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Letters

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Facilitating Cytokine-Mediated Cancer Cell Death by Proteobacterial *N*-Acylhomoserine Lactones

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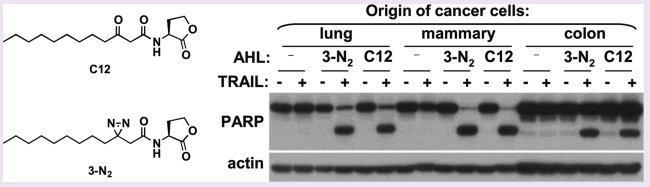
Supporting Information

ABSTRACT: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) preferentially induces apoptosis in cancer cells over normal cells; however, tumor cells may develop TRAIL resistance. Here, we demonstrate that this resistance can be overcome in the presence of bacterial acylhomoserine lactones (AHLs) or AHL-producing bacteria through the combined effect of TRAIL-induced apoptosis and AHL-mediated inhibition of inflammation regulated by NF-κB signaling. This discovery unveils a previously unrecognized symbiotic link between bacteria and host immunosurveillance.



Bacterial metabolites play important roles in inflammation-mediated processes essential for normal development and the pathogenesis of numerous chronic diseases, including cancer.[1,2] Inflammation is typically initiated as an innate immune response to specific bacterial products through receptor-dependent mechanisms, in which induction of the transcription factor NF-κB is required for both activation of the immune system[3] and the control of apoptosis in activated cells.[4−8] For example, in the presence of Gram-negative bacteria, NF-κB activation is initially induced in response to bacterial lipopolysaccharide (LPS), an agonist of the Toll-like receptor 4 (TLR4),[9] leading to the expression of NF-κB-regulated genes encoding pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF) and interleukin-1 (IL-1). After the engagement of TNF or IL-1 receptors, additional rounds of NF-κB activation amplify this LPS-induced inflammatory response.[3,10] NF-κB-dependent processes, in concert with other signaling pathways, up-regulate the expression of pro-apoptotic cancer immunosurveillance effectors,[2,11,12] including the TNF-related apoptosis-inducing ligand (TRAIL), an essential mediator of apoptotic cell death particularly in cancer cells.[13,14] Although the LPS-induced inflammatory response results in the release of pro-apoptotic cytokines such as TNF and TRAIL, cancer cells receiving these death signals can still survive due to the suppressive effects of NF-κB signaling on apoptosis.[5−8,12]

The fine balance between inflammation-induced pro- and antiapoptotic processes is critically dependent on the dynamics of NF-κB signaling, which is autoregulated by the inhibitor of NF-κB (IκB) alpha (IκBα) protein.[3,15] Observations that the bacterial *N*-(3-oxododecanoyl-L-homoserine lactone (C12), a prototypic member of the *N*-acylhomoserine lactone (AHL) family (Figure 1), impairs NF-κB signaling induced by pro-inflammatory stimuli such as LPS and TNF[16] prompted us to ask whether this class of bacterial metabolites could influence the pro-apoptotic activity of death-inducing cytokines. Thus, we examined the Gram-negative bacteria *Pseudomonas aeruginosa*, an opportunistic pathogen that is only able to promote infection in hosts with defective immune system functions.[17] As C12 is a signaling molecule produced in abundance by this bacteria, we examined whether wild-type *P. aeruginosa* or a mutant strain lacking *lasI*, the gene responsible for the synthesis of C12,[17] could render lung cancer cells susceptible to TNF- or TRAIL-induced cleavage of poly(ADP-ribose) polymerase (PARP), an indicative characteristic of apoptosis.[18] Excitingly, PARP cleavage was only detected when cells received a combination of TRAIL and wild-type bacteria (Figure 1b), suggesting that C12 was required for TRAIL-induced apoptosis in cancer cells. Notably, similar results were observed when other AHL-producing bacteria were added to cytokine-stimulated cells, while bacteria that do not possess AHL synthases had no effect (Supplementary Figure 1). Furthermore, titration experiments confirmed that the direct addition of C12 or several naturally occurring AHL analogues resulted in a strong pro-apoptotic response to TNF or TRAIL, and in agreement with our bacterial experiments, the cells were more sensitive to TRAIL (Figure 1c and Supplementary Figure 2).

