Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Oncologia, Ematologia e Patologia

Ciclo XXXIII

Settore Concorsuale: 06/D3 - MALATTIE DEL SANGUE, ONCOLOGIA E REUMATOLOGIA

Settore Scientifico Disciplinare: MED/06 - ONCOLOGIA MEDICA

Caloric Restriction Mimetics, Autophagy, and Anticancer Immunosurveillance.
"The bacterial metabolite prodigiosin inhibits autophagy and suppresses antitumor immunity. Results from a fluorescent biosensor-based screening of bacterial metabolites"

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Esame finale anno 2021

Abstract

Nutrition plays a crucial role in the development and progression of different types of cancer. Dietary components, as well as gut microbiota-derived factors, can exert metabolic and immunomodulatory functions on the host, both locally and systemically. Recent studies highlighted the role of specific gut microbes as predictors of response to immunotherapy. Autophagy has a key function in the elicitation of an immune in response to anticancer therapy. Here, we conducted an automatized fluorescent biosensor-based screening to identify autophagy modulators from a chemical library of host- and bacteria-derived metabolites and found prodigiosin, a red pigment produced by Serratia marcescens, as a potent inducer of LC3 dots in GFP-LC3 biosensor cells. Further autophagic flux analysis in RFP-GFP-LC3 tandem reporter cells and a GFP-Q74 Huntington's disease model revealed that prodigiosin acts as an inhibitor of autophagic flux. Consistent with the described immunosuppressive role of prodigiosin, our in vivo experiment in BALB/c mice transplanted with syngeneic colon cancers suggest that prodigiosin impairs the activity of anti-PD1 immunotherapy.

Acknowledgment

The project was conducted at the INSERM UMR1138 (Kroemerlab) “Metabolism, cancer and immunity” at the Gustave Roussy Institute, Paris, France.

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1. Background

1.1. Introduction

Nutrition plays a crucial role in the development and progression of different types of cancer. [1] [2] Some diet nutrients, as well as gut microbiota-derived compounds, can exert metabolic and immunomodulatory function on the host, both locally on the gut epithelium and systemically. Moreover, fasting and caloric restriction reduce side effects and improve the outcome of conventional anticancer treatment in preclinical studies. [3] Caloric restriction mimetics (CRM) are non-toxic compounds capable of inducing autophagy by decreasing global protein acetylation. These molecules mimic the cellular biochemical changes induced by caloric deprivation. [4] Experimental studies have highlighted the ability of CRM to stimulate or restore anticancer surveillance, crucial for the durable success of antineoplastic therapy. [5] [6]

In parallel, some chemotherapy drugs, such as anthracycline or oxaliplatin, possess "bystander" effects, namely, they are able, beyond their main mechanism of action, to induce immunogenic cell death (ICD) and consequently stimulate an immune response towards residual tumor cells. This kind of cell death is characterized by pre-mortem cellular stress that leads to the immunostimulatory emission of danger-associated molecular patterns (DAMP). [7] [8] Given these premises, the main goal of the project was to detect potential new CRMs, ICD inducers, or autophagy disruptors from a library of bacteria and host derived metabolites with the purpose to identify potential modulator or inductor of response to immunotherapies or other immunogenic anticancer treatments.

1.2. Autophagy modulators and lysosomotropic agents

Autophagy (from the Greek αὐτόφαγος meaning "eating of self") is a stress-induced intracellular machinery through which the cell adjusts its energy requirement and remove damaged molecules and organelles or potential intracellular threats. [9] More simplistically, it could be also described as a diversified trafficking intracellular system capable of sequestrate unrequested molecules into double-membrane surrounded vesicles and vehiculate them to the lysosomes for degradation. [10] Since its discovery [11] and its genetic and molecular characterization in yeasts [12], this process gains attention due to the potential impact on the etiopathogenesis of diseases and drug discovery. Autophagy has been recognized as a leading driver or cofactor in several pathological conditions including metabolic or neurodegenerative disorders, infectious diseases and cancer. [13] To date three distinctive forms of autophagy have been recognized in mammalian cells, according to the lysosome/vacuole membrane onset and cargo takeover: chaperone-mediated autophagy, microautophagy, and macroautophagy. [14] [15] These three processes morphologically differ on the modality of cargo sequestration. [16] Here we focus mainly on macroautophagy, herein cited as autophagy, which is the most widely known and examined pathway. This cellular mechanism consists in the formation of double-layer membrane vacuoles (autophagosomes) which restrict cytosolic portions, or damaged structures, and later vehiculate the cargo to the lysosomes, thus allowing the degradation of the cargo mediated by the lysosome hydrolases. [17]

The whole process figuratively consists of five consecutive steps orchestrated by specialized autophagy-related protein (ATG): initiation; phagophore constitution by double-membrane nucleation; autophagosome generation by phagophore expansion and cargo acquisition; autolysosome genesis by fusion with the lysosome; degradation of cargo. [18] Autophagy can be either defined as selective or non-selective depending on whether it concerns a clear-cut

organelle/microorganisms (e.g. mitophagy or xenophagy, respectively) or not. [19]Autophagy-deficient eukaryotic cells and autophagy-incompetent mice are more sensitive to metabolic stressors and more easily develop chemically or genetically induced neoplasms respectively.[20] [21] This whole evolutionary-preserved catabolic machinery lies on the concerted activity of specialized proteins named ATG (autophagy-related) and it is finely modulated by several signaling pathways. [22] In balanced cellular conditions autophagy is enabled at a basal level thus preserving the cellular homeostasis. [23] The occurrence of stressors upregulates its activation status. The principal inductors of autophagy are nutrient deprivation and cellular metabolic perturbations. Nevertheless, some both synthetic and natural pharmacological compounds can mimic the biochemical and molecular intra-cellular modification induced by nutrient deprivation, thus inducing the autophagic route. This evolutionary preserved machinery is finally regulated by a highly interconnected signal transduction cascade. [17] In both mammalian and yeast cells, many metabolic sensors (AMPK, mTORC1, eIF2α kinases, sirtuins, actetyltransferases, transcription factors, and cell surface receptors) and at least two signaling pathways sense nutrient availability: PI3K Class III/Akt/mTOR/p70S6K and the Ras/Raf/MEK/ERK1/2. [24252617] They mainly act by sensing the accessibility to amino acids, glucose, and ATP cellular reservoir. Nevertheless, the accumulation of metabolic subproducts, such as ammonia, can stimulate autophagy. [27]

Mechanistic target of rapamycin (mTOR) is the key regulator of autophagy and by and large of the homeostasis between catabolic and anabolic processes. [28] mTOR aggregates in two complex mTORC1 and mTORC2, but solely mTORC1 is sensitive to cellular nutrient and energetic amount. [29] Mechanistic target of rapamycin complex 1 (mTORC1) acts by inhibiting autophagy and it can be in turn inhibited by AMP-activated protein kinase (AMPK), which is responsive to cAMP increase as a direct result of a higher ATP expenditure. [30] mTORC1 and AMPK therefore constitute a regulatory entangled interface which modulates autophagy

activation. [29] Several growth factor singling pathways and anabolic inputs inhibit autophagy via mTORC1 activation. [31] In nutrient-rich conditions mTORC1 can directly phosphorylate the ULK complex (ULK1/2, Atg 13, FIP200) thus inhibiting its kinase activity and blocking autophagy. [14] Furthermore, mTORC1 regulates the activation of concurrent signaling pathways which in turn modulate autophagy. [24] Amino acid starvation leads to mTORC1 activation and autophagosome appearance. [32] Glucose or oxygen deprivation can lead to intracellular AMP/ATP ratio increase and AMP-dependent AMPK activation by phosphorylation at Thr-172, ultimately stimulating autophagy. [33] The AMPK phosphorylation is mainly regulated by upstream kinases such as the liver kinase B1 (LKB1) serine/threonine kinase complex (a germline mutation of the tumor-suppressor STK11/LKB1 gene occurs in the Peutz-Jeghers familial cancer syndrome[34]) and Ca2+/calmodulin-activated protein kinases. [35] [33] [36] Metabolic disruptor or reactive oxygen species (ROS) can also induce AMPK activation. ROS can both act by impairing the ATP generation by the mitochondrial respiratory chain and activating the cytoplasmatic pool of ATM (Ataxia-Telangiectasia Mutated). ATM, a PI3K-like kinase, probably causes AMPK activation in an LKB1-depended fashion. [33] Nevertheless, AMPK stimulation of autophagy is not restricted to mTORC1 inhibition. AMPK can activate ULK1 via phosphorylation. This event is normally prevented under nutrient sufficiency conditions by mTORC1, as well as subunits of the BECN1/VPS34 complex. [37] [38] Worthy of note is to mention that the modifications of intracellular pH, namely mild acidification, can trigger the autophagic response. [39] [40] Such event typically occurs during starvation and hypoxia. [41] [42] Other stress stimuli (e.g. protein aggregates, damaged DNA or organelles) can activate autophagy, thus giving evidence to its cytoprotective and homeostatic role. [20]

Besides being regulated by metabolic and cellular stress related signals, autophagy is additionally controlled by cytokine and other innate immune mediators. [43] Several pathogen-

associated molecular patterns (PAMPs) can de facto stimulate autophagy via TLR7-MyD88 pathway. [44] A separate mention goes on intracellular pathogens (discussed below).

ULK1 (or Atg1) complex, once activated, recruits the PI3K complex thereby contributing to the autophagy initiations. [10] **Figure Intro** (Pag. 22) Phosphatidylinositol 3-phosphate (PIns-3P) generated by the PI3K complex interact with a pool of protein which possess PIns-3P-binding motif. These events mediate the autophagosome nucleation. [32]

In yeasts the generation of the autophagosome takes place in specific sites named phagophore assembling sites (PAS). The actual source of the isolation membranes is still unclear. [32] In mammals, it has been proposed that such an event could occur at designated autophagosome formation sites of the ribosome-free region of the ER or Golgi apparatus. [45] [46] However other intracellular organelles (i.e. mitochondria, endosomes and, plasma membrane) seem to be somehow involved and a "de novo" formation of a vacuolar membrane has also been described. [14] [47] At these specific formation sites, the ATG proteins intercede thus enabling the formation of the phagophore which later will rip into an autophagosome. [22] IκB kinase (IKK) complex which acts by phosphorylating the regulatory subunit of phosphoinositide-3-kinase (PI3K) is essential to orchestrate the cellular response to starvation. [48]

After the nucleation, the ATG16L1 complex (composed of ATG5, ATG12, ATG16L1) induces the lipidation of LC3, thus allowing its adhesion to the autophagosome membrane. [32] LC3 lipidation as well as ATG12 conjugation are essential for autophagosome and pre-autophagosome constitution, respectively. [49] LC3/GABARAP family proteins (LC3, GABARAP, GATE-16) are thought to cooperate to the phagophore expansion and constitution of an independent membrane-bounded structure.

1.2.1 Monitoring autophagy in vitro

Different markers have been proposed to monitor the phenomena "autophagy". The entire process can be outlined in subsequent phases and is mediated by more than 30 autophagy-related proteins (encoded by ATG genes). Some of those proteins such as Beclin-1, Lamp-1 and 2 (lysosome-associated membrane proteins), LC3A/B (microtubule-associated proteins 1A/1B light chains 3A/B) are recognized as appropriate markers for assessing established stages of autophagy. The first steps consist of the phagosome initiation, then follow nucleation and expansion. Later, the autophagosome fuse with the lysosomes giving rise to the autolysosomes. Several methods rely on the evaluation of light chains 3 (LC3). The detection of LC3 cytosolic puncta by fluorescence-based microscopy reveals the induction of autophagy. [50] In light of this an automatized screening platform has been organized for high-throughput high-content quantification of pharmacologically induced autophagy in cancer cells. [51] The centerpiece of this platform lies in the use of engineered biosensor cells that detect and quantify the pharmacological potential of each molecular candidate. Undoubtedly, (GFP)-tagged MAP1LC3B/LC3 (microtubule-associated proteins 1 light chain 3 beta) cell lines are by far the most used. The lipidation of GFP-LC3, which occurs as part of the autophagosome nucleation, allows the detection of morphological modification in the fluorescence pattern within the cells. Furthermore, the same model can point out the occurrence of phenotypic signs of toxicities, if any. [51] One major limitation resides in the fact that autophagy flux blockers, such as lysosomotropic agents, can mimic the fluorescence LC3 pattern induced by genuine autophagy stimulators. LC3-II accumulation can be attributable either to the induction of autophagy or to a hindrance to the autophagic flux. [52] To further limit the detection of potential false-positive subsequent steps are needed. Tandem GFP-RFP LC3 reporter cells are widely used to investigate the conversion of the autophagosome into autolysosomes in light of the

susceptibility of GFP fluorescence to the decrease in pH. Another option consists in the use of cell stably expressing GFP-labelled mutated huntingtin protein. This cell engineering allows monitoring the degradation of the autophagic cargo. Furthermore, Western Blotting quantification of LC3-I and LC3-II and p62 forms is applied to estimate the LC3 turnover and monitor the flux. The cargo receptor protein p62 mentioned above is widely used as an indicator of autophagic flux. Differently from almost all the ATG proteins, the knockout (KO) of p62 ($p62^{-/-}$) does not lead to the occurrence of growth retardation and precocious lethality but rather mature-onset of obesity in mice. [53] [54] Autophagy inhibition led to an increase of p62/SQSTM1 as the protein is itself degraded by autophagy. [55] Conversely, under conditions of autophagy activation a decrease of p62 levels is generally encountered.

The autophagic process and the lysosomal biogenesis is coordinated by the transcription factor EB (TFEB), a master regulator of such cellular catabolic processes. [56] Stable as well as transient induced over expression of TFEB induces autophagy in vitro. [56] This transcription factor modulates the lysosomal biogenesis by stimulating the of the Coordinated Lysosomal Expression and Regulation "CLEAR" network which codify for lysosomal constitutive protein and hydrolases as well as components of the vacuolar H^+-ATPase. [57] [58] TFEB is normally present in the cytoplasm in is inactive, phosphorylated forms. [59] TFEB subcellular localization is steadily modulated by mTOR-dependent phosphorylation and thus by the nutrient availability. [60] In its dephosphorylated status, TFEB relocate to the nucleus where it induces the expression of genes involved in lysosomal and autophagy. [61] Through TFEB-GFP engineered cell line it is possible to monitor the activation, witnessed by the nuclear translocation, of this transcript factor. [62]

Of course, all these in vitro techniques are valuable to detect promising molecules nevertheless in vivo testing is mandatory to confirm their activity. The in vivo phase can envisage the use

of GFP-LC3 transgenic mouse models as well as the recourse to increasingly specific design according to the presumed activity and potential application of the investigated compound.

