Pseudoalteromonas haloplanktis TAC125 produces 4-hydroxybenzoic acid that induces pyroptosis in human A459 lung adenocarcinoma cells

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In order to exploit the rich reservoir of marine cold-adapted bacteria as a source of bioactive metabolites, ethyl acetate crude extracts of thirteen polar marine bacteria were tested for their antiproliferative activity on A549 lung epithelial cancer cells. The crude extract from Pseudoalteromonas haloplanktis TAC125 was the most active in inhibiting cell proliferation. Extensive bioassay-guided purification and mass spectrometric characterization allowed the identification of 4-hydroxybenzoic acid (4-HBA) as the molecule responsible for this bioactivity. We further demonstrate that 4-HBA inhibits A549 cancer cell proliferation with an IC₅₀ value ≤ 1 μg ml⁻¹, and that the effect is specific, since the other two HBA isomers (i.e. 2-HBA and 3-HBA) were unable to inhibit cell proliferation. The effect of 4-HBA is also selective since treatment of normal lung epithelial cells (WI-38) with 4-HBA did not affect cell viability. Finally, we show that 4-HBA is able to activate, at the gene and protein levels, a specific cell death signaling pathway named pyroptosis. Accordingly, the treatment of A549 cells with 4-HBA induces the transcription of (amongst others) caspase-1, IL1β, and IL18 encoding genes. Studies needed for the elucidation of mode of action of 4-HBA will be instrumental in depicting novel details of pyroptosis.

Lung cancer is an extremely important health concern that affects millions of people worldwide¹,², and any progress leading to improvement of cancer survival rates is a global priority. Patients with lung cancer generally have a poor prognosis with a 5-year survival¹. Traditional cancer chemotherapy has mainly been based on the use of highly cytotoxic drugs that non-specifically target all dividing cells and may therefore only result in a modest improvement in patients that become immunosuppressed as chemotherapeutics kill all proliferating cells including monocytes and lymphocytes. For this reason, a new trend in anticancer research has arisen focusing on the discovery of new natural drugs that induce specific programmed cell death mediated by immunogenic signals. A recently discovered form of immunogenic cell death is represented by pyroptosis. This pathway differs from that of apoptosis as it is uniquely mediated by caspase-1 (CASP1) activation, which in turn triggers the formation of an "inflammasome", a cytosolic complex with inflammatory features³ linked to interleukin 1β (IL1β) release for immune cell recruitment.

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Many of the anticancer drugs used in clinical practice today are natural products or derivatives thereof and the continued and systematic exploration of natural sources, such as marine microbiota, is expected to lead to the discovery of different and unforeseen compounds with interesting biological activities, including anticancer activity. Marine bacteria have proven to be a unique and promising source of biologically active natural products. The production of anticancer drugs by microorganisms can be advantageous in comparison to other natural sources, such as plants, due to i) the possibility of genetically engineering microbes and ii) their higher production rates.

Amongst marine bacteria, cold-adapted microorganisms represent an untapped reservoir of biodiversity endowed with an interesting chemical repertoire. It has been already shown that cold-adapted bacteria produce valuable bioactive secondary metabolites, such as anti-biofilm molecules, antimicrobials and compounds displaying various other pharmacologically-relevant activities. In this context, polar marine bacteria could likely be a potential source of new molecules with antiproliferative activity.

In the present study we screened ethyl acetate extracts of thirteen different cultivable cold-adapted bacteria on A549 cells, a lung adenocarcinoma cell line, which represents a suitable model for the study of Non Small Cancer Lung Cells having typical characteristics in terms of proliferation index and malignancy. We demonstrate that *Pseudoalteromonas haloplanktis* TAC125 is able to produce an antiproliferative agent. In particular, this bacterium produces 4-hydroxybenzoic acid that specifically activates pyroptosis in A549 cells without affecting viability in normal cells.

**Results**

**Screening for antiproliferative activity of polar bacteria ethyl acetate extracts, and production conditions optimization.** Ethyl acetate crude extracts of thirteen bacterial strains (Table S1) were tested for their antiproliferative activity using the MTT assay on lung adenocarcinoma A549 cells. The ethyl acetate extract of uninoculated GG medium was used as a negative control. A549 tumor cells were treated with different concentrations (1, 10 and 100 μg ml$^{-1}$) of the total extracts for 24 hours (data not shown) and 48 hours, and compared with cells treated with the negative control extract. The highest concentration tested (100 μg ml$^{-1}$) induced a decrease in the percentage of viable cells for most of the extracts (Figure S1). Interestingly, the crude extract of *P. haloplanktis* TAC125 inhibited cell viability in a dose dependent manner, with a calculated half Inhibition Concentration (IC$_{50}$) of about 30 μg ml$^{-1}$. Based on these results, we focused on *P. haloplanktis* TAC125 as a potential source of antiproliferative compounds.