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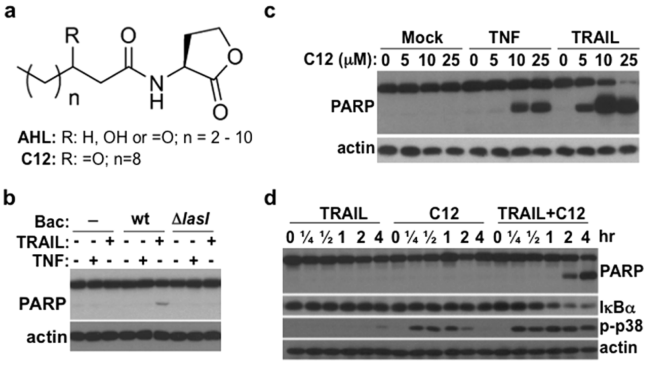

Figure 1. C12-producing bacteria or C12-promoted cytokine-mediated apoptosis in cancer cells. (a) Chemical structures of AHLs examined in this study. (b) Lung cancer cells were incubated with or without *P. aeruginosa* (Bac) wild-type (wt) or a *lasI* mutant strain (Δ*lasI*) in the presence or absence of TNF or TRAIL as indicated. After 2 h, cell lysates were prepared and analyzed by immunoblotting with antibodies specific for PARP or actin as a loading control. (c) Lung cells were untreated (Mock) or treated with TNF or TRAIL in the presence of the indicated doses of C12 for 6 h; cell samples were analyzed as in panel b. (d) Comparison of lung cell responsiveness to TRAIL, C12, or their combination. Western blot analysis of PARP, IκBα, the phosphorylated form of p38 (p-p38), and actin in cellular extracts prepared after treatment with stimuli as indicated.

Since activation of NF-κB signaling inhibits apoptosis, the observed difference in the pro-apoptotic effects of TNF and TRAIL might be linked to the distinct ability of these cytokines to modulate NF-κB activity.[13] Consistent with this interpretation, Western blot analysis for the degradation and resynthesis of IκBα, an indicative biochemical marker of NF-κB signaling,[3] revealed a robust activation of NF-κB signaling in response to TNF but not to TRAIL (Supplementary Figure 3). Although no modulation of NF-κB or apoptotic signaling was induced in response to TRAIL or C12, substantial changes in the levels of IκBα were matched with PARP cleavage in lung cancer cells stimulated with a combination of C12 and TRAIL (Figure 1d). Interestingly, we also observed that the combined action of TRAIL and C12 resulted in a prolonged activation of the mitogen-activated protein kinase (MAPK) p38 as determined by Western blot analysis for the phosphorylated form of p38 (Figure 1d; p-p38). These findings suggest that C12 enhances TRAIL's ability to execute apoptosis in cancer cells through modulation of NF-κB, p38, or both signaling processes.[2,12,19]

Despite the expression of TRAIL receptors, normal cells are resistant to TRAIL-induced apoptosis. Similar to nontransformed cells, many malignant cells are not sensitive or only partially sensitive to the pro-apoptotic action of TRAIL.[13,14] Therefore, in order to assess the selectivity of C12 as a modulator of TRAIL-dependent tumor immunosurveillance, we compared the sensitivity of several cancer cell lines and normal cells to TRAIL and C12. Consistent with our previous observations, substantial induction of PARP cleavage was observed in lung, colon, and breast cancer cells stimulated with a combination of C12 and TRAIL. In contrast, human hepatocytes from normal donors as well as other primary cells from normal tissues were resistant to the same treatment (Figure 2a,c). Importantly, longer treatment of cancer cells with TRAIL plus C12 significantly decreased their viability although no effect on the survival of normal cells was noted (Figure 2b,d).

While these data demonstrate a therapeutic potential of C12 as an enhancer of TRAIL-dependent anticancer activity, questions concerning C12-mediated pro-apoptotic effects on immune cells, such as primary macrophages,[20−22] still needed to be addressed. Considering the potential inherent pharmacokinetic liabilities associated with C12, a small series of C12 analogues was tested against bone marrow-derived macrophages and TRAIL-treated lung cancer cells. From these studies, an AHL lacking the 3-oxo moiety was found to be completely inactive in both assays (Supplementary Figure 4a−c); however, a 3-diazirine-containing derivative of C12 (3-N_2; see the Supporting Information for structure, synthesis, and characterization data) was found to be nontoxic to macrophages (Supplementary Figure 4d), yet exhibited toxicity