1.2.2. Caloric restriction mimetics (CRMs)

Caloric restriction mimetics (CRMs) are nontoxic metabolites or synthetic chemical compounds able to replicate the effect of nutrient deprivation within the cell, thereby eliciting autophagy. They mainly act by inducing deacetylases (e.g. resveratrol), inhibiting acetyl transferases (e.g. spermidine and curcumin), or depleting acetyl coenzyme A (e.g. hydroxycitrate), thus resulting in the deacetylation of cellular proteins. [4] The consequence of the protein deacetylation is the up-regulation of the cellular autophagic flux. [51]

The ability of CRMs to induce the metabolic modification induced by starvation makes them interesting as anticancer and immune eliciting agents. The beneficial effect of fasting in neoplastic disease has been progressively recognized. The rationale of this approach relies on limiting the anabolic demand of the malignant cell, on the induced modification of the cancer microenvironment as well as on delaying the onset of chemoresistance and curtailing the occurrence of treatment-related side effects. [63] A 48h starvation strategy combined with chemotherapy is able to retard the tumor growth in mice. [64]

The use of fasting mimicking diets is one of the potential approaches to overcome the practical limitations of a total fasting approach (not feasible in humans as in experimental mouse models). Another solution is the identification of non-toxic compounds capable of inducing the metabolic effects caused by fasting on cells. The potential benefit of these compounds is not restricted to malignant disease but also degenerative age-associated conditions and ischemic tissue damages. [65]

The activity of these compounds, which mimic the effects of starvation on cells as well as potentially the systemic response to fasting, strongly relies on autophagy induction.

The up-regulation of autophagy in dying cancer cells allow the release of eat-me signals in the extracellular space, as adenosine triphosphate (ATP), in turn able to attract and promote the activity of antigen-presenting cells (APCs). [66] Furthermore, autophagy modulates the exposure of CD39, a protein on cellular surface which convert ATP into AMP. This ecto-enzymes is strongly induced by several stressors (such as hypoxia and tissue damage and is over expressed by various tumor types. [67]

Spermidine, a recognized CRM, is a natural polyamine, such as spermine and putrescine, present in all living organisms. [68] [69] Furthermore, this compound is also largely produced by gut microorganisms. The microbiota composition plays a crucial role in regulating the concentration of these metabolites in the colonic lumen and consequently in the bloodstream. [70] Spermidine and hydroxycitrate, an extract from the fruit Garcinia Cambogia, have shown to improve the efficacy of anticancer immunotherapies.

The administration of hydroxycitrate, spermidine, and alpha-lipoic acid reduces the lung implantation of B16 melanoma cells in C57BL/6 mice. [71] Hydroxycitrate acts as inhibitor of the ATP citrate lyase. In autophagy proficient mouse models this acidic compound is able to induce a depletion of Treg, thus eliciting antitumor immunosurveillance and exerting anticancer effects.

1.2.3. Lysosomotropic agents

Lysosomes are acid vesicles (with an interior pH of 4.5-5.0) that figuratively could be described as the stomach and the intestine of the cell. These organelles house more than 50 distinctive hydrolases able to "digest" all the constituents of the cell. To permit the activity of these hydrolases, lysosomes have to generate and preserve an acidic micro-environment. This condition is achieved through the intercession of the vacuolar-ATPase proton pumps. [72] The V-ATPase pump generates a chemical (pH) and electrical positive potential between the lysosome membrane by actively carrying H^+ ions within the lysosomal compartment against their electrochemical gradient. In the same way as the lysosomes, the interior of most cellular organelles exhibits a lower pH than the surrounding cytosol. [73] On the lipidic bilayer the electrogenic potential is both dissipated through cation and ClC-7 anion channels which mediates the efflux of K^+ cation in the cytosol and the influx of chloride anion (Cl^-) within the lysosome, respectively. [72] This event is crucial to avoid that the electrical potential hinders the activity of the pump itself before reaching a target pH. [73] Although not yet fully understood the activity of V-ATPase is regulated by intrinsic and extrinsic factors. Bafilomycin A1, an inhibitor of V-type H^+-ATPase, can impair the lysosomal acidification, thus blocking the autophagic flux, and induce a caspase-3 and -9 dependent apoptosis. [74] Furthermore, chloride anion (Cl^-) is another key regulator of the lysosomal function. Culturing gastric cancer cells under Cl^- restricted conditions leads to impairment of autophagy (LC3II and p62 accumulation) as a consequence of reduced Cl concentration in the cytosol and the lysosome and concomitant increase of intralysosomal pH. [74] Undoubtedly, lysosomes' cardinal role is to guarantee the degradation of misfolded or damaged intracellular constituents as well as the elimination of potential treating microorganisms and the regeneration of building blocks for the anabolic processes. These organelles further participate in signaling pathways, in the preservation of

cellular homeostasis, and the execution of cell death programs.[75] The term lysosomotropic, according to the definition of C. De Duve, refers to compounds "that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake". [76] Many pharmacological compounds naturally possess this feature, and if not, it can be obtained by coupling them with an appropriate carrier. [76] Such of these compounds are usually weak bases. In their active unprotonated status, they can enter within the acidic organelles and accumulate as protonated form. They finally lead to an increase in the intra-vesicular pH and a disruption of the autophagic functioning. [77] [78] Other molecules are also capable to permeabilize the phospholipid bilayer that constitutes the lysosomal membrane resulting in an alteration of cytosolic pH and release of hydrolytic enzymes. The lysosomal membrane permeabilization (LMP) induces the trigger of apoptotic pathway whereas an extensive lysosomal rupture, similarly to what happens in case of intestinal perforation in humans, leading to massive cellular stress and cell death by necrosis. [79] [80] [75] It is worthy of note to specify that some compounds, e.g. ML.9, can induce the autophagosome formation, in this specific scenario by downregulating the Akt/mTOR pathway, while hindering the lysosome degradation by increasing the intravacuolar pH. [81] Furthermore, ML-9 was showed to enhance the activity of the chemotherapy agent docetaxel by inducing accumulation of autophagic vacuoles and cell death in prostatic cancer. [81]

Other agents, such in the case of prodigiosin, can dissipate the lysosomes H^+ potential by behaving as H^+/Cl^- symporter. [72] Anion transporters can disrupt autophagy and cause a decrease of intracellular pH which in turn can trigger the activation of apoptotic events. [82] [83]

Malignant cells elevate autophagy to face the anabolic requests and survive in a hypoxic microenvironment. [84]

While lysosomotropic agents are reported to exert anticancer activity in vitro or in immunocompromised in vivo models (such as patient-derived xenograft), we speculate that

their activity could be counteracting as immunotherapy adjuvant. This assumption relies on the fact that the impairment to the autophagic machinery can on the one hand minimize the occurrence and the peculiarities of the immunogenic cell death in the cancer cell, on the other hand, some compounds could exert the same activities at a similar concentration on specific immune cells thus failing in stimulating the mounting of a strong immune response.

1.3. Immunogenic cell death

Immunogenic cell death is a peculiar type of cellular demise able to induce inflammatory effect and immune modulation in the tissue microenvironment. It is characterized by modification in protein on the surface membrane of the dying cell and releasing of immune-stimulating factors (DAMPs) in the cell microenvironment. [85] Chemotherapeutic agents such as oxaliplatin and anthracyclines can induce ER premortem stress and consequently stimulate the anticancer immunoresponse. [86]

1.3.1. Main features of ICD

This singular form of cell death is distinguished by a series of pre-apoptotic events: the exposure of endoplasmic reticulum (ER) proteins at the cell membrane (mainly calreticulin), the release of the nonhistone chromatin protein high-mobility group box 1, (HMGB1) and the discharge of ATP in the extracellular space. [85] These happenings suggest the occurrence of 'pre-mortem' cellular distress such as the alteration of ER functioning and induction of autophagy. The late phase of this dying process recapitulates the distinctive events which occur during the apoptosis (e.g. the exposure of phosphatidylserine (PS) on the cell surface). [87] The dying cells expose calreticulin (CRT) on their surface and release ATP and HMGB1 in the extracellular matrix. The first of these processes is independent of nuclear activities while seems strictly related to reactive oxygen species (ROS) and nitric oxide formation. [85] The ATP release, conversely from HMGB1, require an on caspase-dependent activation (accountable for specific cleavage of the plasma membrane channel pannexin 1 (PANX1)[88]) and autophagy proper functioning.[89] [90] Extracellular ATP exerts autocrine and paracrine function. This

molecule can interact with purinergic receptors (P2X and P2Y) and is a strong activator signal for phagocytes chemotaxis. [89] Furthermore, the systemic administration of these compounds reasonably exposes to their effects not only the malignant cells but also the immune cells.

1.3.2. Damage-associated molecular pattern (DAMP)

Damage-associated molecular patterns are both intracellular and extracellular molecules released as a consequence of peculiar types of stressors. This group of molecules constitutes the "alter ego" of "pathogen-associated molecular patterns" PAMPs and differentiate from the latter for their non-infectious origin. Those biomolecules can trigger or sustain an inflammatory response in tissues by interacting with a wide class of innate immune receptors collectively named pattern recognition receptors (PPRs).[91] The pattern recognition hypothesis was originally proposed by Charles Janeway who based on the "self/non-self" Burnet's theory [92] postulated the existence of recognition receptors on the innate immune cells able to detect microbial-derived molecular patterns lately named as PAMPs.[93] [94] The identification of the Toll-Like Receptor (TLR) on the antigen-presenting cells (APC) practically reinforced the assumption. The recognition of endogenous host-derived molecules as a potential modulator of inflammatory response dates back to the nineties. [95] TLR when triggered by the presence of DAMPs or PAMPs act by releasing pro-inflammatory mediators and expressing costimulatory molecules which can activate/modulate the adaptive immune response. [96] At present DAMPs have been recognized as a major player in initiating and perpetuating the inflammatory processes underlying a wide group of diseases such as autoimmune and neurodegenerative diseases, metabolic disorders, and lastly cancer. [94] They can be classified according to their origin and mode-of-emission. [97] The cellular DAMPs can originate from different compartments and organelles (i.e. cytosol, nucleus, mitochondria, endoplasmic reticulum,

plasma membrane, and intracellular granules). Furthermore, these molecules can be categorized into those exposed on the cellular surface or actively and passively secreted. Investigation on new PAMPs, as well as DAMPs released from the host tissues as a consequence of bacterial colonization, may offer the opportunity to identify new strategies to modulate the immune response in the context of malignant diseases.

In a similar way, intratumoral bacteria, as well as mucosal-associated microbiota, can both impact the metabolic reprogramming of malignant cells and the modulation of the immune contexture through the activities of secondary metabolites, toxins, and further mediators.

1.4. The human microbiome

In the last decades the historical idea of humans as being simply constituted by eukaryotic cells and structured in tissue, organs and, systems has been overtaken by the concept of the 'Human Holobiont' which flourished notably after the seminal Metchnikoff's works and had a renaissance recently. [98] All in all the human microbiota has been estimated to contain over one thousand bacterial species (more than one hundred and sixty in each person) and millions of genes. [99] [100] Gut microbes are directly involved in energy metabolisms and play a cardinal role in modulating the cell-to-cell interaction and host immunity homeostasis.[101]

Focusing on oncology, preclinical and clinical findings suggest the crucial role of the gut microbiome in shaping anticancer immunity and the response to cancer chemotherapies and immunotherapies. [102] [103] [104] Three recent studies confirmed these data in humans, reporting the unexpected role of specific members of the gut microbiota as a predictor of response to immunotherapy in a distinctive series of epithelial tumors (NSCLC, renal cell carcinoma, and urothelial carcinoma) and melanoma patients. [105] [106] [107] Moreover, the phenotype of responders or non-responders can be transferred by performing fecal microbiota transplantation, such as utilizing germ-free or antibiotic-pretreated mice as recipients for feces of responder or non-responder patients. [108] Similarly, oral supplementation with specific bacteria seems a feasible option to restore the phenotype of responders in avatar mice carrying the microbiome of non-responder patients. [109] In the new era of immunotherapy-based cancer treatments, these pieces of evidence are undoubtedly of crucial relevance. The predictive role of the microbiome in terms of response and the potential of its manipulation to foster the efficacy of the treatments has a large potential for clinical application.

1.4.1. Bacterial metabolites or derived secondary metabolites as metabolic and immune modulators

Bacteria-derived compounds, both from pathogens and not, can exert a wide range of effect on the host. [101] Furthermore, the power of those molecules can be extremely amplified if directly released within the tissue. Along these lines, the focus on potential intertumoral bacteria-derived compounds deserve attention. In the gut lumen, the metabolites can directly derive from the diet or conversely be released both from the host cell and the gut microbes. Nutrient competition is probably the first discriminant to limit the potential colonization of pathogens.[110] Here I briefly discuss some significant evidence on the most-investigated bacterial metabolites and toxins.

In light of its role in bacterial proliferation, virulence and metabolism iron constitutes one great example.[111] [112] This micronutrient impacts cytokine secretion and transcription of key mediators of immune response.[111] This mineral is an essential nutrient for both bacteria and humans. [113] In mammals, it exerts the most prominent function as a component of oxygen-carrying proteins myoglobin, and hemoglobin. In addition, iron is required for the function of the mitochondrial respiratory chain and DNA synthesis enzymes. Lastly, this metal mediates the generation of oxygen radicals in neutrophils and macrophages, and is required for the clonal expansion of T cells. [111] Several mechanisms of withholding have evolved to restrict the iron availability to intracellular and extracellular microorganisms.

Others bacterial metabolites or bacterial-processed host compounds can exert specific metabolic and immune activities on the host.

Lipopolysaccharide, a constitutive glycolipid of the Gram-negative bacterial membrane, is able to induce inflammatory response through the interaction with different receptors on immune cells. LPS activity is mainly mediated by the activation of the TLR-4 cascade and the

consequent release of proinflammatory cytokines in the microenvironment. Other proteins, namely LPSBP (LPS binding protein) and CD14, facilitate the extraction of LPS monomers and the interaction with the TLR4 on the cellular surface. [114] Several studies suggest the pro-tumorigenic activities of LPS in different types of cancers. [115] [116] α-galactosylceramide, and its analogs, elicit the activity of invariant natural killer T (iNKT) cells, a distinctive subset of T lymphocytes that express an invariant TCRα chain. [117] [118]

Bile acids have shown various activities. Among them, deoxycholate (DC) can activate autophagy in non-cancer colonic cells by increasing the expression of the autophagic protein, beclin-1 in a ROS-dependent manner. [119] DC can also indirectly impact on colonic carcinogenesis by activating the COX-2 signaling pathway in the cancer associated fibroblasts. [120] Lithocholic acid exerts anticancer activities by inducing apoptosis in prostate cancer cell line. In such a context, this secondary bile acid has been shown to induce endoplasmic reticulum stress as well as autophagy (conversion of LC3BI-LC3BII and ATG5 induction) and mitochondrial dysfunction. [121] Ursodeoxycholic acid induces the expression of LC3B in a hepatocellular cell line in vitro and in vivo in nude mice. [122] Taurocholic acid, as well as cholic and chenodeoxycholic acid, induces LC3B and p62 in primary hepatocytes but this effect seems to be attributable to an impairment of the autophagic flux. [123] Glycochenodeoxycholate induces autophagy by activating the AMPK/mTOR pathway in hepatocellular carcinoma cells and interestingly this effect is reversed by chloroquine (an inhibitor of the autophagic flux). [124] [125] Short Chain Fatty Acids are the sub-product of bacterial fermentation of non-digestible carbohydrates. These compounds can reach high concentration in the blood of the host. [126] Many of their activities are mediated by the interaction with G-protein-coupled receptors (GPRs). [127] Among them butyrate is probably the most characterized. In the colon butyrate is able to modulate the inflammatory response by acting both on epithelial cells and immune cells.

