectively.

Quantitative characterization (Fig. 2) showed that the treatment of logarithmic *E. coli* cultures with hBD-2 resulted in the presence of extracellular nucleosides at low- μM range, detected exclusively after peptide addition and not in untreated controls. The variation over time after hBD-2 treatment highlighted differences in the temporal dynamics, the accumulation of Ado being accentuated at longer time-points (Fig. 2A). The dose-response curve (Fig. 2B and 2C) demonstrated that below 10 $\mu\text{g/ml}$ hBD-2 was not able to induce accumulation of the compounds; towards higher doses, the concentration of both compounds increased. Ado was more abundant than Guo at intermediate doses (20 - 30 $\mu\text{g/ml}$). On the other hand, the response also varied according to cell density, the concentration of Ado being higher than Guo when cultures were treated at higher OD values (Fig. 2D and 2E). The growth-curves corresponding to the dose-response and the density-response experiments (Fig. 2C and 2E) corroborated the idea that Ado accumulation correlates to peptide/cell ratio rather than exclusively to growth-inhibition effect. Moreover, Ado is a well-known signaling molecule with a plethora of biological activities in human cells, including important immunomodulatory properties (23). Therefore, we chose to focus in this compound for further investigation.

Different antimicrobial peptides have contrasting abilities to induce extracellular adenosine in E. coli. In addition to hBD-2, two other human defensins were tested (human β -defensin-3 and human α -defensin-5) and two non-human peptides (SMAP and magainin I) were also analyzed as a comparison. At the dose of 20 $\mu\text{g/ml}$, all peptides were equally able to arrest bacterial growth in liquid cultures (data not shown). In contrast, the extracellular concentration of Ado generated by the bacteria after treatment was remarkably different for each peptide, being hBD-2 the most efficient to elicit this response (Table 1). In another comparative approach, the accumulation of extracellular Ado in response to hBD-2 was evaluated in a different strain, the probiotic *E. coli* Nissle 1917, using identical conditions as specified for *E. coli* strain W. The treatment of the probiotic strain with hBD-2 (20 $\mu\text{g/ml}$) also resulted in the presence of extracellular Ado ($0.632 \pm 0.009 \mu\text{M}$) 2 h after peptide addition

Adenosine accumulation did not result from plain cell lysis. Next, bacterial viability and membrane integrity were investigated in the defensin-treated cultures, to evaluate the occurrence of peptide-mediated lysis. Bacterial growth was arrested after treatment with hBD-2 at 20 $\mu\text{g/ml}$ (Fig. 3A); accordingly, metabolic activity was also immediately impaired and CFU counts showed that the cultures were no longer viable shortly after addition of hBD-2 (Fig. 3B). The release of intracellular molecules into the medium was examined as an

indication of lysis (Fig. 3C). Like untreated controls, hBD-2 did not result in the release of ATP, DNA, nor proteins. Fluorescence microscopy confirmed that intracellular material was kept within discrete cells in hBD-2-treated bacteria, in contrast to cultures subjected to freeze-thaw lysis. Importantly, extracellular Ado levels were not significantly increased following lysis protocol. These results indicated that although cellular processes were severely affected by hBD-2, intracellular contents were not indiscriminately released in the extracellular medium.

Structural evidence was obtained by transmission electron microscopy (TEM). Untreated *E. coli* cells (Fig. 3D, left column) showed intact membranes and cytoplasm organization and the presence of small areas of plasmolysis was observed only in few cells (8%). In contrast, in hBD-2-treated cultures (Fig. 3D, right column) 82% of cells presented extensive plasmolysis, with large periplasmic space. Intracellular contents were retained, except for 8% of visually detected lysed cells. Our observations indicate that plain bacterial lysis was not solely responsible for Ado release after hBD-2 treatment.

