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2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine is an anti-inflammatory TLR-2, -4 and -5 response mediator in human monocytes

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Abstract

Objective and design—To elucidate the influence of 2-amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP), a canonical Wnt/ β -catenin pathway activator, on the inflammatory response of TLR-engaged innate cells in vitro.

Material or subject—Primary human monocytes.

Treatment—AMPMB (0–10 μ M), LPS (0–1.0 μ g/ml), Pam3CSK4, FSL-1, or *S. typhimurium* flagellin (0–0.25 μ g/ml).

Methods—TLR-induced cytokine release (TNF, IL-6, IL-12 p40) was monitored by ELISA while Wnt-related signals (GSK3 β , p65, I κ B, β -catenin) were assessed by Western blot, pharmaceutical inhibition and gene silencing.

Results—AMBMP induced the rapid phosphorylation of NF κ B p65 at Ser⁵³⁶ and abrogated total I κ B, accompanied by a subsequent increase in pro-inflammatory cytokine production (TNF, IL-6, IL-12 p40) in otherwise naive monocytes. However, in TLR2, -4 and -5-engaged monocytes, AMBMP-suppressed cytokine production. In the context of LPS stimulation, this occurred concomitant with the phosphorylative inactivation of GSK3 β at Ser⁹, β -catenin accumulation and abrogation of NF κ B p65 phosphorylation. AMBMP-mediated suppression of the TLR4 - induced inflammatory response was reversed by two pharmaceutical Wnt/ β -catenin pathway inhibitors, IWP-2 and PNU-74654 and by Wnt3a silencing.

Conclusions—Herein, we show that AMBMP induces canonical Wnt signaling events and acts as a suppressor of inflammation in surface TLR-engaged primary human monocytes.

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Compliance with ethical standards

Conflict of interests The author(s) declare(s) that they have no competing interests.

Keywords

2-amino-4-(3,4-(methylenedioxy) benzylamino)-6-(3-methoxyphenyl)pyrimidine; β -catenin; Cytokines; Inflammation; GSK3 β ; TLR; Wnt

Introduction

There is a major need for the generation of novel pharmaceuticals for the treatment of acute and chronic inflammatory conditions. Of great interest are the multiple, potentially convergent, endogenous pathways that serve to limit inflammation and promote its resolution, thereby protecting the host from collateral damage. Such innate braking systems include the $\alpha 7$ acetylcholine receptor-dependent cholinergic-; glycogen synthase kinase 3 β - (GSK3 β); serum and glucocorticoid-regulated kinase 1-(SGK1); and Wnt-anti-inflammatory signaling axes [2–6].

2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBMP) is a cell permeable small molecule that is an established canonical Wnt/ β -catenin activator [7–9]. AMBMP is under investigation as a potential treatment for, e.g., ischemic/reperfusion injury, hemorrhagic shock and liver transplantation [10–12]. Wnt proteins are secreted, cysteine-rich, palmitoylated glycoproteins that are best known as regulators of embryogenesis, cell proliferation and migration but recent data have established critical roles for Wnt signaling in the control of inflammation [10, 11, 13–15]. There are at least three Wnt pathways. Activation of the canonical Wnt/ β -catenin pathway has previously been shown to result in phosphorylation and, therefore, inactivation of GSK3 β , thus influencing β -catenin and NF- κ B-dependent gene transcription [13, 16, 17]. More specifically, β -catenin accumulation is known to suppress bacterial-induced inflammation, at least in part, due to physical interactions with NF- κ B, in a similar manner to I κ B [18]. In a second, alternative Wnt cascade, activation of phospholipase C (PLC) leads to Ca⁺⁺ influx, PKC activation and NFAT- and NF- κ B-mediated transcription events [13, 17, 19, 20]. Thirdly, in the Wnt/planar cell (PCP) pathway, small G-proteins, such as Rac1 and RhoA, are activated and, subsequently, MAPK/Jnk1/2, which results in AP-1-driven transcriptional activity [13, 21, 22].