[128] SCFA regulates the function of effector lymphocytes (CD8) as well as the frequency of T-regs and T cell cytokine release during infections. [129] [130]

Indole metabolites, derived from the metabolism of tryptophan, are further relevant mediators able to impact on host-immune functions. [131] As an example, indole-3-propionic acid (IPA), one of these bacterial produced compounds, exerts anti-inflammatory activities in an in-vivo steatohepatitis model by reducing the level of pro inflammatory cytokines via the NF-κB signalling. [132]

Finally, a separate mention should be made on bacterial toxins. Pyocyanin, a well-known Pseudomonas aeruginosa virulence agent, induces an anti-inflammatory response in LPS-activated macrophages. [133]As proposed by the authors, this may subtend an immune-evasion strategy. Similarly, violacein, a quorum-sensing and water-insoluble compound released from *Chromobacterium violaceum* and other gram-negative bacteria, reduces pro-inflammatory cytokine production and induces Tregs in an LPS-induced inflammation model in mice. [134]

1.4.2. Bacterial interactions with the autophagic machinery

Besides its role as degradation machinery of the cell, autophagy exerts a pivotal role among the defenses of the eukaryotic cell against bacteria. This particular type of selective autophagy has been named xenophagy by Levine and can advisedly be classified as part of the innate immune response. [135] Xenophagy is both essential for the clearance of the intracellular treats and the initiation of a pathogen-specific immune response. [136] In this specific autophagic process, the cargo identification necessitates a specific interaction between flag molecules (i.e. ubiquitin) and specific receptors. Those receptors incorporate the ubiquitin-binding domain (UBD) and LC3-interacting region (LIR) motif. The interaction of the LIR region with LC3 guarantees the recruitment of the phagosome membrane nearby the ubiquitinated pathogens. [137] The best known and firstly described among those receptors is p62/SQSTM1 (hereafter p62). [138] p62 is more widely involved in cargo identification over different forms of autophagy, not-only in xenophagy. [55] The function of this adaptor protein has been in-depth detailed in the context of protein aggregation diseases. [139] Nonetheless, its role in the recognition of intracellular pathogens makes it also a crucial player in innate immunity. [138] To date, many other receptors involved in this process have been described: optineurin (OPTN), a neighbor of BRCA1 gene 1 (NBR1), and nuclear domain 10 protein 52 (NDP52). [137] In certain circumstances the ubiquitination, and then the autophagic process, can be triggered "ab initio" by specific inductors such as the S-guanylation of the cysteine of the Group-A streptococcus membrane proteins or the galectin-8 depended flagging of endosomal vesicles damaged by pathogens (e.g *Salmonella, Lysteria or Shigella*). [140] [141] Furthermore intracellular sensors, namely Nod1 and Nod2, are shown to recruit the ATG16L1 at the bacterial entry sites in a NF-κB/RIP2 independent manner. [142] As an evolutionary consequence, some bacteria developed singular strategies to escape the autophagic response. [143] This can be reached through surface

modification or production and release of metabolites and mediators. They can act by interfering with the signaling pathways that modulate autophagy or directly disrupting the autophagic machinery. Certain bacteria species can even modulate autophagy to gain their benefit. Some species prevent the fusion of the autophagosome with lysosome thus avoiding their degradation and obtaining an intracellular niche to settle down. It is crucial to notice how intracellular bacteria evolved singular strategies to modulate autophagy rather than completely hinder it. This evidence once again attests to the fundamental importance of autophagy for the eukaryotic cell, which constitutes "the casket" for intracellular-bacteria replication. Some unique strategies evolved from pathogenic bacteria are discussed below.

Anaplasma phagocytophilum, an intracellular gram-negative bacterium responsible for human granulocytic anaplasmosis, can induce autophagy nucleation and the expression of related markers within the host cell (such as LC3-I/II and Beclin 1) while inhibiting the fusion with lysosomes. Doing it this way, this microorganism can stably segregate within the autophagosome. [144] Similarly, *M. tuberculosis*, *H. pylori*, *L. pneumophila* and *M. avium* prevent through different mechanisms the fusion of autophagosomes with lysosomes. [145] Some of them can act by preventing the lysosome acidification while others by disrupting lysosomal membrane integrity, as for the virulence factor gamma-glutamyltranspeptidase by *H. pylori*. [145] *M. tuberculosis* ESAT-6 effector protein blocks the process at the stage of phagosome-lysosome fusion. [146] [147] *L. pneumophila* hijacks the autophagic chain finally leading to the genesis of endoplasmic reticulum shaped structures. This gram-negative bacterium induces the constitution of singular organelles consisting of smooth vesicles (derived from the ER) that, rather than fuse with lysosomes, are later coated with ribosomes. [148] [149] Intriguingly, this bacterium employs the same trick to hide out in protozoans, which are the primary hosts, implying its capability to act on an evolutionarily conserved process. [150] Genetic analysis on *L. pneumophila* mutant strains leads to the identification of *dot* genes which are required to evade

the fusion with lysosomes. [151] Those genes encode for a Dot/icm transporter which shares similarities with the bacterial type IV secretion system family, owned by certain other microorganisms. [152] Those genes are required for bacterial virulence. [150] The Dot/transporter should be involved in the secretion of putative and still unidentified effector molecules that are injected into the host cell. [148] Other human pathogens, such as *L. monocytogenes* and *C. trachomatis* can induce mitophagy consequently increasing the nutrient availability and curtailing the production of reactive oxygen species by the invaded cell. [153] Infection by *Chlamydia*, obligate intracellular bacteria, replicate within intracellular vacuoles which do not fuse with lysosomes. *Chlamydia trachomatis* infection induces the formation of LC3 cytosolic puncta as well as their colocalization with LAMP1 (a lysosomal marker) however the p62 cellular amount remains stable or increased. [154] [155] Furthermore, *Chlamydia trachomatis* decreases the activity of lysosomal enzyme in macrophages. Intriguingly, the vATPase inhibition (by treatment with Bafilomycin A) exerts an opposite effect by stimulating the bacterial growth in wild type cell while inhibiting it in ATG5-/- autophagy deficient cells. [155] These results suggest a dual activity of *Chlamydia trachomatis* on the autophagic machinery and confirm the relevance of the blockage of the autophagic flux in pathogenic model of this bacterium.

L. monocytogenes, a facultative intracellular gram-positive bacterium, can similarly inhibit the late autophagic events, thus preventing the degradation of the Listeria-containing phagosomes (SLAPs) in the macrophages where it replicates. [156] This event is mediated by the Listeriolysin O, a pore-forming toxin that impedes the evolving of vacuoles into autophagosomes and maintains the intravacuolar pH as neutral. [157]

Given its ability to generate intracellular niches where replicate, *S. typhimurium* represents another well-described model of intracellular infection by pathogens. In order to survive within the phagosomal compartment this bacterium can adapt its metabolism to the extravesicular

conditions. [158] Similarly to *L. monocytogenes*, the effect of this gram-negative bacterium on autophagy is ambivalent. *S. typhimurium* can both induce the autophagic machinery via the T3SS1-depended release of amino acids form the host cell (and consequent mTORC1 inhibition) and escape the autophagic degradation through its SseL deubiquitinase. [159] Furthermore *S. enterica* impairs the activity of the lysosome that fuse with Salmonella vacuoles by reducing the trafficking of hydrolytic enzymes. [160] *Shigella flexneri* achieves the same goal by inducing autophagy by VirG, that binds the ATG5, while escaping it via IcsB secretion that interfere with the autophagic machinery. [161]

The Gram-negative bacterium *Coxiella burnetii*, the pathogenic agent of the Q fever, has adapted to replicate in the harsh acidic environment of the phagolysosome. [162] This feature makes it unique among intracellular bacteria. *C. burnetii* rather than escape the bactericidal environment of phagolysosomes has evolved a pH-depended regulation of its metabolism as a singular parasitic strategy. [163] Autophagy induction by starvation and overexpression of autophagic proteins fosters the replication of this obligate intracellular gamma-proteobacterium by promoting the development of replicative compartments. [164]

S. marcescens, another gram-negative, release the ShlA pore-forming exoprotein which can act as a potent cytotoxin. Furthermore, this protein can stimulate the autophagic response in nonphagocytic cells before the bacterial internalization happens. [165] Bifidobacteria reduce the autophagy activation of intestinal epithelial cells induced by lipopolysaccharide from gram-negative bacteria.[166] [167]

Other bacteria can act on TFEB which is involved in the host response to the infectious threat. In Caenorhabditis elegans, the homolog of TFEB is strongly activated upon *Staphylococcus*

aureus infection. [168] Similarly, *Salmonella typhimurium* induces TFEB activation in mouse macrophages through the activation of the phospholipase C/protein kinase D pathway. [169]

Figure Intro 1

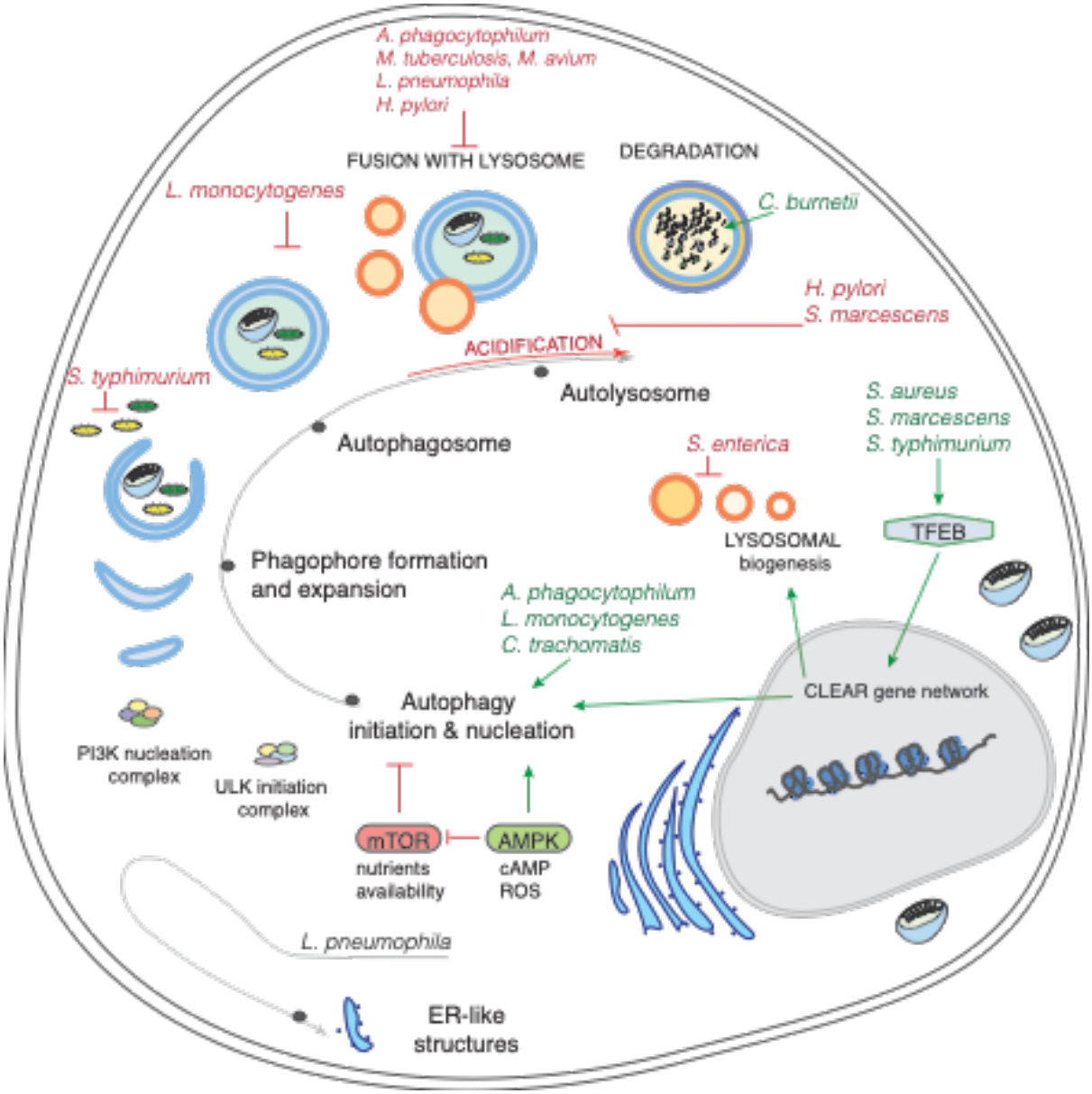


Figure Intro. Bacterial interactions with the autophagic machinery. ULK initiation complex: ULK1 and ULK2, ATG13, FIP200, ATG101. PI3K nucleation complex: Beclin 1, VPS34, p150, ATG14L.

1.4.3. Intra-tumoral bacteria

A growing interest is flourishing in the last years on the identification of intra-tumoral bacteria.

Several research papers are shedding light on the potential implication of intra-tumoral bacteria on the response to anticancer treatments. [170] Geller et all. recently reported that intratumor microbes can impact the efficacy of anticancer therapies. The authors showed that intratumor Gammaproteobacteria which possesses the bacterial enzyme cytidine deaminase can convert gemcitabine into its inactive metabolite. Furthermore, they reported that more than 70% of pancreatic ductal cancer they screened were positive for bacteria (mainly Gammaproteobacteria). [170]

More recently a deeper characterization of the tumor proper microbiome in comparison with the adjacent non-tumoral tissue has been carried out as regards seven types of common cancers (breast, lung, ovary, pancreas, melanoma, bone, and brain). [171] The authors reported both malignant and tumor-infiltrating immune cells can host bacteria. [171] Furthermore, the composition of the tumor microbiome, as well as its plausible metabolic function, quantitatively and qualitatively vary across different tumor types and even subtypes (as for breast tumors). [171] The origin of this microbial tissue ecosystem is still unclear although the leaky tumoral vasculature and the tumor inflammatory microenvironment could play a crucial role. [171] [172]

This evidence does not allow to assert a metabolic dependency or a “symbiotic relationship” between bacteria and tumoral cells yet, but it opens to further investigations. Furthermore, the bacterial composition may impact the response to anti-cancer treatments, particularly immunotherapies, as well as provide an opportunity to identify peculiar tumor metabolic dependencies. It is also worthy of note that being a low biomass microbiome still makes it challenging to dissect at the species level. [173]

In addition to the potential use in the diagnostic field, the more innovative perspective lies in the preclinical development of engineered tumor-targeting bacteria. [174] [175] Some bacteria species can constitutively exert antitumor activities (*e.g. Salmonella Typhimurium*). [176] A detailed portray of tumor colonizing microbiota can also lead to the selection of ideal strains to deliver active compounds and pro-drug converting enzymes or able to foster immune-related reactions against cancer cells. In this context, the selective tumor trophism of systemic administered bacteria constitutes one crucial aspect. [177] [178] To date many details of preferential tumor colonization by specific bacteria remains unclear. Bacterial metabolic properties could play a pivotal role. As an example, the disruption of the aromatic amino acid biosynthetic pathway, widely used to obtain e virulence attenuated bacterial strains, led to relevant changes in bacterial colonization of tumors in mice. [179] Another possible strategy relies on the use of activable synthetic metabolites (e.g. by light irradiation) to maximize the on-target efficiency. [180] Furthermore, the intratumor bacteria are probably closely interrelated with gut microbiota composition. [181] This evidence could strengthen the significance and hide a potential role of gut dysbiosis in several steps of cancer development and treatment.