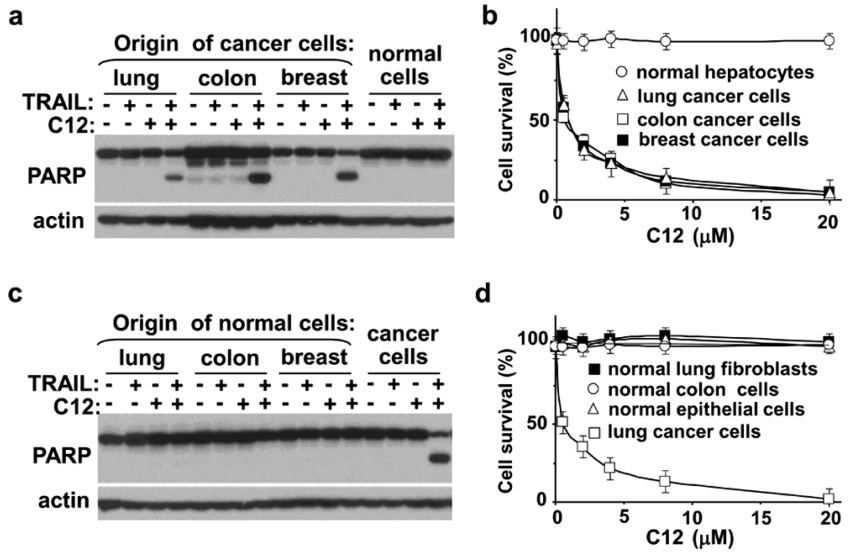

Figure 2. C12 promotes the TRAIL-mediated killing of cancer cells. (a,c) Western blot analysis of PARP cleavage in cancer or normal cells treated for 3 h with TRAIL, C12, or a combination of both, as indicated. (b,d) XTT-based assay monitoring the viability of cancer and normal cells grown for 18 h in media containing TRAIL and the indicated doses of C12. Cell survival was ∼100% in control samples (untreated cells) as well as in samples incubated with TRAIL alone or the same doses of C12 without TRAIL.

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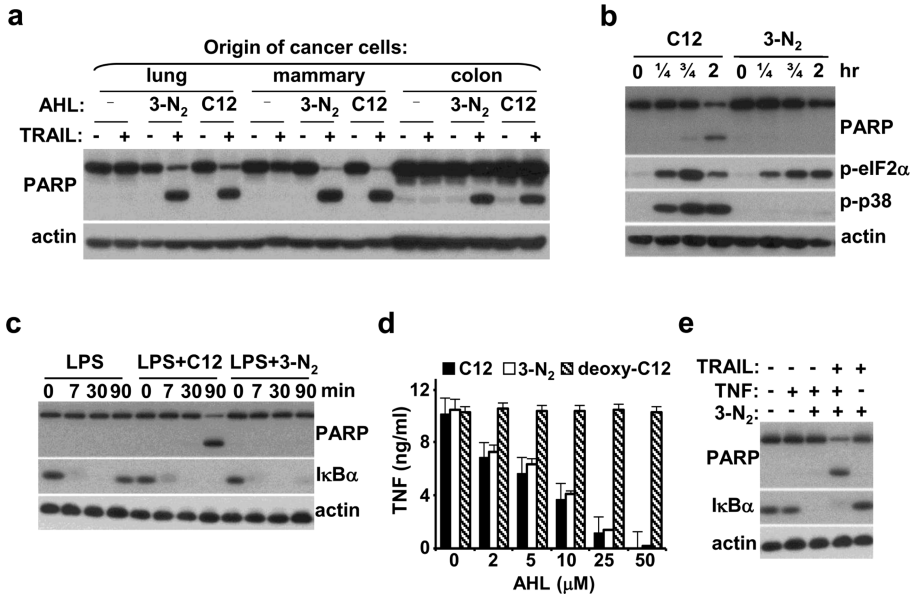

Figure 3. AHL-mediated inhibition of inflammation-induced NF-κB signaling is sufficient for rendering tumors susceptible to TRAIL-induced apoptosis. (a) Western blot analysis of PARP cleavage in cancer cells stimulated for 3 h with TRAIL or its combination with different AHLs as indicated. (b) Western blot analysis of PARP cleavage as well as phosphorylated forms of eIF2α and p38 in extracts from bone marrow-derived macrophages (BMDM) stimulated with C12 or 3-N_2 as indicated. (c) Western blot analysis of PARP cleavage and temporal profiles of IκBα expression in BMDM extracts prepared after treatment with LPS or its combination with C12 or 3-N_2. (d) Inhibitory effect of C12 and its derivatives on LPS-induced TNF production in BMDM. (e) Lung cancer cells were exposed to 3-N_2 (10 μM), TRAIL (0.5 ng/mL), TNF (20 ng/mL), or their combinations as indicated. After 2 h, cell lysates were prepared and analyzed by Western blot for PARP cleavage, IκBα, and actin.

against TRAIL-treated cancer cells in a manner similar to the parental molecule (Supplementary Figure 5). Moreover, we also observed a comparable effect of C12 and 3-N_2 on TRAIL-induced PARP cleavage in TRAIL resistant cancer cells (Figure 3a).