Extracellular adenosine derived from pre-existent bacterial RNA. Isotopic labeling experiments were performed to investigate whether extracellular Ado in hBD-treated *E. coli* cultures originated from pre-existent nucleic acids. Cultures grown either in ^{12}C - or ^{13}C -Glu containing medium were treated with hBD-2 (20 $\mu\text{g/ml}$). The isotopic ratio of extracellular Ado was compared to that of nucleic acids extracted immediately before treatment and was found to be in the same range as the pre-existent RNA in labeled cultures (Fig. 4A). Targeted tandem-MS (Fig. 4B) demonstrated that ribonucleotides and pyrimidine nucleosides were also present in the supernatants of treated cultures, supporting the conclusion that extracellular Ado derived from bacterial RNA. In contrast, deoxyribose compounds were not detected. Interestingly, addition of total RNA purified from *E. coli* increased Ado concentration detected after hBD-2 treatment (Fig. 4C). This was observed exclusively in cell-free supernatants from hBD-treated cultures and was negligible in the corresponding harvested cells, indicating the involvement of an extracellular bacterial factor. Electrophoretic analysis suggested a direct interaction between the peptide and RNA, however not sufficient to cause accumulation of Ado in sterile medium alone.

DISCUSSION

The key question in this study - whether human defensin triggers an immunomodulatory response from enteric bacteria - was approached by searching bacterial metabolites released from *E. coli* into the extracellular medium after hBD-2 treatment. Our results demonstrated that hBD-2 induces a selective accumulation of purine nucleosides, including Ado which is an important and well-studied immunomodulator, validating our initial hypothesis. The experimental setup was outlined considering the effective concentrations reported for hBD-2, ranging from 10 to 50 $\mu\text{g/ml}$ (24-26). In a physiologic context, the precise local concentrations of peptide at the site of defensin action are not known, and it is reported that most of the antimicrobial activity secreted by the intestinal mucosa is retained in the mucus layer, which contributes to an uneven distribution and variable local concentrations (27). In human fecal samples, concentrations of $> 50 \text{ ng/mL}$ were found, estimated to be a dilution reflecting even higher concentrations present in the mucosa (28).

The possible effects of Ado release in the context of the human body, particularly of the intestinal mucosa, are manifold, depending on which of the four known Ado receptors are being stimulated in the host (29). Micromolar concentrations found in our *in vitro* studies are in the range of basal levels of Ado in human tissues ($< 1 \mu\text{M}$), and consistent with EC_{50} values reported for A_1 and A_3 receptors ($0.3 \mu\text{M}$) and $\text{A}_{2\text{A}}$ receptor ($0.7 \mu\text{M}$) (30). $\text{A}_{2\text{A}}$ receptor was shown to be implicated in the anti-inflammatory activity of Ado (31) On the other hand, the predominant receptor-type expressed in intestinal epithelial cells, $\text{A}_{2\text{B}}$, has a lower potency ($\text{EC}_{50} 24 \mu\text{M}$) and can trigger pro-inflammatory signals (30, 32, 33). The final *in vivo* outcome of the phenomenon reported here is difficult to predict. Nevertheless, exploring localized production of Ado at the site of contact with the microbiota represents a valuable asset to the therapeutic manipulation of Ado signaling, which is a promising approach for the treatment of inflammatory disorders, although often limited by the risk of side-effects due to the broadness of Ado activities (23).

Our study demonstrated that the ability to induce Ado accumulation in *E. coli* is not a general feature of different antimicrobial peptides, nor is it exclusive for β -sheet molecules. Secondary structure of antimicrobial peptides is important for membrane-targeted effects but interactions with other cellular targets and alternative mechanisms of action are found across different structural classes (34). The most efficient peptide to induce accumulation of Ado was hBD-2. This peptide is produced by epithelial cells in the intestinal mucosa, being

upregulated in inflamed tissue (5). Furthermore, the probiotic *E. coli* Nissle 1917, tested under identical conditions, responded to hBD-2 by accumulating extracellular Ado at even higher levels. Taken together, these data are consistent with a possible therapeutic relevance for this interaction in the inflamed gut. Further studies including other Gram-negative and also Gram-positive bacteria would be interesting to investigate the species-specificity of the response. Nevertheless, our observations with *E. coli* Nissle 1917 are noteworthy, as this probiotic strain has been successfully employed in the treatment of intestinal inflammatory disorders and interestingly, its ability to induce hBD-2 production by intestinal epithelial cells was reported as a potential beneficial effect for patients (35-37).