Recently, some pieces of evidence on the potentiality of those approaches are increasingly coming out. A research team reported the feasibility and the efficiency of radioactive Listeria-32P strain in vitro and KPC mice. [182] Another team reported encouraging both in terms of efficacy and toxicity data by combining live-attenuated *Listeria monocytogenes* with GM-CSF–secreting allogeneic pancreatic tumor cells in highly pretreated patients. [183]

1.4.4 Prodigiosin and *S. marcescens*

Prodigiosines are secondary metabolites produced by many strains of both Gram-negative and Gram-positive bacteria (i.e. *S. marcescens, V. psychoerythrus, S. rubrireticuli,* and other eubacteria). [184] These red-pigmented compounds are constituted by a linear methoxytripyrrole skeleton. Those molecules are deemed capable of exerting various biological activities among which antifungal, antibacterial, antiprotozoal, antimalarial, immunosuppressive, and anticancer activities. [185] Due to its chemical structure, prodigiosin can act as a chloride anion binder. Each prodigiosin molecule in its protonated form can carry a chloride ion (Cl^-) through three hydrogen bonds tush behaving as a H^+/Cl^- symporter. [72] Furthermore the amphiphilic nature makes it excellent as a transporter across the lipidic membranes. [72] Its biological function remains unclear but some hypotheses have been postulated. [184] It is known for its antimalarial, antineoplastic, and antibiotic activity. [186] Prodigiosin and its synthetic analogs Obatoclax have been shown an interesting large-spectrum cytotoxic activity in vitro on cancer cells and Obatoclax is currently investigated in more than one clinical trial on hematologic diseases.

The proapoptotic activity of these molecules appears ascribable to induced modification of the cytosolic pH (cyt-H) and lysosomal pH (lys-pH).

Serratia marcescens, a gram-negative bacillus, is an important cause of nosocomial infection. [187] This saprophytic enterobacterium is quite common in the environment and has been found in food, particularly starchy substances. [187] It has been considered non-pathogenic for a long time until the report of nosocomial infection in humans and animals began to appear in the latter half of the 20th century. [188] [189] [190] [191] *S. marcescens* has been reported as a microbial agent implicated in respiratory and urinary tract infection, as well as septicemia and wound infection. [192] [193] [194] [195] Furthermore, some reports described infection sustained from prodigiosin

producing strains. [196] Another common feature, is that the infection mainly, but not exclusively, affects patients with chronic disorders; a condition that earned him the title of opportunistic bacterium. A case series showed how Serratia marcescens was detectable in up to 30% of specimens obtained through bronchoscope procedures. [197]

Prodigiosin has been accredited with distinctive properties on cells. This toxin can induce lysosome de-acidification and consequently cytosolic acidification and apoptosis.[72] It acts as a chloride anion binder able to convey the Cl^- anion across the lysosome bilayer membrane thus decoupling the intra-vacuolar H^+ ion potential generated by the vacuolar-ATPase proton pumps. [72] Based on this assumption we support the hypothesis that these bacterial metabolites could facilitate the bacterial settlement within the cell. As an example, recently a new light on the veritable function of the Shiga toxin from enterohemorrhagic Escherichia coli (EHEC) has been proposed. In the present case, the researchers showed how Shiga toxin was required for suppression of inflammasome responses to cytosolic LPS thus impairing the intracellular immune response against the bacterium. [198] Along similar lines, probably prodigiosin is a molecule released by some bacterial strains to enhance the potential of this bacterium to invade the cells and live intracellularly. The activity in alkalinizing the lysosome and the probable induction of lysosome genesis should confirm such a theory. Doing that the bacterium can create intracellular niches to survive. [199] One plausible explanation of this phenomenon could be linked to the antigenicity of the bacterium. Along similar lines, an interesting paper reported the tumor-immunotherapeutic efficacy of the *S. marcescens* extracts. [200] This assumption is further supported by the evidence of some activity of the Coley's toxin, which consists of streptococcal and *Serratia marcescens* extracts, against Bone and Soft-Tissue Sarcomas. [201]

On tumoral cells, it can induce apoptosis in hematopoietic cell lines with no marked toxicity in non-tumoral cells. [202] The apoptotic death of B-cell chronic lymphocytic leukemia (B-CLL)

cells occurs by reaching an IC50 of 116±25 nM. [203] Prodigiosin showed similar activity on colon cancer cell lines. The IC50 was 275 nM in SW-620 cells. [204]

Noteworthy, cycloprodigiosin hydrochloride, uncedylprodigiosin (UP), and prodigiosin exert their effects also on immune cells. UP blocks the proliferation of T and B lymphocytes as well as the T cell activation at lower concentrations (IC50 7-20 nM) than those required to inhibit the proliferation of leukemic cells.[205] Conversely from Cyclosporin A, UP activity relies on the hindrance to phosphorylation of the retinoblastoma protein (RB) and the inhibition of cell-cycle genes (cyclin E, cyclin A, cdk2, and cdk4). Those events result in a block of T cell in the mid to late G1 phase. Furthermore, other authors showed that prodigiosin can interfere with the IL-2/IL-2R signaling pathway by inhibiting IL-2Ralpha expression. In doing so prodigiosin impedes the T lymphocytes differentiation both into effector T helper and effector cytotoxic T cell. [206] This compound showed promising activity as an immunosuppressive agent. [207]

2. The bacterial metabolite prodigiosin inhibits autophagy and suppresses antitumor immunity. Results from a fluorescent biosensor-based screening of bacterial and host-derived metabolites.

2.1. Introduction and aim of the project

During the last few years, the gut microbiota has gained increasing attention as a consequence of its emerging role as modulators of the immune system. [208] [209] Fascinating studies are shedding light on the impact of the gut microbiome in shaping the response to anticancer immune treatments. [106] Along similar lines, a growing interest is flourishing in deepen the knowledge on the settlement and function of intracellular tumor-specific bacteria.[171]

In such a context, our project aimed to screen a library of microbial metabolites by in vitro automatized biology approaches to select potential candidates able of acting as autophagy modulators and/or ICD inducers, to eventually evaluate their efficacy in combination with immunotherapy in vivo.

More in detail, we performed a further automatized fluorescence-based screening of a list of bacterial and host-derived metabolites to identify compounds able to interfere with the autophagic machinery. The compounds of the library are summarized in the **Appendix Table1**

Appendix table 1

Name	Effect	Reference
Acetate	Induces apoptosis in colon rectal cancer cells	Oliveira et al., 2015 [210]
Butyrate	Prevents autophagy in colonocytes	Donohoe et al., 2011[211]
Propionate	Triggers autophagy in colon cancer cells	Tang et al., 2011[212]
Trimethylamine	Scarce available data	Falony et al., 2015[213]
Trimethylamine N-oxide (TMAO)	Inhibits ATG16L1, LC3-II and p62 expression	Yue et al., 2017[214]
Dimethylamine	Lysosomotropic compound	Kuzu et al., 2017[215]
Monomethylamine	Blocks lysosome acidification	Sobota et al., 2009[216]
Indole	Up-regulates autophagy in Flp-In 293/SH-SY5Y cells	Lin et al., 2014[217]
Indoxyl sulfate	Activates the autophagic machinery. Inhibits the autophagic flux	Sun et al., 2017[218] Rodrigues et al., 2020 [219]
L-Leucine	Inhibits autophagy. Stimulates mTOR signaling	Meijer et al., 2015[220]
Isoleucine	Isoleucine deprivation activates autophagy	Sheen et al., 2011[221]
Valine	Limited effect	Alvers et al., 2009[222]
P-cresol	Activates the autophagic machinery	Sun et al., 2017[218]
Phenylacetic acid	No activity	Peraro et al., 2017[223]
Phenylacetyl glutamine (PAG)	anti-inflammatory activity via inhibition of T cell activation and Toll-like receptor 4 signaling	Hazekawa et al., 2018 [224] Nicklin et al., 2009[225]
Indoleacetic acid	Inhibits the autophagic flux	Rodrigues et al., 2020[219]
Tryptamine	Induces cell death with ultrastructural features of autophagy	Herrera et al., 2006[226]
α-galactosylceramide	Invariant natural killer T (iNKT) cells activation	Keller et al., 2017[117]
Linaclotide	Selective guanylate cyclase C agonist. Analgesic activity on irritable bowel syndrome (IBS)	Castro et al., 2013[227]
Deoxycholic acid	Activates autophagy in non-cancer colonic cells. Increased ROS-dependent expression of beclin-1	Payne et al., 2009[119]
Lithocholic acid	Induces autophagy, ER stress and mitochondrial dysfunction in prostate cancer cell line	Gafar et al., 2016[121]
Ursodeoxycholic acid	Induce LC3B in hepatocellular carcinoma cell line and in nude mice	Wang et al., 2017[122]
Nor-ursodeoxycholic acid	Moderate increase in hepatic autophagy and antiapoptotic effects	Tang et al., 2016[228]
Taurocholic acid	Increases LC3II and p62 in hepatocyte by impairing autophagosomal-lysosomal fusion	Manley et al., 2015[123]

Glycochenodeoxycholate	Induces autophagy by the AMPK/mTOR pathway in HCC cells	Gao et al., 2019[124]
Benzoic acid	Inhibits autophagy by intracellular acidification and disrupting the membrane trafficking	Hazan et al., 2004[229]
Hippuric acid	Scarce available data	
Succinate	Scarce available data	
Urolithin A	Elevates the autophagic flux in macrophages	Boakye et al., 2018[230]
Kynurenic acid	Induces the production of IL-6	Van der Leek et al., 2017[231]
Kynurenin	Inhibits autophagy in bone marrow mesenchymal stem cells (LC3B-II and autophagolysosomal reduction/p62 increase)	Kondrikov et al., 2020[232]
Anthranilic acid	Scarce available data	
Quinolinic acid	Induces the expression of damage-regulated autophagy modulator, beclin 1, and LC3-II	Wang et al., 2009[233]
Serotonin	Induces autophagy in an AKT/mTOR independent fashion in liver cancer cell line	Niture et al., 2018[234]
Melatonin	Enhances autophagy response via SIRT1 deacetylation	Nopparat et al., 2017[235]
Indolepiruvic acid (IPA)	Scarce available data	
Nicotinic acid	Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) increases levels of LC3II, beclin-1 and acidic vesicular organelles in astrocytes	Pereira et al., 2011[236]
2-picolinic acid (PA)	Scarce available data	
N-methyl-d-aspartate (NMDA)	Induces autophagosomes accumulation and autophagic death in rat organotypic hippocampal slices	Borsello et al., 2003[237]
3-hydroxykynurenine (3-HK)	Scarce available data	
3-hydroxyanthranilic acid (3-HAA)	Scarce available data	
GYY4137	Hydrogen sulfide (H2S) induces autophagy via AMPK pathway in colon cancer cell lines	Wu et al., 2012[238]
Taurine	Induces autophagy in adipocytes via TFEB nuclear translocation. Induces autophagy in Leyding cells.	Kaneko et al., 2018[239] Yahyavy et al., 2020[240]
Valeric acid	Modulation of the autophagic machinery by activation of mTOR pathway in Parkinson's disease rat model.	Jayaraj et al., 2020[241]
1-methylnicotinamide	The related enzyme, Nicotinamide N-methyl transferase (NNMT), negatively regulates autophagy	Shin et al., 2018[242]

Dopamine	Induces autophagy (LC3-II activation) and exerts toxic effect on neuroblastoma cells	Giménez-Xavier et al., 2009[243]
Gamma-aminobutyric acid (GABA)	GABAergic signaling promotes antibacterial autophagy	Kim et al., 2018[244]
3-hydroxybutyrate (beta-hydroxybutyrate)	Stimulates the autophagic flux in cortical neurons cultured under glucose deprivation	Camberos-Luna et al., 2016 [245]
Indolepropionic acid (IPA)	IPA suppresses NF-κB signaling and decreases the release of proinflammatory cytokines in a rat model of high-fat diet	Zhao et al., 2019[246]
Violacein	Impairs the autophagic process in RAS- and RAF-mutated melanoma cells	Gonçalves et al., 2016[247]
1-phenyl-1,2-propanedione	Scarce available data	
Acide 3-(4-hydroxyphényl) propionique	Scarce available data	
3,4-dihydroxymandelic acid (DHMA)	Scarce available data	
GG-lysine	L-Lysine stimulates Akt/mTOR and inhibits Autophagic Proteolysis	Sato et al., 2015 [248]
Ferulic acid	Activates basal autophagy by inhibiting mTOR (TORC1) in mouse primary hepatocytes	Bian et al. 2013[249]
Vitamin B1 (thiamine)	Thiamine deficit induces up-regulation of autophagic markers (LC3-II, Beclin1, Atg5) and autophagosome accumulation	Meng et al., 2013[250]
Vitamin B2 (riboflavin)	Scarce available data	
Vitamin B3 (nicotinamide)	SIRT1-dependent induction of autophagy and modulation of mTOR pathways	Maiese, 2020[251]
Vitamin B5 (pantothenic acid)	Vit B5 is an obligatory precursor of acetyl-CoA. Depletion of acetyl-CoA induces autophagy	Mariño et al. 2014[252]
Vitamin B6 (pyridoxine)	Pyridoxine improves the immunogenicity of cisplatin-induced ICD (mechanism still unclear)	Aranda et al., 2014[253]
Vitamin B6 (pyridoxal)	Pyridoxine improves the immunogenicity of cisplatin-induced ICD (mechanism still unclear)	Aranda et al., 2014[253]
Vitamin B6 (pyridoxamine)	Pyridoxine improves the immunogenicity of cisplatin-induced ICD (mechanism still unclear)	Aranda et al., 2014[253]
Vitamin B7 (biotin)	Inhibits autophagy and elicits endoplasmic reticulum stress in adipocytes	Selvam et al., 2019[254]
Vitamin B9 (folic acid)	FA deficiency induces autophagy enhancement (autophagosome accumulation and LC3 and Beclin1 overexpression) in neuronal cells	Zhao et al., 2016[255]