To test whether the composition of growth medium affected the production of antiproliferative compound(s), the Antarctic bacterium was grown in different media: a rich medium (TYP); a synthetic medium (GG); and a medium containing alternatively L-glutamate or D-glucurate as source of carbon and nitrogen. Extract of *P. haloplanktis* TAC125 grown in GG medium displayed the highest antiproliferative activity on A549 cells (data not shown).

To increase production of antiproliferative compound(s), *P. haloplanktis* TAC125 was grown in GG medium in an automatic bioreactor. The cell extract of *P. haloplanktis* TAC125 grown in the bioreactor displayed an IC$_{50}$ value of about 1 μg ml$^{-1}$, indicating a clear enhancement in production yields under this growth condition (Figure S2).

**Bioactivity guided purification and identification of the antiproliferative compound from *Pseudoalteromonas haloplanktis* TAC125.** Pre-fractionation of the *P. haloplanktis* TAC125 extract was achieved on a reversed phase column, and fractions were tested by the MTT assay on A549 lung cancer cells (Fig. 1, panel A). Fractions 1 and 4 were shown to inhibit A549 cells in a dose dependent manner and both fractions displayed IC$_{50}$ values of about 10 μg ml$^{-1}$. Fraction 4 was discarded as it was also shown to exert a high cytotoxicity towards the non transformed lung epithelial WI-38 cells (Fig. 1, panel B).

The active fraction 1 was subjected to a further purification step and the resulting fractions were tested for antiproliferative activity with the MTT assay on A549 cells (Fig. 1, panel C). Fraction 1E was identified as the most active with an IC$_{50}$ value of about 0.8 μg ml$^{-1}$ and very interestingly this fraction displayed no toxic effect towards the WI-38 normal cell line (Fig. 1, panel B).

The active fraction 1E was analysed by Ultra-high performance liquid chromatography-diode array detection-high-resolution mass spectrometry (UHPLC-DAD-HRMS) with tandem MS/HRMS fragmentation that revealed a single compound which was tentatively identified as 4-hydroxybenzoic acid (4-HBA) from a search in a comprehensive in-house standard collection of microbial metabolites.

The identification of 4-HBA was verified by comparison of retention time, monoisotopic mass for the pseudomolecular ion [M-H]$^{-}$, MS/HRMS spectra (10 eV, 20 eV and 40 eV) from negative electrospray ionization (ESI) mode and UV spectrum with a commercially available standard (Fig. 2).

A comparable cytotoxic effect was displayed for the isolated pure compound (1E) and the commercial standard of 4-HBA when tested in the same experiment using the MTT assay on A549 cells (Figure S3). To investigate the structural features essential for this antiproliferative activity, the effect of the hydroxybenzoic acid structural isomers (2-, 3-, and 4-HBA) were analyzed. The results of the MTT assay on A549 cells indicated that 4-HBA was the most active isomer, with an IC$_{50}$ ≤ 1 μg ml$^{-1}$ (Figure S4).

**Mechanism of action for 4-hydroxybenzoic acid.** The specific molecular pathway activated by 4-HBA was defined through a PCR array experiment aimed to evaluate changes in expression of the main genes involved in the most common signaling pathways of cell death (Table 1). A549 cells were treated with 1 μg ml$^{-1}$ of 4-HBA (IC$_{50}$ concentration) and after 2 hours of incubation, cells were recovered and subjected to a real time qPCR analysis. Only two-fold difference in expression values with respect to the control (untreated cells) were used to identify up- and/or down-regulated genes, respectively (Table 1). We found that the expression of the following key genes involved in pyroptotic cell death signalling were all up-regulated including the Caspase Recruitment...
Domain-Containing Protein 5 (PYCARD), Bcl2 Modifying Factor (BMF), Caspase-1 (CASP1), Interleukin-1β (IL1β) and Interleukin-18 (IL18) (Fig. 3, panel A, Table 1).