Both pro- and anti-inflammatory characteristics have been assigned to Wnt proteins [13]. For example, Wnt5a-Fzd5 interaction augments pro-inflammatory cytokine production in human macrophages cells exposed to *Mycobacterium tuberculosis* or the classic TLR4 agonist, LPS, in an autocrine manner [13, 23], while the Fzd1 ligand, Wnt3a, induces β -catenin and suppresses pro-inflammatory cytokine production in *M. tuberculosis* or LPS-stimulated epithelial cells [13, 24]. Schaale et al. [13] point out that Wnt proteins may not signal independently of each other and propose Wnt5a and Wnt3a as prototypic Wnt proteins that represent mediators and suppressors of the innate response, respectively. They further suggest that Wnt crosstalk may be important in the maintenance of homeostasis [13].

Here we report that stimulation with AMBMP alone induces a pro-inflammatory response in naïve primary human monocytes, but in innate cells exposed to agonists of cell surface TLRs, AMBMP suppresses TLR-induced inflammation, a phenomenon likely mediated, at

least in TLR4-engaged cells, via canonical Wnt signaling events involving modulation of GSK3 β , β -catenin, and NF- κ B activities.

Materials and methods

Materials

Monocyte isolation kits came from Miltenyi Biotec (Auburn, CA). RPMI Complete (RPMI 1640 medium supplemented with 10 % FBS, 50 μ M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 U/ml penicillin, and 50 μ g/ml streptomycin) was purchased from Invitrogen Life Technologies (Carlsbad, CA). Ultrapure LPS from *E. coli* 0111:B4 and other human TLR agonists were purchased from InvivoGen (San Diego, CA). 2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine was bought from Calbiochem (San Diego, CA). All antibodies came from Cell Signaling Technology (Danvers, MA). Enhanced chemiluminescence kits were purchased from Thermo Scientific (Rockford, IL). Cytokine ELISA kits (TNF, IL-6 and IL-12/23 p40) were purchased from eBioscience (San Diego, CA). The Wnt pathway inhibitors, *N*-(6-methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-*d*]pyrimidin-2-yl)thio]-acetamide (IWP-2) and benzoic acid, 2-phenoxy-,2-[(5-methyl-2-furanyl)methylene]hydrazide (PNU-74654), came from Tocris Biosciences (Minneapolis, MN). The GSK3 inhibitor, SB216763, was bought from Sigma-Aldrich (St. Louis, MO). Human Monocytes Nucleofector kit, including pCMV-GFP, came from Amaxa AG (Köln, Germany), while SMARTpool siRNA came from Dharmacon (Lafayette, CO).

Preparation of human monocytes

Primary human monocytes were purified from anonymized, citrated whole blood by indirect magnetic monocyte isolation, as we have previously reported [25, 26] and as approved by the University of Louisville Institutional Review Board (529.06). This procedure routinely results in >95 % pure CD14⁺ cells, as shown by flow cytometry. Human monocytes were cultured at 37 °C and 5 % CO₂ atmosphere in complete RPMI plus or minus stimulating agents, as described below.

Cytokine release by human monocytes in the context of TLR4

Primary human monocytes were stimulated or not with AMBMP (0–100 μ M) and/or LPS (0.1 mg/ml), as described in the figure legends. Organic solvent controls (0.01 % DMSO) were included, as appropriate. Cell-free supernatants were harvested at 20 h and assayed for cytokine levels by ELISA, according to the manufacturer's instructions.

TLR specificity of AMBMP-mediated inflammatory suppression

Human monocytes were stimulated with and without AMBMP (10 μ M) and with or without human surface exposed TLR-specific agonists (Pam3CSK4 [TLR2/1]; FSL-1 [Pam2CDGDPKHPKSF, TLR2/6]; and *Salmonella typhimurium* flagellin [TLR5]), at the concentrations noted in the figure legends. Organic solvent controls were included, as appropriate. TNF and IL-6 concentrations were determined in 20 h cell-free supernatants by ELISA.