Regarding the possible mechanisms leading to increased concentrations of extracellular Ado, our results implicate a distinctive non-lytic kind of membrane damage, resulting in extensive plasmolysis. To our knowledge, such effect was not yet reported for hBD-2, but retraction of cytoplasmic membrane after sublethal treatment with porcine β -defensin was described in *Salmonella typhimurium* (38). Studies in model vesicles revealed that hBD-2 caused release of a small marker, but not of molecules of 3,000 Da or more, and it is unlikely to arrange in channel forming oligomers (39). This model supports the conclusion that small metabolites could be released from *E. coli* following hBD-2 treatment independently of a lysis event. Moreover, stress-induced plasmolysis is known to cause selective release of periplasmic enzymes from *E. coli*, including a nucleotidase activity able to convert AMP into Ado (40). In this sense, periplasmic nucleotidase could be the bacterial factor contributing to Ado accumulation in defensin-treated *E. coli*, and the response would be influenced by membrane architecture and subcellular distribution of the enzyme.

In addition to membrane-targeted effects, it is possible that other hBD-2 activities are involved in the generation of extracellular Ado. Our data indicated that Ado accumulation was favored at lower peptide/cell ratios, and we speculate that these conditions would promote plasmolysis and avoid general lysis. It has been reported that sublethal concentrations of antimicrobial peptides can cause selective responses from bacteria, not necessarily involved in their killing-mechanism (41), and the use of peptide concentrations close to the lethal dose may favor the identification of alternative mechanisms and targets (42). Such mechanisms, already described for some cationic peptides, include direct binding and/or degradation of target nucleic acids (11, 43, 44), being in agreement with our observations. While the nature of hBD-2-RNA interaction is not yet clear, this work represents to our knowledge the first suggestion of a nucleic acid-targeted activity for hBD-2.

Conclusion. The central finding of this study - the identification of adenosine as an extracellular bacterial metabolite following defensin treatment - represents a so far undisclosed link between known players in intestinal inflammation: epithelial antimicrobials and Ado signaling. While the immunomodulatory properties of Ado are well-studied in diverse inflammatory conditions, the view of indigenous bacteria as a direct source of Ado has only recently started to be appreciated. In conclusion, besides contributing to the understanding of host-microbial interactions, this work offers a new perspective to the mechanism of action of human β -defensins, to be considered beyond bacteriocidal effects, as a component of the inter-kingdom signaling network.

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Table

Table 1 – Concentration of adenosine in *E. coli* supernatants in response to different antimicrobial peptides.

Peptide	Description	Extracellular Ado (μM) ¹
human β -defensin -2	epithelial β -sheet defensin	0.426 \pm 0.013
human β -defensin -3	epithelial β -sheet defensin	0.127 \pm 0.013
human α -defensin -5	Paneth cell β -sheet defensin	0.023 \pm 0.002
sheep myeloid antimicrobial peptide (SMAP-29)	mammalian α -helical cathelicidin	0.265 \pm 0.006
magainin I	frog skin α -helical defensin	\leq 0.015

¹ Mean \pm SD of three biological replicates, analyzed 2 h after peptide addition (20 $\mu\text{g}/\text{ml}$).