Regarding other death cell signaling pathways\textsuperscript{19}, the observed up-regulation of the gene encoding X-linked inhibitor of apoptosis (XIAP) indicated that the 4-HBA treatment inhibited the canonical apoptotic pathway in A549 cells. Furthermore, microarray results highlighted that genes involved in the extrinsic apoptotic pathway were strongly down-regulated: CD40 ligand (CD40L), Fas ligand (FasL), Myelin associated glycoprotein (MCL1) and Tumor necrosis factor receptor superfamily, member 11b (TNFRS11B). As for the expression of key genes involved in the necrotic pathway, they were also down-regulated: Forkhead box I1 (FOX1), Olfactory receptor, family 10, subfamily J, member 3 (OR10J3), S100 calcium binding protein A7A (S100A7A) and Junctophilin 3 (JPH3). Finally, homeostatic and detoxification cell pathways such as autophagy were also strongly down-regulated.

Figure 1. Panel (A): Cell viability of lung adenocarcinoma cells (A549) treated for 48 hours with three different concentrations (1, 10 and 100 µg ml\(^{-1}\)) of 9 fractions of \textit{Pseudoalteromonas haloplanktis} TAC125 obtained on a C\(_{18}\) column by a Isolera One purification system. Panel (B): Cell viability of lung normal fibroblast cells (WI-38) treated for 48 hours with three different concentrations (1, 10 and 100 µg ml\(^{-1}\)) of three samples: Fraction 4 and Fraction 1 from fractionation on a C18 column by Isolera One system; 1E from following purification step by HPLC of the Fraction 1. Panel (C): Cell viability of lung adenocarcinoma cells (A549) treated for 48 hours with three different concentrations (1, 10 and 100 µg ml\(^{-1}\)) of 7 fractions (from 1A to 1G) of \textit{Pseudoalteromonas haloplanktis} TAC125 obtained from Fraction 1 on a semipreparative HPLC. In all the above experiments, red bar represents untreated cells (control). Three independent assays were performed in triplicate; viability data are shown as mean ± S.D.
down-regulated: Interferon gamma (IFNG), Estrogen receptor 1 (ESR1), Huntingtin (HTT), Insulin-like growth factor 1 (IGF1), Insulin (INS), Immunity-related GTPase family M (IRGM), Kv channel interacting protein 1 (KCNIP1), RAB25 member RAS oncogene family and Synaptonemal complex protein 2 (SYCP2). The genes involved in other death cell signaling pathways were not differently expressed in A549 cells treated with 4-HBA (Table 1).

Since caspase-1 plays a crucial role in the pyroptotic cell signaling pathway, its protein levels in A549 treated cells were analyzed by immunoblot (Figure S5). 4-HBA treatment was carried out under the same conditions used for PCR array analysis (1 µg ml⁻¹), but the cells were collected after 24 hours, since changes in protein levels were more evident at this time point. In Fig. 3, the increase in pro-caspase-1 levels (panel B) and appearance of the P20 caspase-1 protein cleavage fragment (panel C) can be seen only in treated samples, thus confirming that A549 cell death induced by 4-HBA treatment was due to caspase-1 activation.

Discussion

Marine organisms represent an attractive source of marine natural products in view of the high hit rates of marine molecules as antitumor and antibiotic drug leads. Especially, polar marine bacteria are an unexploited hoard of biodiversity equipped with an interesting chemical repertoire. In this study, we evaluated the antiproliferative activity on tumor cells of ethyl acetate crude extracts of cold-adapted bacteria belonging to the genera Pseudoalteromonas, Pseudomonas, Psychrobacter and Psychromonas. Even though nine out of the thirteen analysed strains belong to the same genus (Pseudoalteromonas), their recent genome-wide comparison attested a quite remarkable diversity, with a large proportion of unique genes. This analysis prompted us to use all thirteen strains in the initial screening. The P. haloplanktis TAC125 crude extract was shown to be the most active in inhibiting cell proliferation. The bioactivity guided purification scheme highlighted the presence of more than one fraction endowed with anti-proliferative activity when tested on A549 cells. The fraction that was non toxic on WI-38 cells was subjected to a further fractionation step. 4-Hydroxybenzoic acid (4-HBA) was identified as the single compound responsible for the observed antiproliferative activity. 4-HBA is a primary metabolite as it is one of the products of chorismate lyase, which converts chorismic acid into pyruvate and 4-HBA. The latter molecule is then addressed towards the ubiquinone biosynthetic pathway. It is interesting to note that another Pseudoalteromonas strain, Pseudoalteromonas flavipulchra, was reported to produce 4-HBA, to which an antimicrobial activity was assigned. In this paper, we demonstrate that 4-HBA inhibits A549 cancer cell proliferation with an IC₅₀ value ≤1 µg ml⁻¹. Although we have not currently identified the cellular target(s) of 4-HBA, we show that its effect is specific, since the other HBA isomers (i.e. 2-HBA and 3-HBA) were unable to induce any
| Unigene  | Refseq   | Symbol           | Description                                                                 | Fold Regulation | St. deviation |
|---------|----------|------------------|-----------------------------------------------------------------------------|----------------|---------------|
| Hs.249227 | NM_130463 | PYCARD           | Caspase Recruitment Domain-Containing Protein 5                             | 9.1022         | 0.009952      |
| Hs.591104 | NM_033503 | BMP              | Bcl2 modifying factor                                                        | 8.2385         | 0.009952      |
| Hs.2490   | NM_033292 | CASP1            | Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) | 13.0597        | 1.382987      |
| Hs.743398 | NM_213607 | IL1B             | Interleukin 1-beta                                                          | 15.6858        | 5.424528      |
| Hs.472860 | NM_001250 | IL18             | Interleukin-1-18                                                            | 3.523          | 3.141189      |
| Hs.32949  | NM_005218 | DEFB1            | Defensin, beta 1                                                            | 16.3404        | 4.718064      |
| Hs.569598 | NM_002647 | PIK3C3           | Phosphoinositide-3-kinase, class 3                                          | 2.0217         | 0.291856      |
| Hs.356076 | NM_001167 | XIAP             | X-linked inhibitor of apoptosis                                              | 2.0656         | 0.551179      |