Expression of canonical-Wnt signaling and GSK-3 β anti-inflammatory pathway molecules

Levels of total β -catenin, total I κ B, Ser⁵³⁶ phosphorylated NF- κ B p65, Ser⁹ phosphorylated GSK3 β , as well as β -actin, were determined by Western blot using whole-cell lysates (20 μ g protein). Images were visualized by enhanced chemiluminescence and acquired using ImageQuant LAS 4010 (GE Healthcare Life Sciences, Pittsburg, PA). Expression levels are presented as mean intensity ratios relative to β -actin.

siRNA and pharmacological inhibition of Wnt3a and GSK3 β

Transfection of human monocytes was carried out by electroporation using a Nucleofector device (Amaxa) according to the manufacturer's protocols. Briefly, 4×10^6 primary monocytes were re-suspended in 100 μ l of Nucleofector solution together with 2 μ g of a green fluorescent protein (GFP)-coding plasmid (pCMV-GFP) and 2 μ g of Wnt3a-specific ON-TARGET SMARTpool siRNA. Immediately after electroporation, 400 μ l of pre-warmed M-199 containing 10 % FCS was added to cells which were then transferred into culture plates containing pre-warmed M-199 with 10 % FCS. At 48 h post-transfection, cells were exposed to LPS for 24 h and the supernatants were harvested for cytokine analysis by ELISA. The levels of total Wnt3a were assessed by Western blot at 48 h post-transfection. The contribution of the canonical Wnt pathway to the inhibition of TLR4-induced cytokine induction in human monocytes was further explored through the use of two pharmaceutical Wnt signal inhibitors, IWP-2 (100 nM) and PNU-74654 (1 μ M), cells being pre-treated for 2 h prior to LPS (1 μ g/ml) stimulation. A 0.01 % DMSO organic solvent control was also employed. Cell-free supernatants were assayed for cytokine levels by ELISA 20 h after the addition of LPS.

Statistical analyses

Statistical significance between groups was evaluated by the analysis of variance and the Turkey multiple comparison test using the InStat program (GraphPad, San Diego, CA). Differences between groups were considered significant at the level of $p < 0.05$.

Results

AMBMP augments p65 phosphorylation, abrogates I κ B and induces pro-inflammatory cytokines in naïve monocytes

As shown in Fig. 1a–c, low doses (0.1 and 1 μ M) of AMBMP induced the release of the pro-inflammatory cytokines TNF, IL-6, and IL-12/23 p40 from naïve primary human monocytes. However, higher concentrations of AMBMP were not pro-inflammatory (data not shown). As shown in Fig. 1d–g, AMBMP stimulation rapidly increased phosphorylation of NF κ B sub-unit p65, at residue Ser⁵³⁶ while abrogating the total I κ B signal. Levels of β -catenin in naïve innate cells were not influenced by stimulation with AMBMP alone (data not shown). Thus, we have established for the first time that AMPMB can be pro-inflammatory in primary innate cells that have not been microbe-associated molecular pattern (MAMP)-primed and that AMBMP leads to release of multiple inflammatory cytokines, likely by facilitating the translocation of active p65 to the nucleus.

AMBMP chronically suppresses pro-inflammatory cytokine production in TLR-primed monocytes

In contrast to naïve cells, AMBMP suppressed the concentrations of TNF, IL-6, and IL-12/23 p40 detected in 20 h cell-free supernatants of LPS-stimulated monocytes (see Fig. 2). Furthermore, this inhibition was prolonged and occurred at multiple time points (2, 4, 8, 12, 16, and 24 h), as presented in Fig. 3. The ability of AMBMP to suppress TNF and IL-6 secretion upon engagement of surface TLR-specific cognizant ligands was also determined and, indeed, AMBMP (10.0 μ M) also suppressed TNF secretion in TLR2/1-, TLR2/6- and TLR5-engaged innate cells, as shown in Fig. 4.