Figures

Fig 1

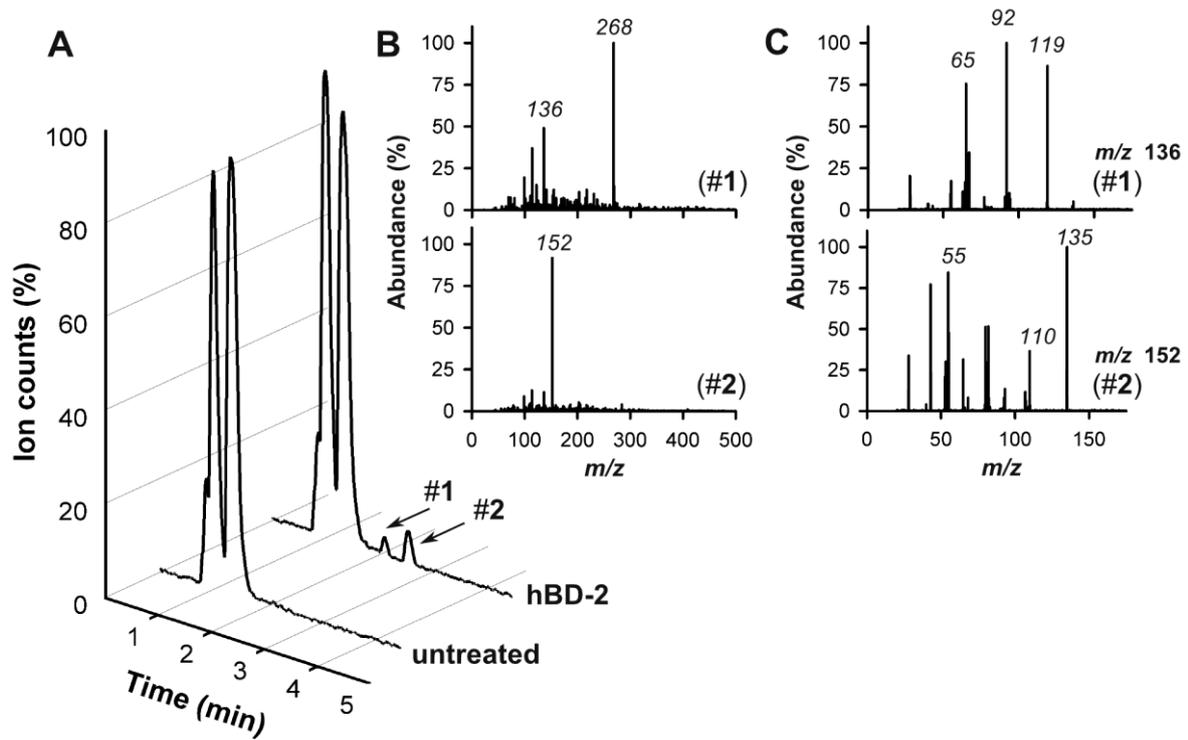


FIG 1 – hBD-2 induces extracellular accumulation of purine nucleosides. LC-MS analysis of supernatants from *E. coli* cultures untreated or treated with hBD-2 (20 $\mu\text{g/ml}$). A: Ion base peak chromatogram. B: Characterization of differential peaks (#1 and #2) by mass spectra in scan mode. C: Fragmentation pattern of target ions by tandem-MS at collision energy 50 V.