**Genes up-regulated by 4-HBA treatment**

| Unigene  | Refseq   | Symbol           | Description                                                                 | Fold Regulation | St. deviation |
|---------|----------|------------------|-----------------------------------------------------------------------------|----------------|---------------|
| Hs.249227 | NM_130463 | PYCARD           | Caspase Recruitment Domain-Containing Protein 5                             | 9.1022         | 0.009952      |
| Hs.591104 | NM_033503 | BMP              | Bcl2 modifying factor                                                        | 8.2385         | 0.009952      |
| Hs.2490   | NM_033292 | CASP1            | Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) | 13.0597        | 1.382987      |
| Hs.743398 | NM_213607 | IL1B             | Interleukin 1-beta                                                          | 15.6858        | 5.424528      |
| Hs.472860 | NM_001250 | IL18             | Interleukin-1-18                                                            | 3.523          | 3.141189      |
| Hs.32949  | NM_005218 | DEFB1            | Defensin, beta 1                                                            | 16.3404        | 4.718064      |
| Hs.569598 | NM_002647 | PIK3C3           | Phosphoinositide-3-kinase, class 3                                          | 2.0217         | 0.291856      |
| Hs.356076 | NM_001167 | XIAP             | X-linked inhibitor of apoptosis                                              | 2.0656         | 0.551179      |

**Genes down-regulated by 4-HBA treatment**

| Unigene  | Refseq   | Symbol           | Description                                                                 | Fold Regulation | St. deviation |
|---------|----------|------------------|-----------------------------------------------------------------------------|----------------|---------------|
| Hs.249227 | NM_130463 | PYCARD           | Caspase Recruitment Domain-Containing Protein 5                             | 9.1022         | 0.009952      |
| Hs.591104 | NM_033503 | BMP              | Bcl2 modifying factor                                                        | 8.2385         | 0.009952      |
| Hs.2490   | NM_033292 | CASP1            | Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) | 13.0597        | 1.382987      |
| Hs.743398 | NM_213607 | IL1B             | Interleukin 1-beta                                                          | 15.6858        | 5.424528      |
| Hs.472860 | NM_001250 | IL18             | Interleukin-1-18                                                            | 3.523          | 3.141189      |
| Hs.32949  | NM_005218 | DEFB1            | Defensin, beta 1                                                            | 16.3404        | 4.718064      |
| Hs.569598 | NM_002647 | PIK3C3           | Phosphoinositide-3-kinase, class 3                                          | 2.0217         | 0.291856      |
| Hs.356076 | NM_001167 | XIAP             | X-linked inhibitor of apoptosis                                              | 2.0656         | 0.551179      |