Vitamin B12 (cobalamin)	Vit B12 and FA prevent autophagic inhibition induced by hyperhomocysteinemia	Tripathi et al., 2016[256]
Sarcosine	Activates autophagy and enhances the autophagic flux in cultured cells	Walters et al. 2018[257]
Equol	Seems to reverse the effects of zearalenone (among which there is an autophagy induction) on ovarian preantral follicles	Silva et al., 2019[258]
Prodigiosin	Inhibits autophagy and induces apoptosis of K562 leukemia cell line	Ji et al., 2019[259]
Phenazine-1-carboxylic acid	Impairs vesicular trafficking and autophagy in Saccharomyces cerevisiae	Zhu et al., 2017[260]
Pyocyanin	Induces autophagy by EIF2AK4/GCN2 pathway in Beas-2B cells	Yang et al., 2016[261]
1-hydroxyphenazine	Inhibition of autophagy protects astrocytoma cells against 1-hydroxyphenazine induced toxicity	McFarland et al., 2011[262]
2-aminoacetophenone (2AA)	Induces oxidative stress and apoptosis in murine skeletal muscle	Bandyopadhaya et al., 2016[263]
1,4-dihydroxy-2-naphthoic acid (DHNA)	Scarce available data	
O-desmethylangolensin (ODMA)	Scarce available data	
Cyclic-di-AMP sodium salt	The cytosolic DNA sensor, cyclic GMP-AMP synthase, promotes the autophagic targeting of M. tuberculosis	Waston et al., 2015[264]
5-hydroxy-L-tryptophan	Serotonin has been hypothesized to inhibits autophagy thus enhancing gut inflammation	Haq et al., 2019[265]
5-hydroxyindole acetic acid (5HIAA)	Induces apoptotic cell death of prostate and bladder cancer	Jeong et al., 2011[266]
Indole-3-butyric acid	It is converted to indole 3-acetic acid in a peroxisomal β-oxidation process	Damodaran et al., 2019[267]
Queuine	Scarce available data	
L-(+)-Ergothioneine	Antioxidant cryoprotection	Paul and Snyder, 2010[268]
Pyrroloquinoline quinone	Protects cell from autophagy-dependent doxorubicin-induced apoptosis	Jiang et al., 2019[269]
GG-leucine	Amino acids are feedback inhibitors of autophagy. Leucine acts in a GLUD1-dependent fashion	Lorin et al., 2013[270]
GG-tryptophan	Induces mTOR activation	Osawa et al., 2011[271]
GG-tyrosine	Inhibits autophagy	Meijer et al.,2015[220]

Sodium hydrosulfide	Suppress overactivated autophagy in an ischemia/reperfusion model in rats	Jiang et al., 2017[272]
Rapamycin	Inhibits mTOR. Induces autophagy	Sarkar et al. 2008[273]
Torin-1	Inhibits mTOR. Induces autophagy	Zhou et al., 2013[274]
Thapsigargin	Induces ER stress	Li et al., 2000[275]
Tunicamycin	Induces ER stress	Zhang et al., 2014[276]
Brefeldin A	Reversibly disrupts the Golgi apparatus	Sciaky et al., 1997[277]
Crizotinib R	Induces autophagy and immunogenic cell death	You et al., 2015[278] Liu et al., 2019[279]
Spermidine	Induces autophagy	Eisenberg et al., 2009[280]

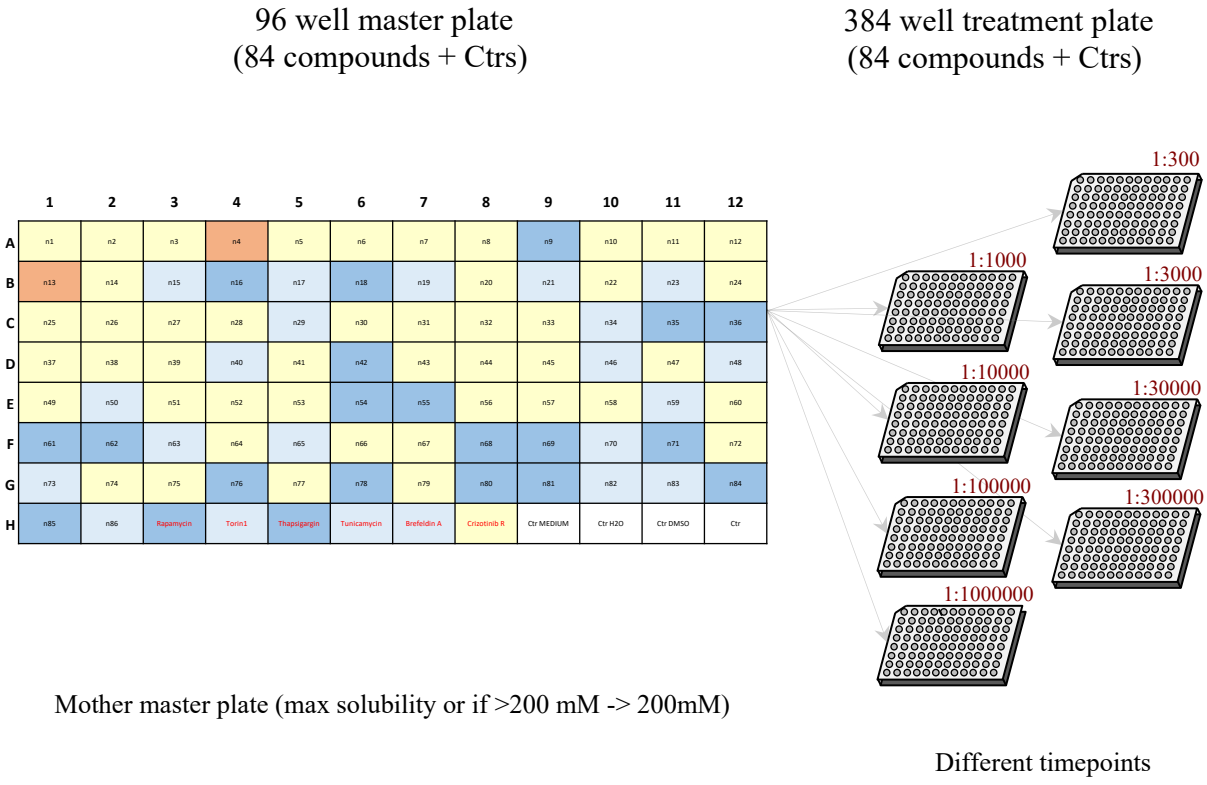
Appendix Table 1. All bacterial metabolites screened with one ref. for each of them (name, effect, reference). The compounds used as positive controls are reported in bold.

2.2 Methods

2.2.1 Screening program

We determined the maximum solubility known for each compound (or 200 mM if the maximum solubility exceeded this value) and prepared the stock solution by diluting them in DMSO (mostly) or water. We firstly organized a mother 96-wells master plate with our compounds and controls. Subsequently we treated GFP-LC3 U2OS cells in 384 well plates at different dilutions (1/300*; 1/1000; 1/3000; 1/10000; 1:30000; 1:100000; 1:300000; 1:1000000*; * the first and last dilution only when indicate according to the compound concentration in the master plate). **Suppl. Fig. 1.**

Supplementary Figure 1



Supplementary Figure 1. Scheme of the design of the screening. Cell death essay (Hoechst/PI) and LC3 dots essay by treating U2OS wt and U2OS L3-GFP cells, respectively with a wide range of dilutions of the mother stock (1/300*, 1/1000, 1/3000, 1/10000, 1/30000, 1/100000, 1/300000, 1/1000000*). *only when indicate according to the compound concentration in the master plate

2.2.1 Cell lines and culture conditions

U2OS wild-type cells and their derivatives (U2OS LC3-GFP; U2OS LAMP-GFP; U2OS GALT-GFP, U2OS SMAC-GFP; U2OS Calr-GFP H2B-RFP; U2OS TFEB-GFP) were cultured in DMEM supplemented with supplemented with 10% heat-inactivated fetal bovine serum and 10 mM HEPES buffers. PC12 GFP-Q74 cells were cultured in RPMI-1640 containing 5% fetal bovine serum and 10% horse serum. Murine colon carcinoma CT26 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffers, 10 U/ml penicillin sodium and 10μg/ml streptomycin sulfate. PC12 cells stably expressing doxycycline-inducible Q74-GFP were maintained in (RPMI)-1640 containing 5% fetal bovine serum and 10% horse serum. [281]

2.2.2 Automatized fluorescent biosensor-based screening

Human osteosarcoma U2OS cells stably expressing green fluorescent protein (GFP)-tagged LC-3 were seeded in 384-well black microplates (Greiner-bio-one, Kremsmünster, Austria) and allowed to attach for 24 h (37 °C, 5% CO2 atmosphere). After 24h the cells were treated with the compounds of a custom arrayed library of microbial compounds at different concentrations and for different durations. After treatment cells were fixed with 4%

paraformaldehyde (PFA) in PBS containing 2µM (10µg/mL) Hoechst 33342 (Thermo Scientific, Waltham, MA, USA) for 30 minutes and washed three times with PBS. Four view fields/well were acquired by ImageXpress automated widefield microscope (Molecular Devices, Sunnyvale, CA, USA). High-content image analysis was used to determine markers of autophagy such as the generation of autophagy-associated GFP-LC3 puncta in the cytoplasm. Following images were processed and segmented with the MetaXpress software (Molecular Devices) to analyze GFP-LC3 granularity. Data were analyzed using the freely available software R (https://www.r-project.org).

2.2.3 Cell death essay

U2OS wild-type cells were seeded (2,000 cells/well) in 384-well clear cell culture plates and let adhere in a humidified incubator with 5% CO2 at 37°C for 24 h before treatment. Cells were treated with a wide range of concentrations for each compound for 24 h and then co-stained by the addition of 1µg/ml propidium iodide (PI) and 2µg/ml Hoechst 33342 for 30 min at 37°C before acquisition. Images were acquired on ImageXpress Micro XL automated microscopes (4 view fields per well). Before being acquired the plates were centrifuged in order to drive detached cells to the bottom of the wells. If the cell nucleus was stained by both Hoechst and PI signals (fluorescence co-localization), the cell was considered as dead. [282]

2.2.4 Autophagic flux analysis

The autophagic flux analysis was conducted by using the U2OS GFP-RFP-LC3 tandem reporter cells and PC12 GFP-Q74 cells.

U2OS cells stably expressing LC3 fused with tandem fluorescent GFP-RFP proteins (GFP-RFP-LC3) were treated with top hits compounds that emerged from the first and second screening, for 6 h. After fixation, GFP-LC3 and RFP-LC3 dots were evaluated by automated image acquisition and analysis.

The PC12 GFP-Q74 cell is a cell line engineered to express a doxycycline-inducible autophagic cargo (namely a pathogenic huntingtin protein that contains 74 glutamine repeats, Q74, fused with GFP). [281] Different concentrations of our compounds were evaluated. Rapamycin (20 μM), Torin-1 (0.3 μM) and Bafilomycin (0.1 μM) were utilized as controls.

PC12 GFP-Q74 cell were treated for 6 h according to the experimental scheme attached.

2.2.5 Immunoblotting

U2OS WT cells were treated with the different concentrations of our compound for 6 hours. Then, cells were washed twice with cold phosphate-buffered saline (PBS) and then dislodged by using a cell scraper and collected into microcentrifuge tubes. After centrifugation the lysis buffer with protease inhibitor cocktail was added and the cells were incubated for 30 minutes on ice followed. After removal of insoluble material by centrifugation, the supernatant was transferred into a new tube and the concentration of protein was assessed by using a spectrophotometer. For each sample, 20 μg of protein was resolved on polyacrylamide gel electrophoresis gel (Invitrogen). After migration protein were transferred to polyvinylidene difluoride membranes (Merck Millipore). LC3 and p62 protein levels were measured by SDS–PAGE and immunoblot. Beta-actin was measured as loading control. Band intensities of p62, LC3-I, and LC3-II were measured, and ratios of p62 or LC3-II vs. Beta-actin. (LC3-II/Actin, p62/Actin) were calculated.

2.2.5 TFEB translocation and cotreatments with protein synthesis inhibition or lysosome inhibitors in vitro

U2OS cells stably expressing GFP-TFEB fusion protein were treated with different concentrations of our compound for 6 h. GFP intensities in nuclei and cytoplasm were measured. The ratio of GFP intensities (intensity in nuclei/intensity in the cytoplasm) was estimated to assess TFEB translocation into the nuclei.

We further evaluated the ability of our compound to still induce LC3-II in the presence of transcription or protein synthesis inhibitors (with actinomycin D or cycloheximide) and inhibitor of the lysosomal proton pump (Bafilomycin) in vitro.

2.5×10^3 U2OS-GFP-LC3 cells/well were plated in a 384 well plate and cotreated (after 24h) with our selected compound in combination with 1 μM dactinomycin (DACT), 50 $\mu g\, ml^{-1}$ cycloheximide (CHX) and Bafilomycin (0.1 μM) respectively, for 6 h. After fixation, GFP-LC3 dots were evaluated by automated image acquisition and analysis.

2.2.6 Co-localization experiment:

Prodigiosin is well-known for its autofluorescence. Prodigiosin signal was measured at an excitation of 561 nm and an emission of 594 nm (Texas Red). Briefly, for the co-localization experiment human osteosarcoma U2OS biosensors cells expressing the fluorescent fusion GALT1-GFP (for the Golgi apparatus), CALR-GFP/H2B-RFP (for the ER), LAMP1-GFP (for the lysosomes) and SMAC-GFP (for the mitochondria) were plated on 384-well black microplates (Greiner-bio-one, Kremsmünster, Austria). Image segmentation and colocalization of auto-fluorescent compounds with the above-mentioned organelles were performed by the ColocalizR analysis algorithm. [283]

2.2.7 In vivo experiment

Syngeneic BALB/c female mice were implanted with CT26 mouse colon cancer cell line. Mice were used between 6 and 8 weeks of age. 1×10^6 CT26 cells were mixed with 100 µL PBS and injected subcutaneously into one flank. Once visible, tumors were measured across two diameters, and volumes were calculated. Body weights were also recorded. When tumors reached 20 to 35 mm² in size mice were treated according the scheme reported in **Figure 6a** with isotype control, clone 2A3, plus prodigiosin vehicle (controls); anti-PD-1 mAb (250µg/mouse; clone RMP1-14) plus vehicle; Prodigiosin plus isotype control; Prodigiosin plus anti-PD-1 mAb; anti-PD1 plus anti-CTLA-4 (100µg of anti-CTLA-4 mAb (clone 9D9). The size of the tumor was monitored after three days from the treatment by means of a caliper and mice were then sacrificed. The tumor size was calculated as: length(mm) x width(mm) = tumor size (mm^2). The heart, the liver, the spleen, the colon, the ileum, and the tumors were collected after the sacrifice. 1% DMSO/12%PEG 400 in normal saline was chosen as drug delivery vehicle for prodigiosin according to previous report. Firstly 1 mg prodigiosin was dissolved in 1 mL of dimethyl sulfoxide in a 20µL DMSO/240µL PEG 400 solution. Then 1740 normal saline was added to obtain the primary emulsion. [284] A fixed volume of 200µL was administered intraperitoneally in each mouse to achieve a dose of 5 mg/kg. Data analyses were performed either with the Prism 8 (GraphPad, San Diego, CA, USA) software. Tumor size differences were calculated either using two-way analysis of variance (ANOVA) and post hoc T-test with Bonferroni Correction (to compare the group treated with Prodigiosin plus Anti-PD-1 mAb versus Prodigiosin alone). To date, only one experiment has been performed.