AMBMP enhances TLR4-induced Ser⁹ phosphorylation of GSK3 β and augments TLR4-induced β -catenin accumulation and abrogates TLR4-mediated activation of NF- κ B p65

As presented in Fig. 5a and b, LPS alone mildly stimulated the phosphorylation, and thus inactivation, of GSK3 β at Ser⁹. However, AMBMP further enhanced the GSK3 β Ser⁹ phosphorylation signal in LPS-stimulated innate cells. As presented in Fig. 5c and d, LPS-stimulated the accumulation of β -catenin in monocytes over 150 min. Again, AMBMP further enhanced the β -catenin signal in LPS-stimulated innate cells. As presented in Fig. 5e and f, LPS induced the rapid phosphorylation of the transcriptionally active NF κ B sub-unit, p65, at residue Ser⁵³⁶. However, LPS-induced p65 activation was, essentially, abrogated by AMBMP. Thus, while LPS-stimulation alone results in the accumulation of small amounts of β -catenin (Fig. 5c, d), this is insufficient to suppress TLR-mediated pro-inflammatory events that result, in balance, in p65 activation (Fig. 5e, f), I κ B degradation (Supplemental Fig. 1) and the induction of pro-inflammatory cytokine release (Fig. 2). Furthermore, monocytes pre-treated with SB216763 exhibited reduced phosphorylation of the GSK3-specific substrate, glycogen synthase, at S640/641 (Fig. 5g) concomitant with suppression of the release of multiple pro-inflammatory cytokines (TNF, IL-12 p40 and IL-6) in LPS-stimulated cells (Fig. 5h). Thus, AMBMP augments TLR-4-induced GSK3 β inactivation and β -catenin accumulation but abrogates NF κ B p65 phosphorylation.

Wnt pathway inhibitors and Wnt3a knockdown rescue TLR4-mediated pro-inflammatory cytokine induction

Constitutively active GSK3 β contributes to the induction of NF- κ B transcription and, thus, is critical for the development of a robust inflammatory response to TLR engagement [25]. β -catenin accumulation is known to suppress bacterial-induced inflammation, at least in part, due to physical interactions with NF- κ B, akin to I κ B [18]. AMBMP, therefore, may suppress pro-inflammatory cytokine production in LPS-stimulated monocytes via both the inactivation of GSK3 β and the consequential accumulation of β -catenin. As AMBMP is a Wnt-related β -catenin agonist and IWP-2 blocks Wnt-dependent phosphorylation of Dvl2 proteins and, thus, inhibits β -catenin accumulation, we examined if Wnt inhibition influenced AMBMP activity. As shown in Fig. 6a–c, the use of IWP-2 rescued the AMBMP-suppressed pro-inflammatory response of monocytes to LPS. PNU-74654 binds to β -catenin and, subsequently, prevents β -catenin-transcription factor interactions. Likewise, the use of PNU-74654 also rescued the AMBMP-suppressed pro-inflammatory response of

monocytes to LPS Fig. 6a–c. Successful siRNA Wnt3a gene silencing (Fig. 6d, e) also enhanced LPS-induced proinflammatory cytokine production (Fig. 6f).

Discussion

In LPS-stimulated innate cells, the production of multiple pro-inflammatory cytokines (TNF, IL-6 and IL-12 p40) is simultaneously suppressed by AMBMP. Suppression of TNF and IL-6 release in response to the TLR2/1, TLR2/6 and TLR5 agonists, Pam3CSK4 (a synthetic diacylated lipoprotein), FSL (a synthetic triacylated lipoprotein) and *S. typhimurium* flagellin, respectively, was also noted. We have previously shown such a pan-TLR phenomenon for cotinine, the primary metabolite of nicotine, an $\alpha 7$ acetylcholine receptor ligand and potent GSK3 β -related cholinergic anti-inflammatory pathway activator [3, 27].