**Genes not regulated by 4-HBA treatment**

| Unigene  | Refseq   | Symbol           | Description                                                                 | Fold Regulation | St. deviation |
|---------|----------|------------------|-----------------------------------------------------------------------------|----------------|---------------|
| Hs.249227 | NM_130463 | PYCARD           | Caspase Recruitment Domain-Containing Protein 5                             | 9.1022         | 0.009952      |
| Hs.591104 | NM_033503 | BMP              | Bcl2 modifying factor                                                        | 8.2385         | 0.009952      |
| Hs.2490   | NM_033292 | CASP1            | Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) | 13.0597        | 1.382987      |
| Hs.743398 | NM_213607 | IL1B             | Interleukin 1-beta                                                          | 15.6858        | 5.424528      |
| Hs.472860 | NM_001250 | IL18             | Interleukin-1-18                                                            | 3.523          | 3.141189      |
| Hs.32949  | NM_005218 | DEFB1            | Defensin, beta 1                                                            | 16.3404        | 4.718064      |
| Hs.569598 | NM_002647 | PIK3C3           | Phosphoinositide-3-kinase, class 3                                          | 2.0217         | 0.291856      |
| Hs.356076 | NM_001167 | XIAP             | X-linked inhibitor of apoptosis                                              | 2.0656         | 0.551179      |

Continued
Table 1. Transcriptional modulation of a subset of genes involved in human death cell signalling pathways in 4-HBA treated A549 cells. Gene transcription is considered unaffected by compound treatment if fold regulation is in the range ± 2.0.

| Unigene | RefSeq | Symbol | Description | Fold Regulation | St. deviation |
|---------|--------|--------|-------------|----------------|--------------|
| Hs.181301 | NM_004079 | CTSS | Cathepsin S | −1.5347 | 0.507273 |
| Hs.578973 | NM_015247 | CYLD | Cylindromatosis (turban tumor syndrome) | −1.157 | 0.156494 |
| Hs.654567 | NM_005848 | DENND4A | DENN/MADD domain containing 4A | −1.0189 | 0.299697 |
| Hs.484782 | NM_004401 | DFFA | DNA fragmentation factor, 45kDa, alpha polypeptide | −1.1597 | 0.394894 |
| Hs.100058 | NM_006426 | DPPSL4 | Dihydropyrimidinase-like 4 | −1.5649 | 1.078326 |
| Hs.158688 | NM_015904 | EIF5B | Eukaryotic translation initiation factor 5B | −1.4494 | 0.055674 |
| Hs.667309 | NM_000043 | FAS | Fas (TNF receptor superfamily, member 6) | 1.137 | 0.229523 |
| Hs.1437 | NM_000152 | GAA | Glucosidase, alpha; acid | 1.0603 | 0.682058 |
| Hs.80409 | NM_001924 | GADD45A | Growth arrest and DNA-damage-inducible, alpha | 1.1215 | 0.288813 |
| Hs.269027 | NM_014568 | GALNT5 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GaNAc-T5) | 1.8231 | 0.996225 |
| Hs.444356 | NM_002086 | GRB2 | Growth factor receptor-bound protein 2 | 1.3273 | 0.242409 |
| Hs.29169 | NM_024610 | HSPBAP1 | HSPB (heat shock 27 kDa) associated protein 1 | 1.3892 | 1.706257 |
| Hs.431420 | NM_000875 | IGF1R | Insulin-like growth factor 1 receptor | −1.1904 | 0.095898 |
| Hs.322273 | NM_181509 | MAP1L3A | Microtubule-associated protein 1 light chain 3 alpha | −1.0059 | 0.049965 |
| Hs.138211 | NM_002750 | MAPK8 | Mitogen-activated protein kinase 8 | 1.0652 | 0.595847 |
| Hs.632486 | NM_021960 | MCL1 | Myeloid cell leukemia sequence 1 (BCL2-related) | 1.1497 | 0.49134 |
| Hs.618430 | NM_003998 | NFKB1 | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | −1.0531 | 0.547016 |
| Hs.513667 | NM_003946 | NOL3 | Nucleolar protein 3 (apoptosis repressor with CARD domain) | −1.7116 | 0.206745 |
| Hs.177766 | NM_001618 | PARP1 | Poly (ADP-ribose) polymerase 1 | −1.0047 | 0.048371 |
| Hs.409412 | NM_005484 | PARP2 | Poly (ADP-ribose) polymerase 2 | 1.0899 | 0.160191 |
| Hs.171844 | NM_006505 | PVR | Poliovirus receptor | −1.0185 | 0.2427 |
| Hs.636442 | NM_003161 | RPS6KB1 | Ribosomal protein S6 kinase, 70 kDa, polypeptide 1 | 1.1352 | 0.310545 |
| Hs.21374 | NM_000345 | SNCA | Synuclein, alpha (non A4 component of amyloid precursor) | −1.7882 | 0.009952 |
| Hs.48513 | NM_006038 | SPATA2 | Spermatogenesis associated 2 | −1.3653 | 0.694708 |
| Hs.587290 | NM_003900 | SQSTM1 | Sequestosome 1 | −1.073 | 0.076573 |
| Hs.189782 | NM_018202 | TMEM57 | Transmembrane protein 57 | −1.0805 | 0.149693 |
| Hs.241570 | NM_000594 | TNF | Tumor necrosis factor | −1.7491 | 0.089952 |
| Hs.5191834 | NM_003844 | TNFRSF10A | Tumor necrosis factor receptor superfamily, member 10a | 1.2277 | 0.200162 |
| Hs.713833 | NM_001065 | TNFRSF1A | Tumor necrosis factor receptor superfamily, member 1A | −1.4144 | 0.047858 |
| Hs.347460 | NM_000546 | TP53 | Tumor protein p53 | −1.1421 | 0.259099 |
| Hs.525206 | NM_021138 | TRAF2 | TNF receptor-associated factor 2 | −1.4742 | 0.036574 |
| Hs.134406 | NM_017853 | TXN1L4B | Thioredoxin-like 4B | −1.2299 | 0.083412 |
| Hs.47061 | NM_003565 | ULK1 | Unc-51-like kinase 1 (C. elegans) | −1.7787 | 0.146981 |