To better define the structure−activity relationship between AHL-mediated effects on the pro-apoptotic action of TRAIL and the agonistic potential of C12 or 3-N_2, the responses of macrophages to these compounds were examined by Western blot analysis for PARP cleavage, activation of p38,[22] and the phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), a distinct feature of mammalian cell activation in response to C12 and its 3-oxo-analogues.[21] A comparison of the agonistic activities of C12 and 3-N_2 revealed that both compounds induced eIF2α phosphorylation in a similar fashion; however, 3-N_2 did not induce p38 phosphorylation and PARP cleavage (Figure 3b and Supplementary Figure 6), suggesting that activation of the p38 pathway promotes AHL-induced apoptosis in addition to stimulating phagocytic activity, as previously disclosed.[22]

Besides its agonistic activities, C12 also inhibits inflammatory responses to TNF, LPS, and other TLR ligands in a wide variety of cell types.[16,23] In macrophages, the anti-inflammatory activity of C12 interferes with the inducible expression of NF-κB target genes, such as *IκBα* and *TNF*.[16] Our results also suggest that AHL-mediated disruption of IκBα-dependent NF-κB signaling renders cancer cells susceptible to TNF- and TRAIL-induced apoptosis (Supplementary Figure 7; see also Figure 1d). Therefore, to examine whether 3-N_2 affects stimulus-induced NF-κB signaling, we compared the dynamics of IκBα expression in macrophages activated by LPS or its combination with C12 or 3-N_2. Curiously, although the expected pro-apoptotic cleavage of PARP was observed in the presence of C12 but not 3-N_2, both compounds were equally effective in blocking LPS-induced resynthesis of IκBα (Figure 3c). Moreover, similarity between the anti-inflammatory activities of C12 and 3-N_2 were also evident from comparison of their inhibitory effects on LPS-induced production of TNF (Figure 3d). These experiments indicate that 3-N_2 is nontoxic to resting or inflammation-activated macrophages; however, it retains the ability of C12 to modulate LPS-induced NF-κB signaling.

TNF is a key inflammatory mediator responsible for LPS-induced tumor growth, and the growth-promoting activity of TNF is dependent on NF-κB activation.[24,25] Importantly, experiments using a mouse model of LPS-induced tumor growth suggest that inhibition of TNF-mediated NF-κB signaling in cancer cells converts inflammation-induced tumor growth to inflammation-induced tumor regression mediated by endogenous TRAIL.[12] To address whether an AHL is able to enhance the anticancer activity of TRAIL in inflammation-activated cancer cells, we examined the effect of a suboptimal concentration of TRAIL on PARP cleavage in cancer cells treated with a combination of TNF and 3-N_2. Western blot analysis revealed that TRAIL-dependent PARP cleavage was manifested in cancer cells stimulated with TNF in the presence of 3-N_2, and significantly, the TRAIL-mediated apoptotic response coincided with disruption of TNF-induced NF-κB signaling (Figure 3e).

In summary, the identification of C12 and its analogues as TRAIL-enhancers and the ability of these compounds to inhibit pro-inflammatory responses through modulation of NF-κB signaling provides a proof-of-principle application for the selective killing of cancer cells. Notably, the synergistic effects of 3-N_2 on TRAIL-induced apoptosis in cancer cells were comparable with those for an anticancer agent bortezomib; however, in contrast to bortezomib, 3-N_2 alone or in combination with TRAIL was nontoxic to human hepatocytes

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derived from tissues of normal donors (Supplementary Figure 8). A linkage of cancer and inflammation suggests a substantial benefit can be gained from using anti-inflammatory agents, such as inhibitors of NF-κB, in cancer therapy and prevention.[2] Moreover, our findings also highlight future thought provoking ideas extending host-bacterial relationships from traditional nutritional benefits[1] to cancer promotion or prevention.[2]

■ ASSOCIATED CONTENT

Supporting Information

General materials and methods, synthetic details and characterization data for 3-N_2, biochemical and cellular assay details, Supplementary Figures S1−S8. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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