Statistical analyses

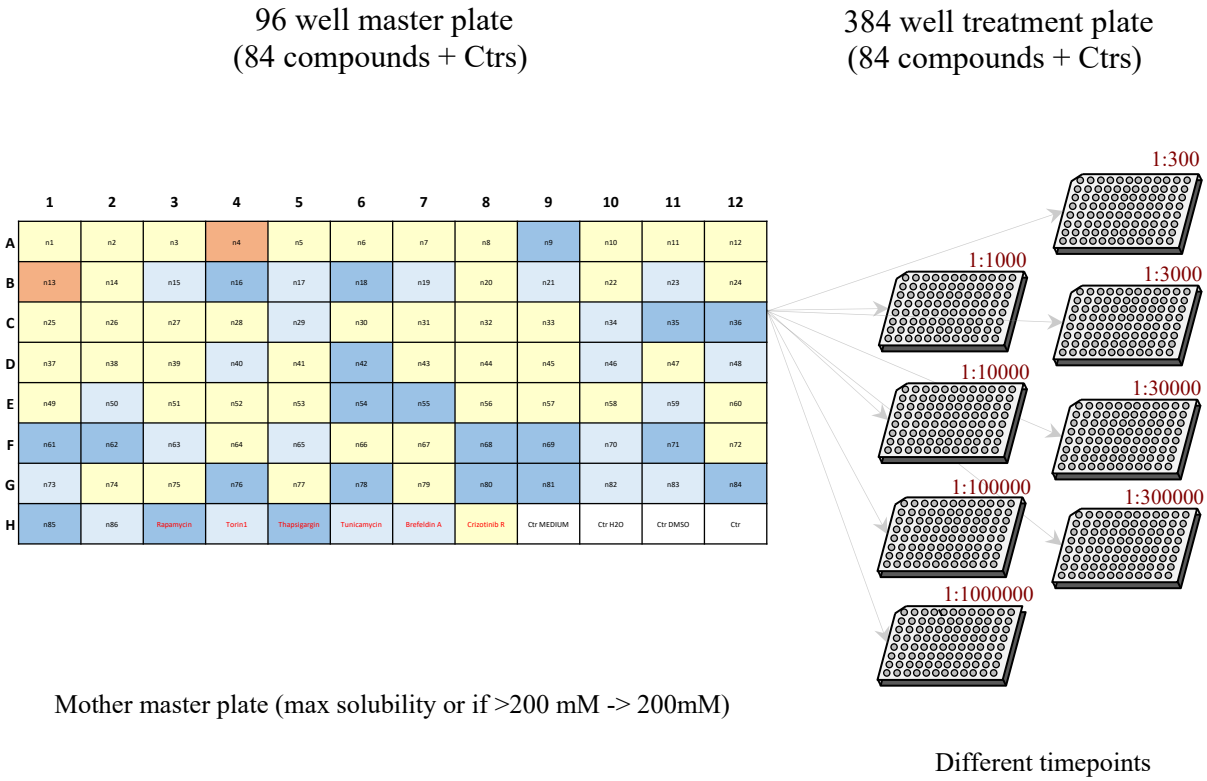
Unless otherwise specified, data are reported as mean ± SD of at least three independent experiments. Data were analyzed using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) and R software. Statistical significance was analyzed by means of two-tailed Student's t-test or ANOVA tests, as appropriate. Differences to negative controls were assessed to be significant if $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

2.3. Results

2.3.1 Identification of prodigiosin as a '*bona fide*' autophagy disruptor

In an effort to identify new autophagy inducers, we initially screened a library of 85 bacterial metabolites (**Appendix Table 1** in the introduction) for their capacity to stimulate the generation of cytoplasmic GFP-LC3 dots in human osteosarcoma U2OS GFP-LC3 cells.

The screening approach is summarized in **Suppl. Fig. 1** (description in the methods subsection).



Supplementary Figure1. Scheme of the design of the screening.

From our first screen we selected the bacterial metabolites that showed the strongest effect. **Fig 1a** We further validate the results by a second validation screening. **Fig 1b**

Fig 1a

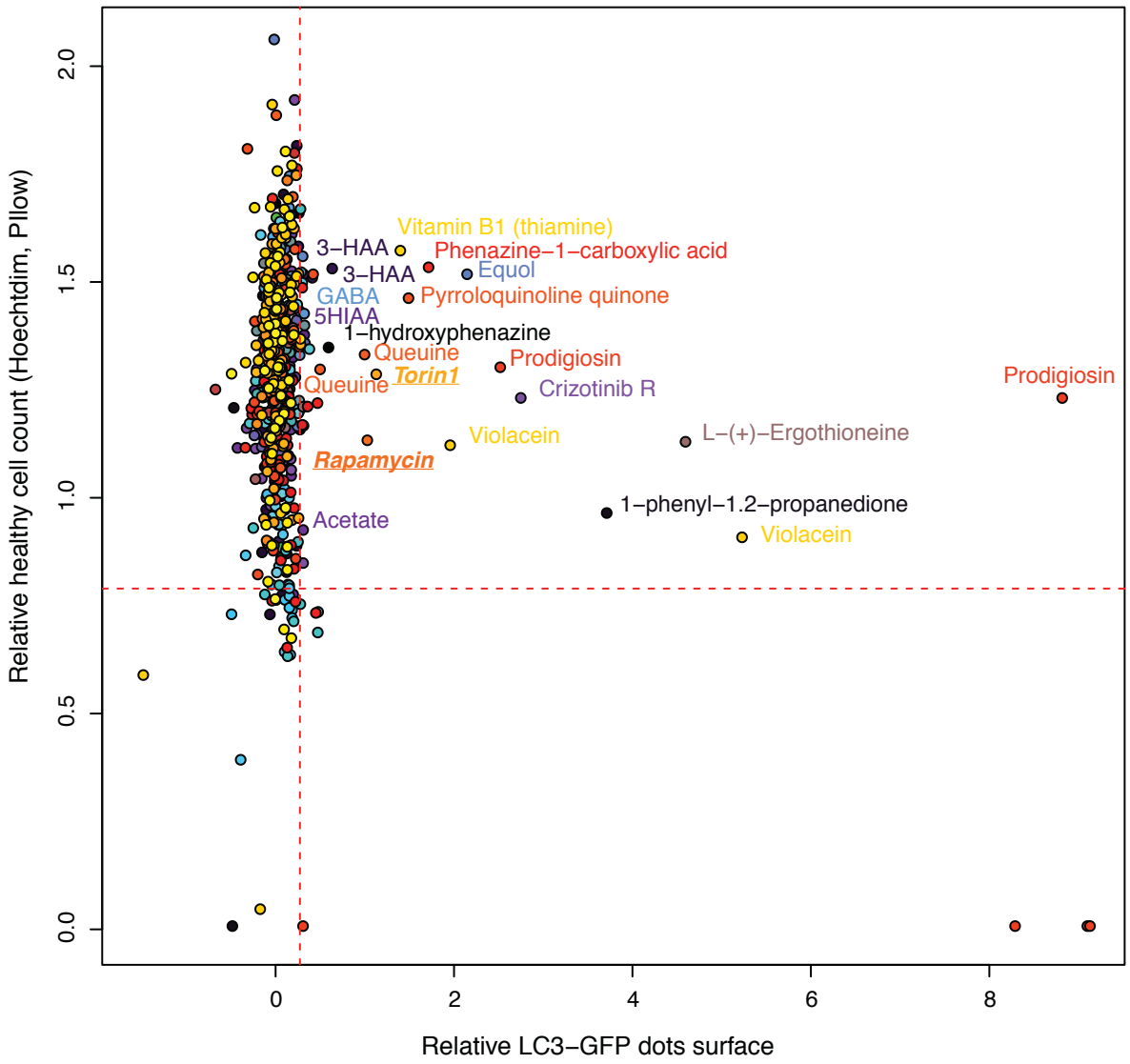


Figure 1a. Human osteosarcoma U2OS GFP-LC3 cells and U2OS wt cells were treated with a library of bacterial metabolites for 6 h (compounds are listed in Appendix Table 1). After the treatment U2OS GFP-LC3 cells were fixed while U2OS wt cells were stained with Hoechst and PI for the cell death essay. GFP-LC3 dots were counted to measure autophagy activity. Scatter plots depicting the relative healthy cell count versus the LC3-GFP dots surface.

Fig 1b

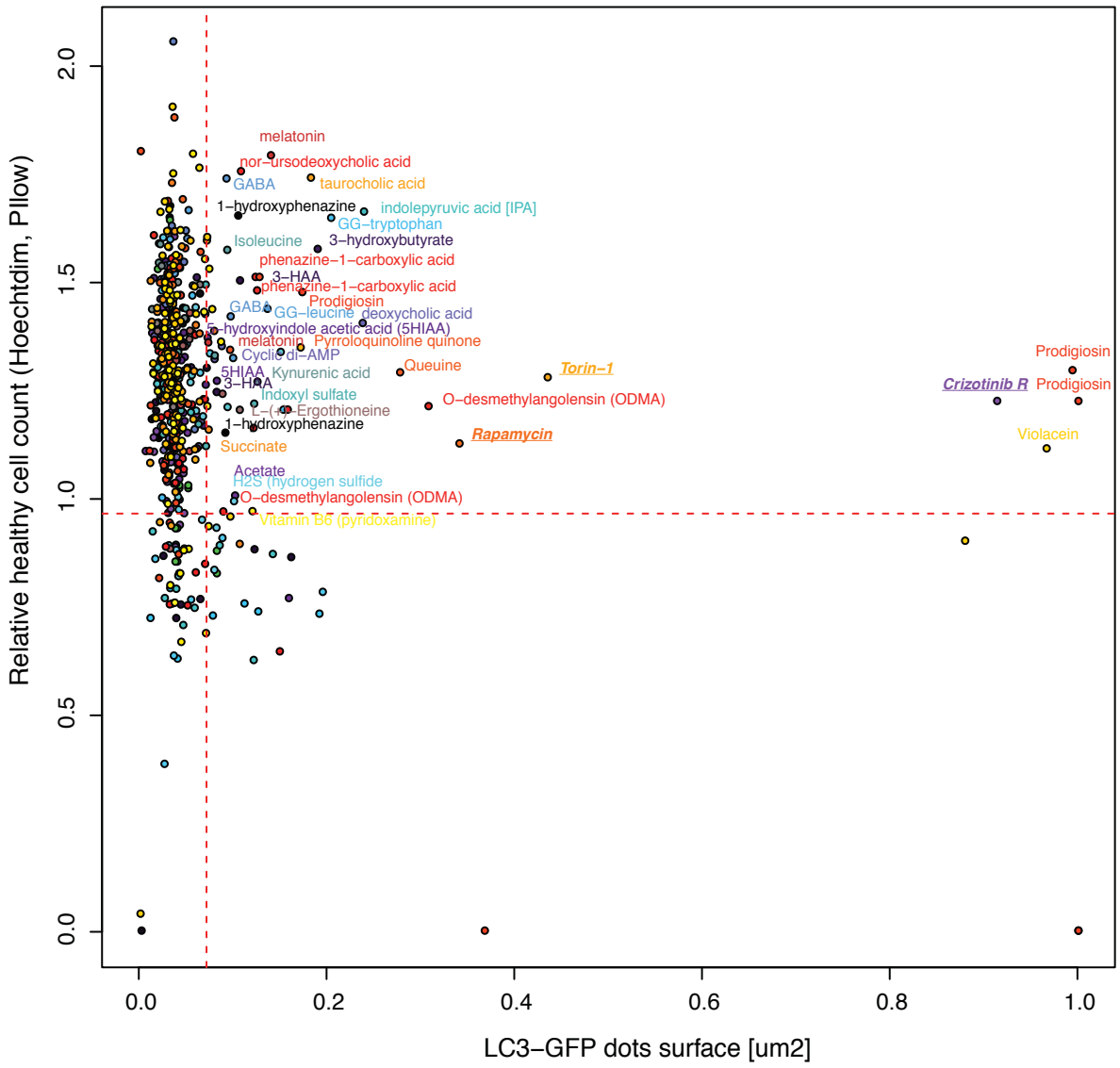


Figure 1b. Human osteosarcoma U2OS GFP-LC3 cells and U2OS wt cells were treated with a library of bacterial metabolites for 6 h (compounds are listed in Appendix Table 1). After the treatment U2OS GFP-LC3 cells were stained with DAPI and fixed, while U2OS wt cells were stained with Hoechst and PI for the cell death essay. GFP-LC3 dots were counted to measure autophagy activity. Scatter plots depicting the relative healthy cell count versus the LC3-GFP dots surface.

We then rescreened the hits with the U2OS GFP-LC3 (for a third time) **Fig 1c** and with U2OS GFP-RFP-LC3 tandem reporter cells to deeper investigate their activity on the autophagic flux. **Fig 1d-e**

Fig 1c

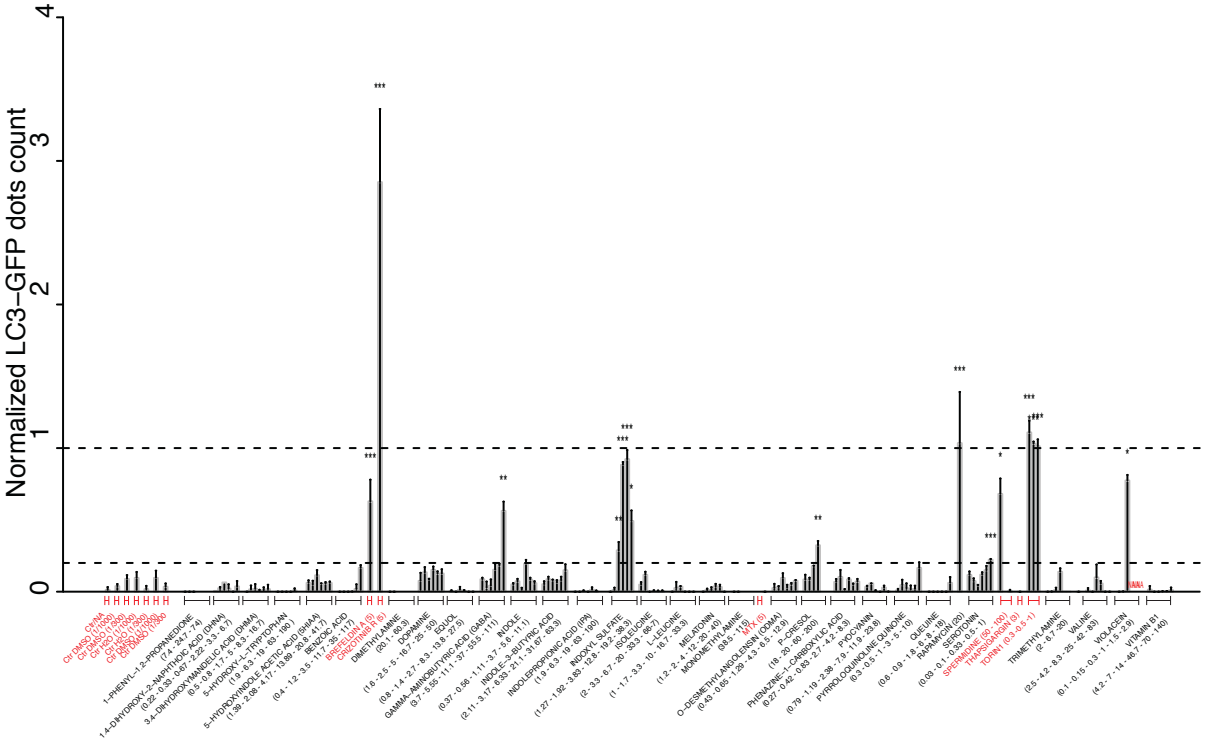


Figure 1c. Detection of GFP-LC3 puncta from compound-mediated autophagy in U2OS GFP-LC3 cells. Human osteosarcoma U2OS GFP-LC3 cells were treated with the hits compound of the previous screening. After the treatment U2OS GFP-LC3 cells were stained fixed. GFP-LC3 dots were counted to measure autophagy activity. Bar chart the quantitation of LC3-GFP dots.