We demonstrate that at gene and protein levels, 4-HBA is able to activate pyroptosis in A549 cells. Pyroptotic cell death was reported as a caspase-1-dependent cell death of macrophages when infected by Salmonella typhimurium4,12. In contrast to apoptosis, pyroptosis is a pro-inflammatory type of cell death due to the activation of caspase-1 leading to the formation of the inflammasome, resulting in the release of the pro-inflammatory cytokines IL-1β and IL-18. Our results show that treatment of A549 cancer cells with 4-HBA induces the transcription of (amongst others) caspase-1, IL1β, and IL18 encoding genes. Furthermore, a clear accumulation of caspase-1 and its activated form (pro-caspase-1) was observed at the protein level. Interestingly, the only death signaling pathway activated was pyroptosis, as demonstrated by the down-regulation of key genes involved in apoptosis, necrosis and autophagy. This clearly indicates that 4-HBA is specifically recognized by tumor cells that fall into the inflammasome cascade. At the morphological level, 4-HBA treated cells showed a typical membrane swelling ascribable to cell lysis occurring during pyroptosis. The observation that the viability of WI-38 was not affected by treatment with 4-HBA suggests that this microbial derived metabolite cannot be considered a bacterial virulence factor. On the other hand, its selective anti-proliferative activity against cancer cells (at least the tested in the present study) indicates a molecular target mainly present in transformed cells. To test if 4-HBA was also able to inhibit the proliferation of other transformed cell lines, colon rectal adenocarcinoma (HT29) cell line was
selected and subjected to treatment with all HBA chemical isomers (Figure S6). Again only 4-HBA was able to inhibit HT29 cell proliferation.

Data reported here disclose the role of 4-HBA as a novel inducer of pyroptosis. We forecast that 4-HBA will become a useful tool for elucidating still unknown molecular relationships between pyroptosis and cancer.

**Methods**

**Bacterial strains and culture conditions.** Each Antarctic bacteria strain (see Table S1) were grown aerobically in flasks at 15 °C in GG defined medium \(^{16}\) (10 g L\(^{-1}\) L-Glutamate, 10 g L\(^{-1}\) D-Gluconate, 1 g L\(^{-1}\) K\(_2\)HPO\(_4\), 10 g L\(^{-1}\) NaCl, 1 g L\(^{-1}\) NH\(_4\)NO\(_3\), 200 mg L\(^{-1}\) MgSO\(_4\)\(_7\)H\(_2\)O, 5 mg L\(^{-1}\) FeSO\(_4\)\(_7\)H\(_2\)O, 5 mg L\(^{-1}\) CaCl\(_2\)\(_2\)H\(_2\)O pH 7.5) or TYP medium \(^{17}\) (16 g L\(^{-1}\) yeast extract, 16 g L\(^{-1}\) bacto Tryptone, 10 g L\(^{-1}\) NaCl). At the end of the exponential phase cells and spent medium were harvested.