Fig 2

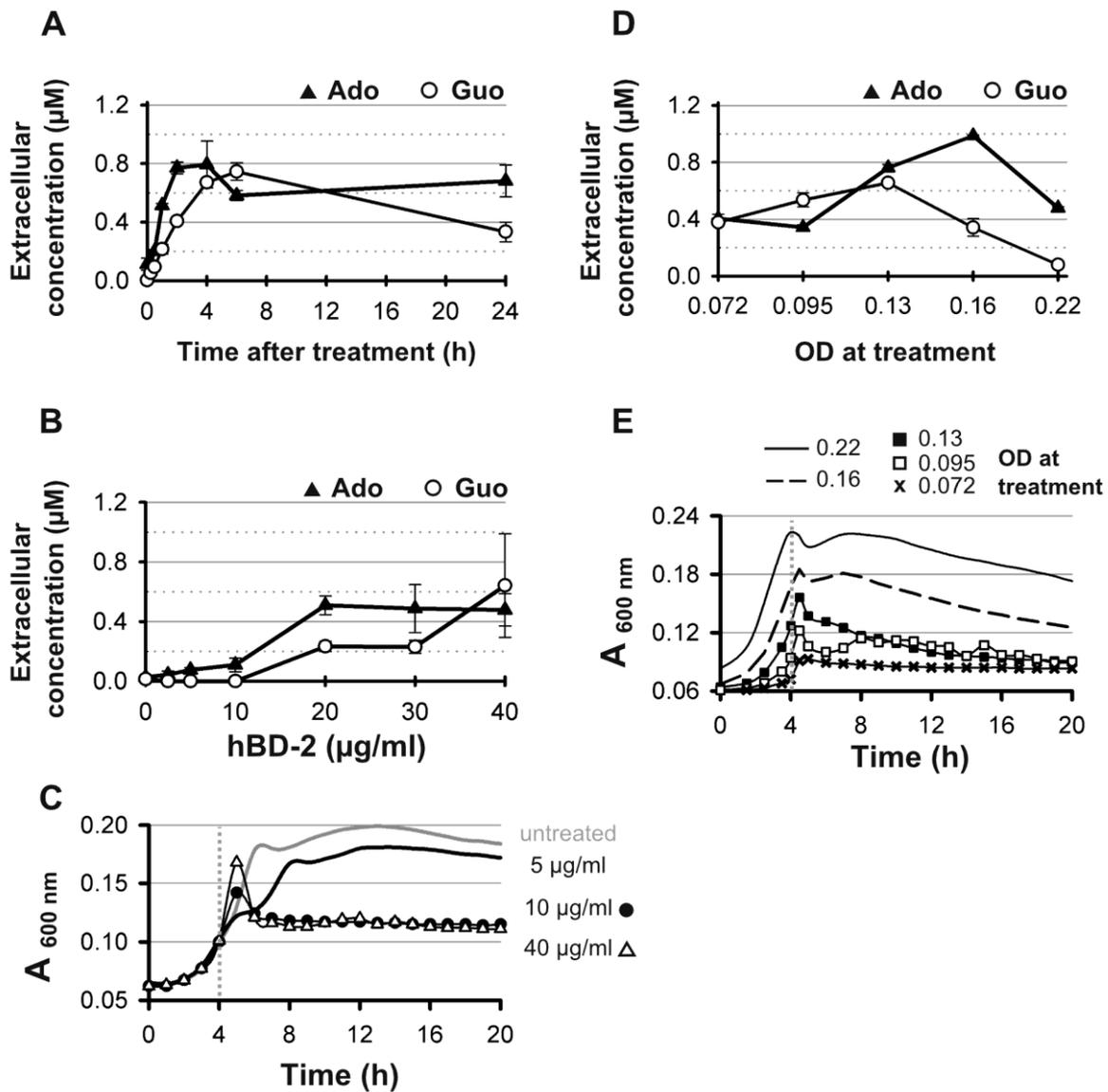


FIG 2 – Quantitative characterization of extracellular nucleoside accumulation in response to hBD-2. Samples were analyzed by tandem-MS (quantitative MRM mode). Mean \pm SD from three biological replicates. A: Variation in the extracellular concentration of Ado and Guo in *E. coli* cultures over time after treatment with hBD-2 (20 μ g/ml). B: Extracellular concentration of Ado, and Guo in *E. coli* cultures 2 h after treatment with different doses of hBD-2. C: Representative growth curves corresponding to cultures analyzed in the experiment described in B. D: Extracellular concentration of Ado and Guo in *E. coli* cultures of increasing cell densities, 2 h after treatment with hBD-2 (20 μ g/ml). E: Representative growth curves corresponding to cultures analyzed in the experiment described in D. Broken vertical lines in C and E indicate treatment point.