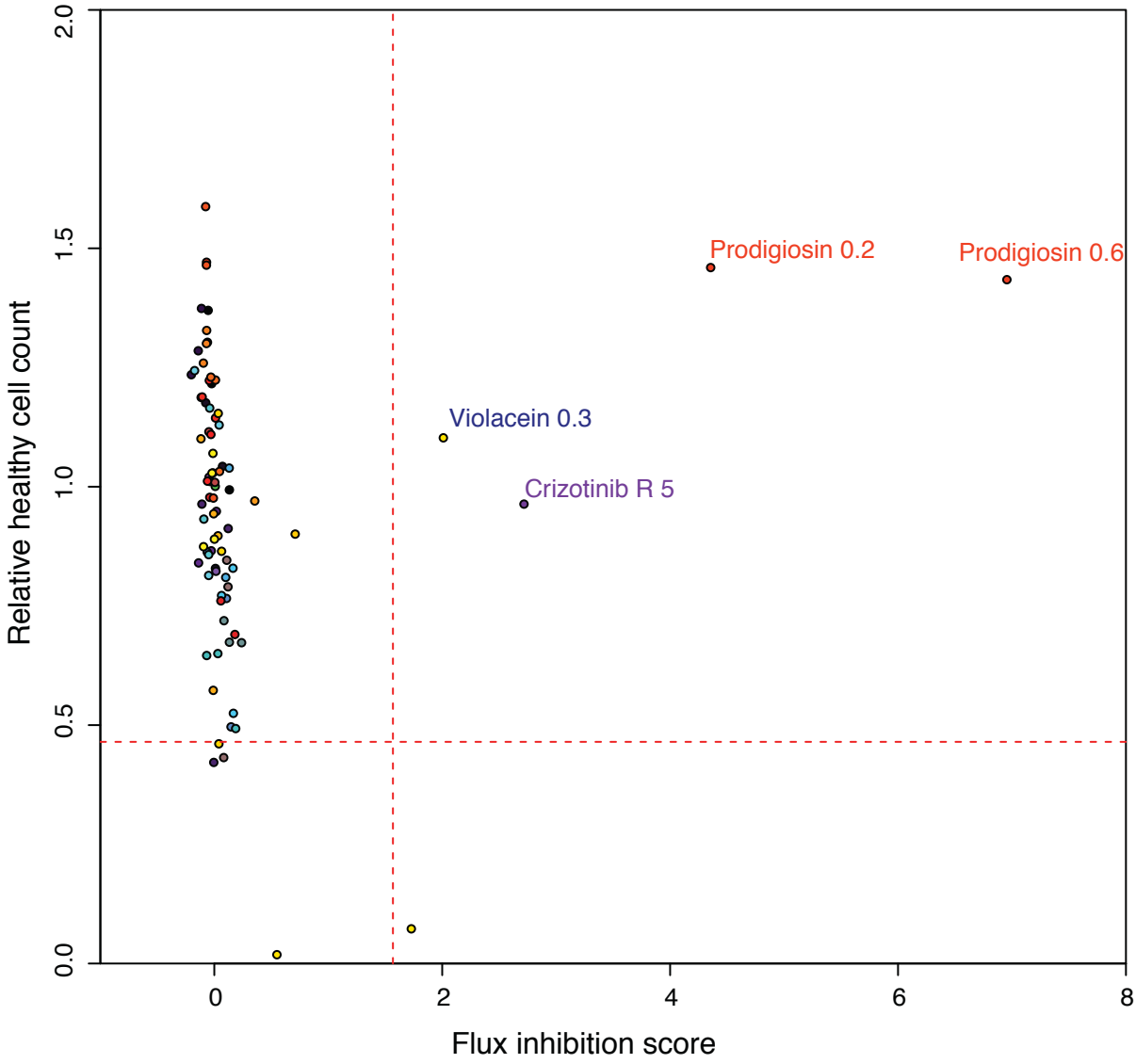


Figure 1d. Human osteosarcoma U2OS GFP-LC3 cells were treated with the hits compound of the previous screening. After the treatment U2OS GFP-LC3 cells were stained fixed. GFP-LC3 dots were counted to measure autophagy activity. Scatter plots depicting the relative healthy cell count versus the flux inhibition score. Scatter plots depicting the relative healthy cell count versus the flux inhibition score.

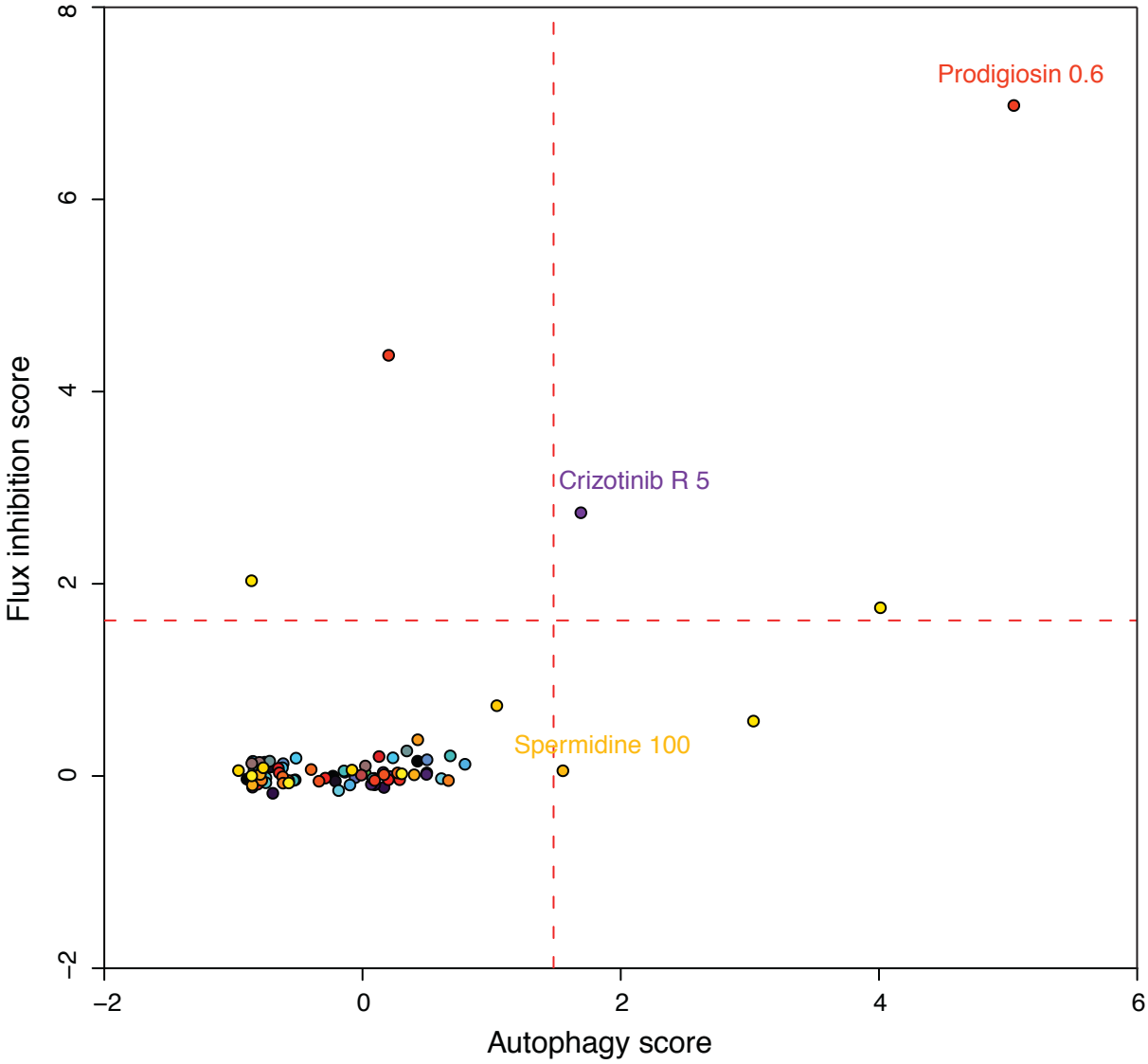


Figure 1e. Human osteosarcoma U2OS RFP-GFP-LC3 tandem report cells were treated with the hits compound of the previous screening. After the treatment U2OS RFP-GFP-LC3 cells were stained fixed. GFP-LC3 and RFP-LC3 dots were counted to measure autophagy activity. Scatter plots depicting the flux inhibition score versus the autophagy score.

Given the pronounced activity of this compound even at low concentration we selected prodigiosin from our screen. The pattern of GFP fluorescence induced by prodigiosin was slightly different from that induced by torin1. The GFP-LC3 puncta tended to fuse at one district around the nucleus. We decided to further investigate on this compound.

We repeated the experiment on U2OS GFP-RFP-LC3 tandem reporter cells for Prodigiosin and violacein. In U2OS cells stable expressing a tandem GFP-RFP-LC3 fusion protein [285], prodigiosin increased the abundance of the autophagosomes (RFP and GFP fluorescence) but not the autolysosomes (RFP fluorescence only), partially contrasting with the autophagic flux inhibitors bafilomycin A1 (BafA1), which induced an increase of GFP fluorescence by interfering with the autolysosome generation. **Fig 1f and Fig 1g**

Fig 1f

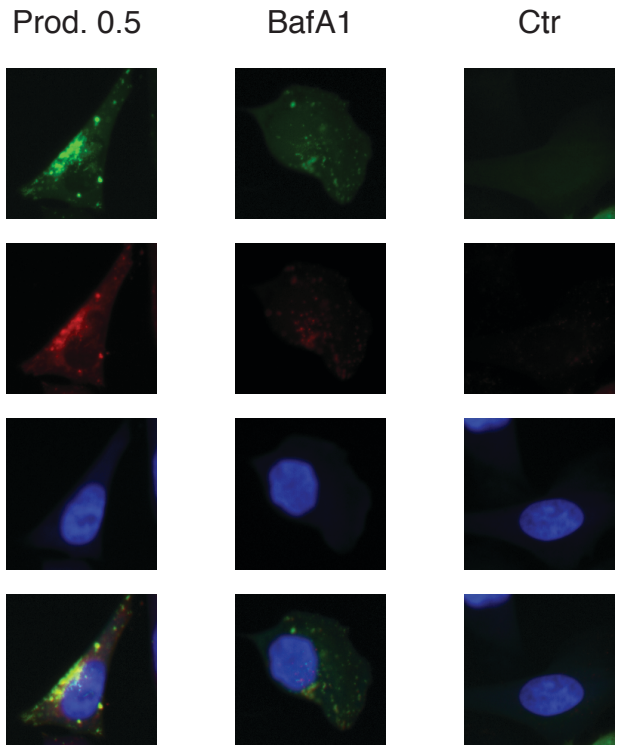


Figure 1f. Representative images of U2OS LC3 GFP-RFP tandem report cell treated with prodigiosin (Prod.) 0.5μM, bafilomycin A1 (BafA1) 0.1μM, and controls.

Fig 1g

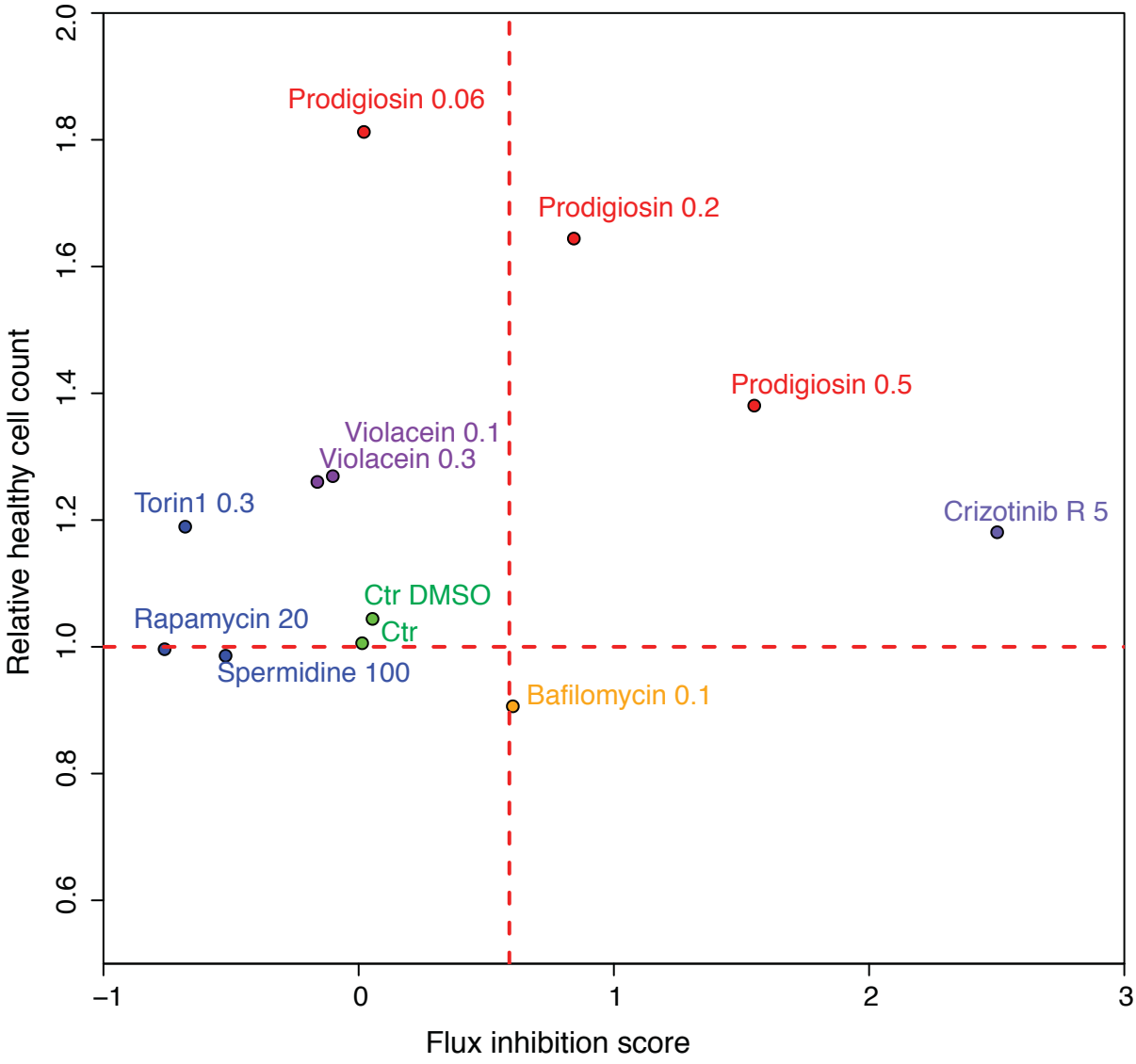


Figure 1g. Human osteosarcoma U2OS RFP-GFP-LC3 tandem report cells were treated with the hits compound of the previous screening. After the treatment U2OS RFP-GFP-LC3 cells were stained fixed. GFP-LC3 and RFP-LC3 dots were counted to measure autophagy activity. Scatter plots depicting the relative healthy cell count versus the flux inhibition score.