**Bacterial fermentation.** *Pseudoalteromonas haloplanktis* TAC125 growth was performed in a Stirrer Tank Reactor 7L Bioreactor Techfors S (INFORS HT) connected to an IRIS 5.0 software with a working volume of 7L. Fermentation was performed in 5L of GG defined medium. The bioreactor was equipped with the standard pH, pO\(_2\), and temperature sensor for bioprocess monitoring. The culture was carried out at 15 °C for 31 hours in aerobic conditions DOT (dissolved oxygen tension) \(\geq\) 20%, using an airflow of 1.5 L/hour, and a stirrer speed of 250 rpm.

**Total bacterial extracts preparation.** Cold-adapted bacterial cultures (cells and spent medium), previously frozen at –80 °C, without adding cryoprotectants, were thawed and stirred with an equal volume of ethyl acetate (Assay Percent Range \(\geq\) 99.5%) (Sigma-Aldrich) and mixed with 1% formic acid (Assay Percent Range = 90%) (JT Baker). Each solution was stirred at least for 30 min and then centrifuged at 3000 rpm for 30 min. The resulting two phases were separated and the organic phases were recovered and dried using a rotary evaporator, Rotavapor (Buchi R-210) at 40 °C. The resulting total organic extracts were dispensed and stored at –20 °C.

**Anticancer compound purification and identification.** *Sample purification.* The crude extract was pre-fractionated on a reversed phase C\(_18\) flash column (10 g, 15 ml) using an Isolera One automated flash system (BIOTAGE, Uppsala, Sweden). The gradient was 10% stepwise (15 column volumes) from 30–100% methanol.
(MeOH) buffered with 20 mM formic acid with a flow of 15 ml/min. Nine fractions were collected manually every 10% step. MeOH was of HPLC grade and water was purified and deionized using a Millipore system through a 0.22 µM membrane filter (Milli-Q water).

4-Hydroxybenzoic acid (fraction 1E) was purified from the Isolera fraction (fraction 1) on a Waters semi-preparative HPLC, with a Waters 600 Controller (Milford, MA, USA) coupled to a Waters 996 Photodiode Array Detector. Separation was achieved on a Luna II C18, 5 µm, 250 × 10 mm column (Phenomenex, Torrance, CA, USA) with a flow of 5 ml min⁻¹ using a linear gradient 5% MeCN in Milli-Q water with 50 ppm TFA going to 35% MeCN in 24 min, from 35–45% MeCN in 2 min, 45–100% MeCN in 2 min, kept for 5 min at 100% MeCN and down to the starting conditions in 2 min. MeCN was of HPLC grade.

4-HBA identification. The identification of 4-HBA was performed using ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-QTOFMS) with tandem HRMS fragmentation on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD and an Agilent 6550 iFunnel QTOF MS (as previously described18) and comparing results obtained with spectra acquired using the commercial standards.

Commercial standards. 4-hydroxybenzoic acid, 3-hydroxybenzoic acid and 2-hydroxybenzoic acid (salicylic acid) were purchased at Sigma-Aldrich (Steinheim, Germany).

Treatment of Human Cells. The adenocarcinoma human alveolar basal epithelial cell line A549 was purchased from the American Type Culture Collection (ATCC® CCL-185™) and grown in DMEM-F12 (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) of fetal bovine serum (FBS), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. The normal diploid human lung fibroblasts WI-38 were purchased from the American Type Culture Collection (ATCC® CCL-75™) and grown in MEM supplemented with 10% (v/v) of fetal bovine serum (FBS), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, 2 mM of L-glutamine and non-essential amino acids (NEAA, 2 mM). The human colorectal adenocarcinoma cell line HT29 was purchased from the American Type Culture Collection (ATCC® HTB38) and maintained in McCoy’s 5 A medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM of L-glutamine and 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. The medium was renewed every 3 days, and the cells were detached via trypsinization when they reached confluence. Before the experiments, cells were seeded in 96-well plates (2 × 10⁴ cells well⁻¹) and kept overnight for attachment. For viability assays, the extracts, fractions and pure compound(s) were dissolved in dimethyl sulfoxide at a final concentration of 1% (v/v) for each treatment.

MTT- Viability assay. The effect of extracts, fractions and pure compound(s) on cell viability were determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Applichem A2231). A549, HT29 and WI-38 cells, seeded in 96-well plates, after treatment times (24 and 48 hours), were treated with 10 µl (5 mg ml⁻¹) of MTT and incubated for 3 hours. After the incubation time, isopropanol was used to dissolve purple formazan crystals. The absorbance was recorded on a microplate reader at a wavelength of 570 nm (Multiskan FC, THERMO SCIENTIFIC). The effect on cell viability was evaluated as percent of cell viability calculated as the ratio between mean absorbance of each sample and mean absorbance of controls.