Fig 3

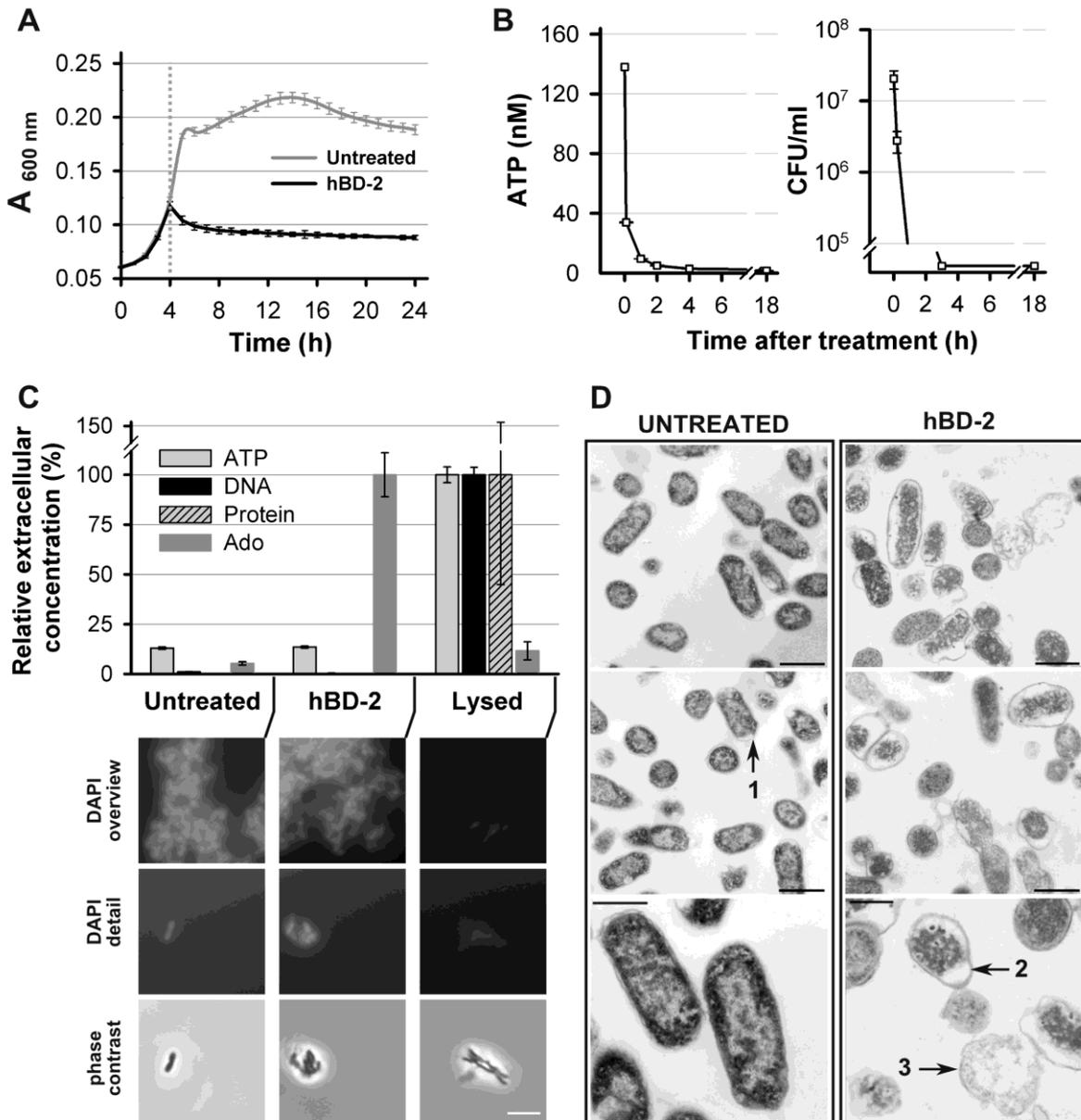


FIG 3 – hBD-2 did not cause indiscriminate lysis. **A**: Growth curve of *E. coli* untreated or treated with hBD-2 (20 $\mu\text{g/ml}$). Broken vertical line indicates treatment point. Mean \pm SD from three biological replicates. **B**: Viability of *E. coli* cultures after treated with hBD-2 (20 $\mu\text{g/ml}$). Left chart: ATP-dependent metabolic activity. Right chart: Viability in agar plates. Mean \pm SD for four determinations. **C**: Assay comparing *E. coli* cultures untreated, treated with hBD-2 (20 $\mu\text{g/ml}$) or lysed by conventional freeze-thaw protocol, regarding the presence of marker molecules in the supernatants (bar chart) and imaging of intracellular material (DAPI) with reciprocal phase contrast micrographs. Samples were collected 2 h after treatment (or immediately for ATP measurements). Mean \pm SD for four determinations. Scale bar: 2.5 μm . **D**: Transmission electron microscopy comparing untreated cultures and hBD-2 (20 $\mu\text{g/ml}$) treated cultures. Arrows indicate: 1) mild plasmolysis; 2) severe plasmolysis with enlarged periplasmic space; 3) lysed bacteria. Scale bars: 1 μm (upper and middle images) or 0.5 μm (bottom images).