Several lines of evidence show that the canonical Wnt signaling pathway is critical for the suppression of host-MAMP cytokine induction and AMBMP is a small molecule inhibitor activator of the Wnt/ β -catenin signaling axis. Concomitant with AMBMP-induced cytokine suppression is the differential expression of several integral components of the canonical pathway. That is, AMBMP augments the LPS-induced phosphorylation of GSK3 β at residue Ser⁹, thus inactivating this constitutively active kinase; AMBMP augments the LPS-induced accumulation of β -catenin; and AMBMP abolishes the Ser⁵³⁶ phosphorylated p65 signal, which would be expected to abrogate p65 nuclear translocation and the associated activation of multiple pro-inflammatory genes. Furthermore, we present the novel finding that two molecules that inhibit different critical signaling events within the canonical Wnt pathway, each rescue the inflammatory response to TLR4 engagement. IWP-2 targets Wnt-dependent phosphorylation of LRP6 and Dvl2 and, thus, inhibits β -catenin accumulation. PNU-74654 binds to β -catenin and, subsequently, prevents β -catenin-transcription factor interactions. Interestingly, while there are 19 known human Wnt proteins, silencing of Wnt3a efficiently rescues AMBMP-induced immune suppression, suggestive of a potential AMBMP-induced Wnt3a-mediated autocrine feedback loop.

Lee et al. have previously shown that LPS induces GSK3 β phosphorylation and β -catenin accumulation in the RAW264.7 macrophage cell line and that β -catenin-specific siRNA transfection results in the down-regulation of LPS-induced IL-6 production [28]. Others have shown that β -catenin signaling in murine intestinal dendritic cells is required for the expression of the anti-inflammatory mediators, IL-10 and TGF- β [29]. Neumann et al. reported that, in *M. tuberculosis*-infected murine macrophages, there is a TLR-dependent induction of Fzd1 and that Wnt agonism suppresses the TNF response to *M. tuberculosis* [24]. These data have established that β -catenin acts as a negative regulator of the inflammatory response. Our data establish that the Wnt-related agonist, AMBMP acts in a similar manner, and provides initial insight into the anti-inflammatory potential of AMBMP in MAMP-stimulated primary human innate cells.

The Wnt/ β -catenin signaling axis has been shown to be involved in the development of degenerative diseases, including sclerosis [30, 31] and inflammatory conditions, not limited to keloids [32], inflammatory bowel disease [33] and asthma [28]. Therefore, the context-

specific immune regulation by AMBMP may have relevance even beyond the manipulation of host-pathogen interactions. Of particular interest, however, remains the therapeutic potential of AMBMP as differential regulator of inflammatory cytokine production. Clearly, appropriate toxicological screening will be required prior to any trial of AMBMP—or functionally analogous compounds—while the use of AMBMP may be inappropriate for some populations, particularly developing children and pregnant women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Michael Martin, a leading light in oral and systemic inflammation research, sadly passed away on February 22nd 2011, aged 37 years [1]. Mike initiated this project. The paper is dedicated to his family—Jayme, Jack and Clara. This work was supported by the US Department of Health and Human Services (National Institute for Dental and Craniofacial Research grants DE023633 (HW) DE017921 (RJL) and DE017680 (DAS).

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Fig. 1. AMBMP stimulation induces pro-inflammatory cytokines, augments phosphop65 and suppresses total IκB. AMBMP was added to human monocytes and **a** TNF, **b** IL-6, and **c** IL-12/23 p40 measured in 20 h cell-free supernatants by ELISA. Data represent the mean $\pm$ SD of three separate experiments. *** $p < 0.001$. AMBMP led to **(d)** a rapid increase in the p65 (Ser⁵³⁶) signal and **(e)** a reduction in total IκB, measured by Western blot in cell lysates (20 μg protein). The densitometric ratio of phospho-65 and IκB to β-actin is shown in **e** and **f**, respectively. Typical data are presented