RT² profiler PCR-array analysis for cell death pathway identification. A549 (2 × 10⁶) cells used for RNA extraction and analysis, were seeded in Petri dishes (100 mm diameter) to obtain the control condition without any treatment and cells treated with the IC₅₀ concentration of the pure compound (1 µg ml⁻¹). After 2 hours of exposure time, A549 cells were washed directly in the Petri dish by adding cold Phosphate-Buffered Saline (PBS) and rocking gently.

Cells were lysed in the Petri dish by adding 1 ml of Trisure Reagent (Bioline, cat. BIO-38033) per 100 mm dish diameter. RNA was isolated according to the manufacturer’s protocol. RNA concentration and purity was assessed using the nanophotomer Nanodrop (Euroclone).

About 200 ng RNA was subjected to reverse transcription reaction using the RT² first strand kit (Qiagen, cat. 330401) according to the manufacturer’s instructions. The qRT-PCR analysis was performed in triplicate using the RT² Profiler PCR Array Kit (Qiagen, cat. 330231), in order to analyze the expression of death cell signaling genes on A549 cells. Plates were run on a ViiA7 (Applied Biosystems 384 well blocks), Standard Fast PCR Cycling protocol with 10 µl reaction volumes. Cycling conditions used were: 1 cycle initiation at 95.0°C for 10 min followed by amplification for 40 cycles at 95.0°C for 15 sec and 60.0°C for 1 min. The cycle threshold (Ct) values were analyzed with PCR array data analysis online software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php, Qiagen). Control genes for Real-Time qPCR were actin-beta (ACTB), beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1) and ribosomal protein, large subunit P0 (RPLP0), the expression of which remained constant in A549 cells.

Protein extraction and immunoblot analysis. A549 cells (2 × 10⁶), were seeded in Petri dishes (100 mm diameter) and cultured without any treatments (untreated control) or with the IC₅₀ concentration of the pure compound (4-HBA, 1 µg ml⁻¹). A549 cell lysates were prepared after 24 hours of treatment by scraping the cells of each Petri dish into 1 ml of Radio Immune Precipitation Assay buffer (RIPA, Cell Signaling, cat. 9806), supplemented with 1 µM of protease inhibitor PMSF (Cell Signaling, cat. 8533). The lysates were incubated on ice for 15 min and then clarified by centrifugation at 14000 × g, for 20 min. Total protein concentrations were determined according to the Bradford method using the Protein Assay Reagent (Applichem, cat. A6932) with bovine serum albumin (BSA, Sigma Aldrich, cat. A2058) as standard. The protein extracts were stored at −20°C until
use. Before electrophoresis, protein samples were incubated at 100°C for 5 min. Following 10% SDS-PAGE, gels were stained with Comassie or blotted onto nitrocellulose membrane (Biorad, cat. 170–4159). Membranes were incubated for 1 hour in blocking reagent (1 × Tris Buffered Saline-TBS), with 0.1% Tween-20 with 5% w/v nonfat dry milk, and incubated overnight at 4°C with the primary antibodies diluted in 1 × TBS, 0.1% Tween-20 with 5% BSA (CASPI, 1:1000, Biorbyt orb10232).

After incubation, membranes were washed three times for 10 min each with 15 ml of TBS/Tween and then incubated with HRP-conjugated secondary antibody with gentle agitation for 1 h at room temperature. For β-actin, we used HRP-conjugated secondary antibody anti-mouse (1:10000, Santa Cruz Biotechnology); for CASPI we used HRP-conjugated secondary antibody anti-rabbit (1:10000, Jackson ImmunoResearch).

After incubation, membranes were washed three times for 10 min each with 15 ml of TBS/Tween. Blotted membranes were immunodetected using clarity Western ECL (Biorad, cat. 170-5060). Proteins were visualized with Fuji medical X-ray film (cat. 47410). Densitometric analysis of immunopositive bands was performed using ImageJ software.

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Author Contributions

F.S., C.S., E.P., G.R., and M.L.T. conceived the experiments, F.S. conducted the polar bacteria growth, fermentation and extracts preparation, F.S. and P.T. conducted the preliminary purification work, F.S. and S.K. conducted the purification and structural determination of active compound, C.S. and C.G. conducted the MTT bioassays, the qPCR microarray analysis and western blotting experiments, F.S., C.S., C.G., S.K., TOL, E.P., G.R., R.F., G.M., D.d.P., A.I., and MLT analysed the results and defined results presentation. All authors reviewed the manuscript and accepted the manuscript final version.

Additional Information

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