Fig 4

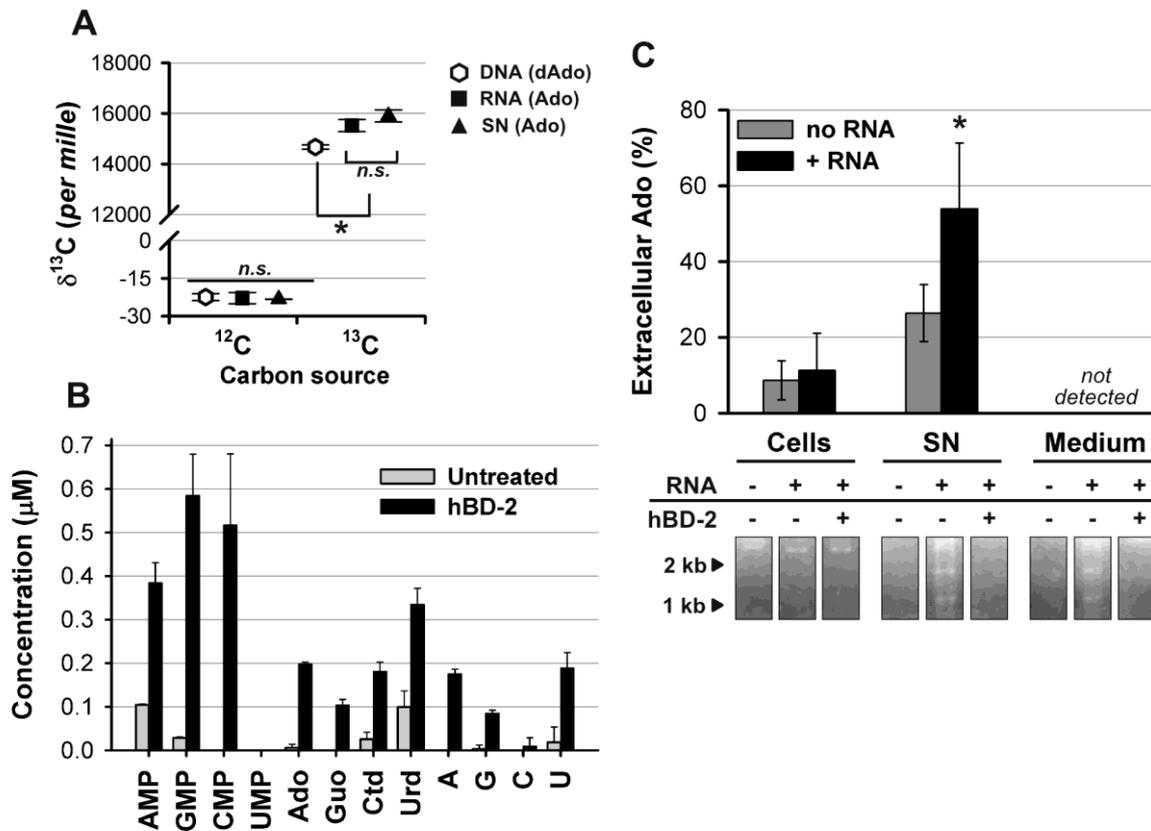


FIG 4 – Extracellular Ado derived from pre-existent RNA. **A:** Isotopic (^{13}C) enrichment in adenine nucleosides in total DNA and RNA and in extracellular Ado 2 h after hBD-2 treatment. *E. coli* was grown either in unlabeled or in [^{13}C]Glu-containing M4 medium and treated with hBD-2 (20 $\mu\text{g}/\text{ml}$). Mean \pm SD for three replicates. **B:** Extracellular concentration of different ribonucleosides and related compounds, released by *E. coli* untreated or treated with hBD-2 (20 $\mu\text{g}/\text{ml}$). Compounds were quantified by tandem-MS 2 h after treatment. Mean \pm SD for triplicate measurements from three biological replicates. **C:** Influence of extracellularly added RNA in Ado accumulation. Cells and supernatants from *E. coli* cultures treated with hBD-2 (20 $\mu\text{g}/\text{ml}$) were separated immediately after peptide addition. Harvested cells resuspended in fresh medium (Cells), cell-free supernatants (SN) and sterile medium were further incubated at 37 $^{\circ}\text{C}$ for 2 h in the absence or presence of total RNA purified from *E. coli* (1 $\mu\text{g}/\text{ml}$). Bar chart shows the relative concentration of extracellular Ado generated in each condition. Values are expressed relative to extracellular Ado levels from *E. coli* cultures after the same hBD-2 treatment. Mean \pm SD for five biological replicates. Bottom images show electrophoretic profile of samples in 2 % agarose gel stained with EtBr. * $p < 0.001$; n.s.: not significant.