Fig. 2. AMBMP suppresses TLR-4 induced pro-inflammatory cytokine production. AMBMP (10.0 μ M) was added to LPS-stimulated (100 ng/ml) human monocytes and **a** TNF, **b** IL-6, and **c** IL-12/23 p40 measured in 20 h cell-free supernatants by ELISA. Data represent the mean $\pm$ SD of three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to cells stimulated with LPS only

Fig. 3. Kinetics of AMBMP suppression of TLR-4 induced pro-inflammatory cytokine production. AMBMP (10.0 μ M) was added to LPS-stimulated (100 ng/ml) human monocytes and cytokine release measured in cell-free supernatants at multiple time points (2, 4, 8, 12, 16, and 24 h) by ELISA. Data represent the mean $\pm$ SD of three separate experiments

Fig. 4.

AMBMP suppression of TLR-2/1, -2/6 and -5-induced pro-inflammatory cytokine production. AMBMP (10.0 μ M) was added to TLR agonist [Pam3CSK4; FSL-1; or *S. typhimurium* flagellin (all, 0.25 μ g/ml)] stimulated human monocytes and cytokine release measured in cell-free supernatants at 20 h by ELISA. Data represent the mean $\pm$ SD of three separate experiments. * p < 0.05, ** p < 0.01 compared to solvent controls. Other than the TNF signal under AMBMP and flagellin co-stimulation, all conditions exhibited significantly higher cytokine release compared to the unstimulated controls (p < 0.05). AMBMP also suppressed pro-inflammatory cytokine production in innate cells stimulated with TLR3 (Poly(I:C) and Poly(I:C) LMW, 1 μ g/ml) and TLR7 (Imiquimod, 1 μ g/ml) agonists (*data not shown*)

Fig. 5.

AMBMP augments TLR-4-induced GSK3 β inactivation and β -catenin accumulation but abrogates NF κ B p65 phosphorylation. Purified human monocytes were treated with LPS (100 ng/ml) in the presence and absence of AMBMP (10.0 μ M) or pre-treated with GSK3 β inhibitor, SB216763 (25 μ M) for 2 h and then stimulated with LPS (100 ng/ml). Levels of (a) phosphorylated GSK3 β (Ser⁹), (c) total β -catenin and phosphorylated p65 (Ser⁵³⁶) (e) were determined in cell lysates (20 μ g protein) by Western blot. Total levels of β -actin were also monitored to ensure equivalent protein loading of cell lysates. The densitometric ratios of phospho-GSK3 β , total- β -catenin and phospho-p65 to β -actin are shown in b, d and f, respectively. g Monocytes pre-treated with SB216763 exhibited reduced phosphorylation of the GSK3 specific substrate, glycogen synthase (GS) (S640/641). Typical data are presented (a–g). h GSK3 inhibition significantly decreased the production of TNF, IL-12, IL-6 in LPS stimulated cells. Data represent the mean $\pm$ SD of three separate experiments. *** p < 0.001, compared to LPS-stimulated controls

Fig. 6.

Blockade of the canonical Wnt pathway rescues the inflammatory response to LPS. Purified human monocytes were pre-treated with two different pharmacological inhibitors of the canonical Wnt pathway, IWP-2 (100 nM) and PNU-74654 (1 μ M), for 2 h or transfected with Wnt3a siRNA and then stimulated with LPS (100 ng/ml). Cell-free supernatants were collected after 24 h and the production of **a** TNF, **b** IL-6 and **c** IL-12 p40 analyzed by ELISA. siRNA-mediated knockdown of Wnt3a protein levels (**d**) enhanced the production of IL-12, TNF, and IL-6 in LPS-stimulated cells (**f**). The densitometric ratios of Wnt3a to β -actin (**e**) were assessed to ensure the equivalent loading of cell lysates. Data represent the mean $\pm$ SD of three separate experiments. * p < 0.05, *** p < 0.001, compared to LPS stimulated cells