As mentioned above, prodigiosin can cross the lysosomal membrane, reach its protonated configuration due to the high intralysosomal H^+ concentration and export a Cl- ion from the lysosome to the cytosol together with three H^+. Inducing de-acidification of the lysosomes it acts as an autophagy disruptor. [72].

We then evaluated the effect of prodigiosin cotreating cell with the inhibitor of protein synthesis cycloheximide (CHX) and the RNA synthesis inhibitor actinomycin D (ActD), and with the V-ATPase and autophagic flux blocker bafilomycin A1 (BafA1). Cycloheximide (CHX) and Actinomycin D (ActD) did not prevent the induction of GFP-LC3 dots by prodigiosin. **Fig 2a-c**

Fig 2a

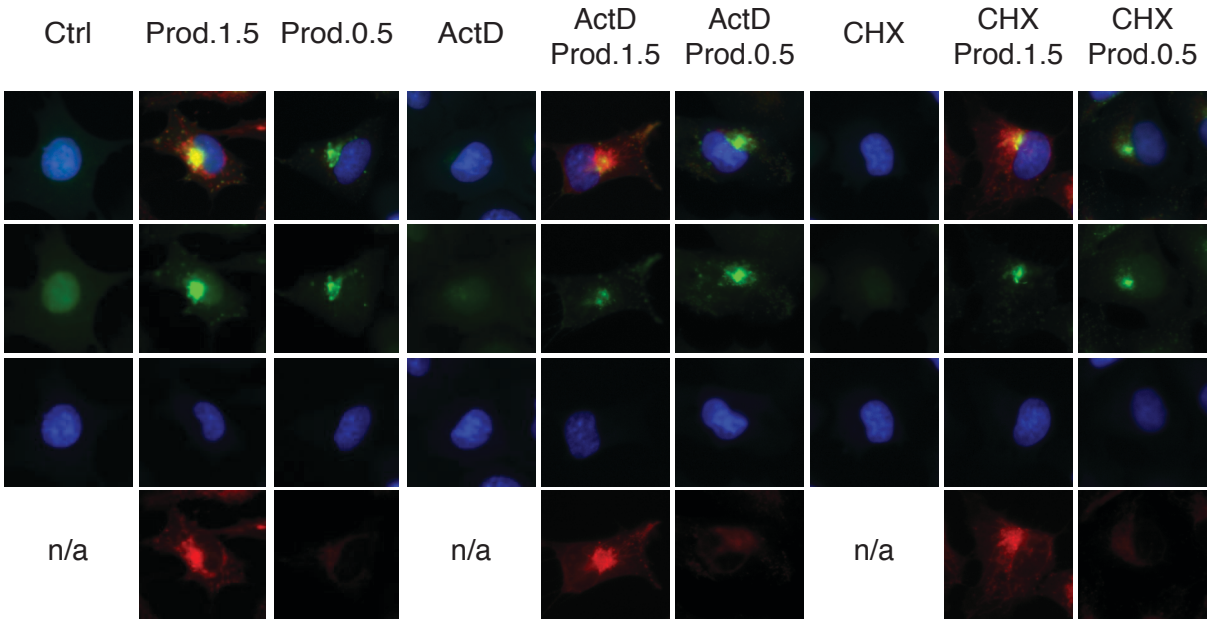


Figure 2a. Representative images of U2OS GFP cell treated with prodigiosin (Prod.) 0.5µM and 1.5µM alone or in combination with actinomycin D (ActD) and Cycloheximide (CHX).

Fig 2b

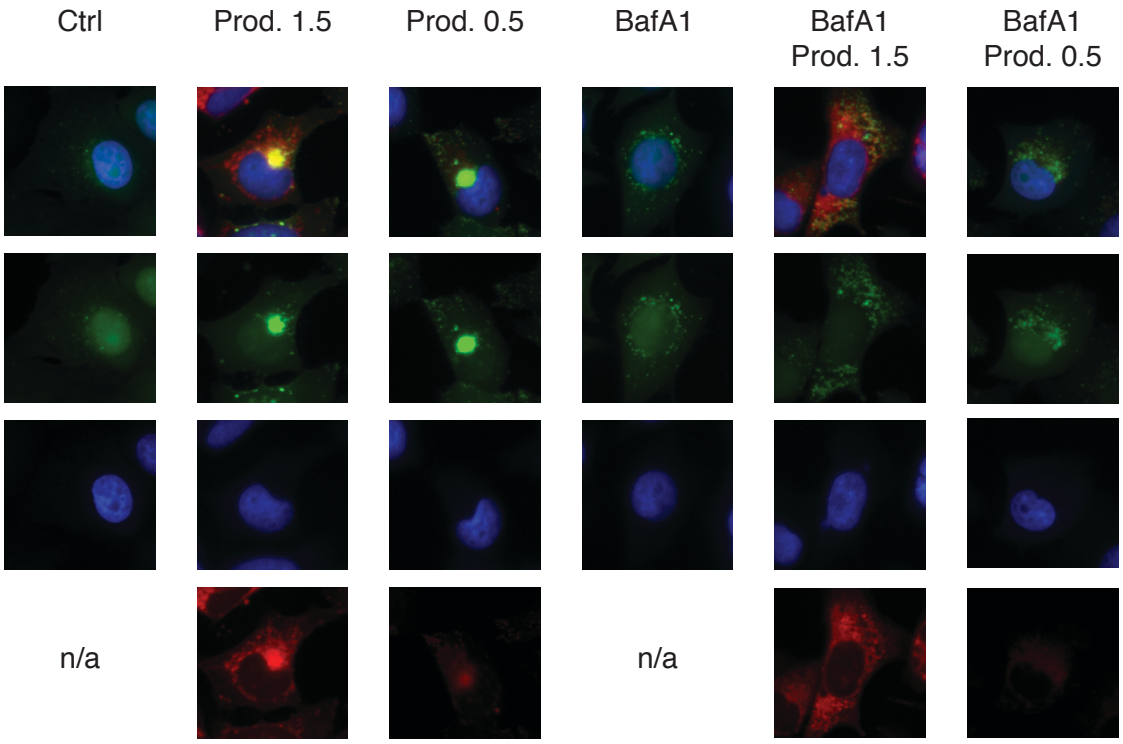
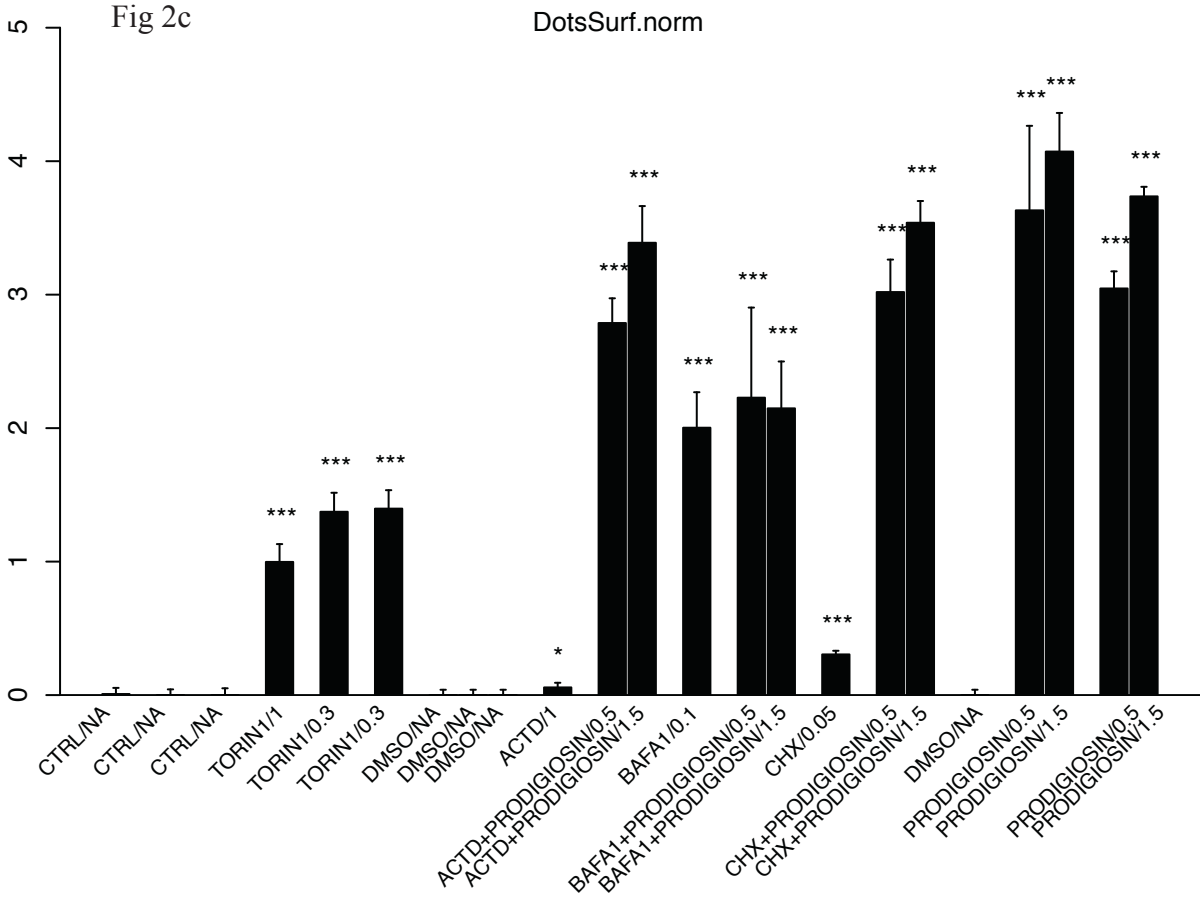


Figure 2b. Representative images of U2OS GFP cell treated with prodigiosin (Prod.) 0.5μM and 1.5μM alone or in combination with bafilomycin A1 (BafA1) 0.1μM.

Fig 2c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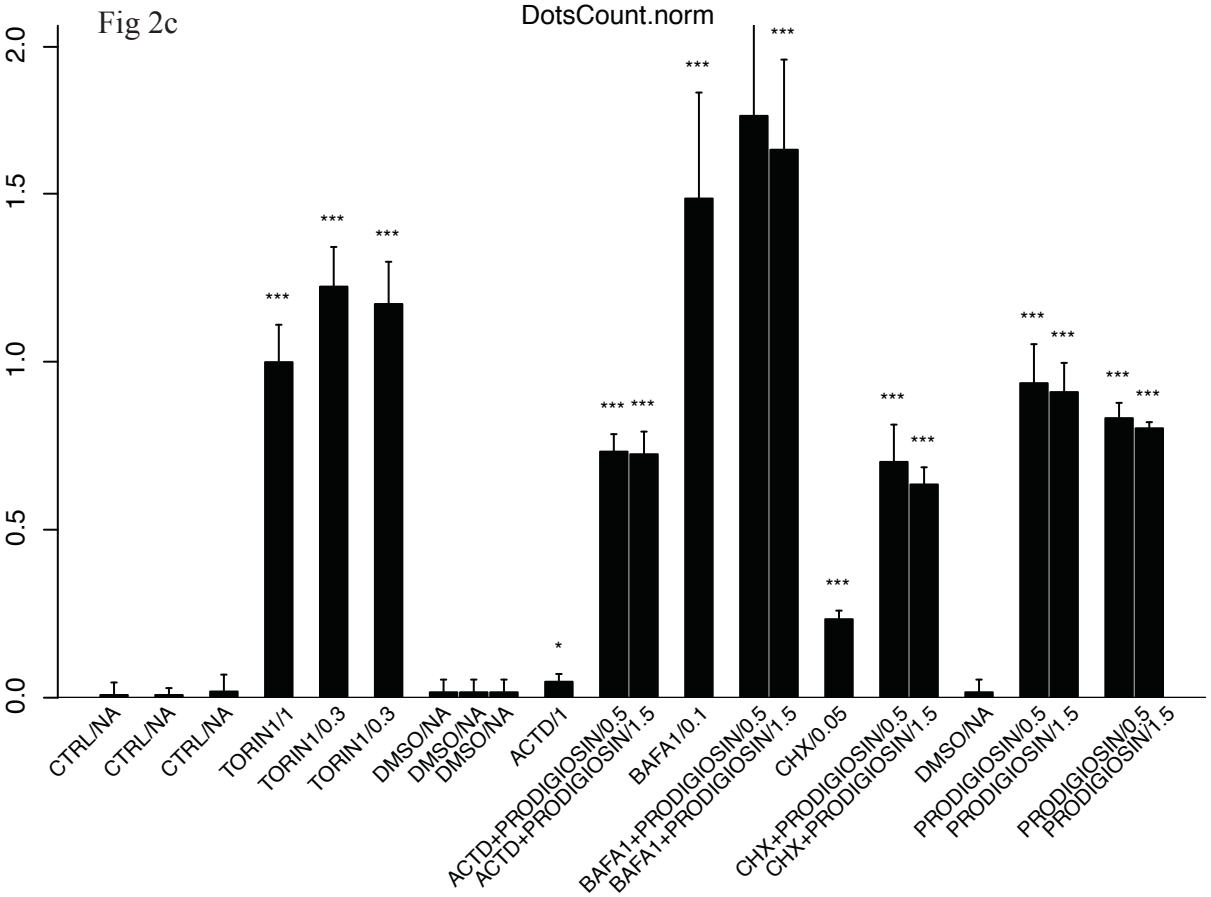
1
2
3
4
5
CTRL/NA
CTRL/NA
CTRL/NA
TORIN1/1
TORIN1/0.3
TORIN1/0.3
DMSO/NA
DMSO/NA
DMSO/NA
ACTD/1
ACTD+PRODIGIOSIN/0.5
ACTD+PRODIGIOSIN/1.5
BAFA1/0.1
BAFA1+PRODIGIOSIN/0.5
BAFA1+PRODIGIOSIN/1.5
CHX/0.05
CHX+PRODIGIOSIN/0.5
CHX+PRODIGIOSIN/1.5
DMSO/NA
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5

Fig 2c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1.0
1.5
2.0
CTRL/NA
CTRL/NA
CTRL/NA
TORIN1/1
TORIN1/0.3
TORIN1/0.3
DMSO/NA
DMSO/NA
DMSO/NA
ACTD/1
ACTD+PRODIGIOSIN/0.5
ACTD+PRODIGIOSIN/1.5
BAFA1/0.1
BAFA1+PRODIGIOSIN/0.5
BAFA1+PRODIGIOSIN/1.5
CHX/0.05
CHX+PRODIGIOSIN/0.5
CHX+PRODIGIOSIN/1.5
DMSO/NA
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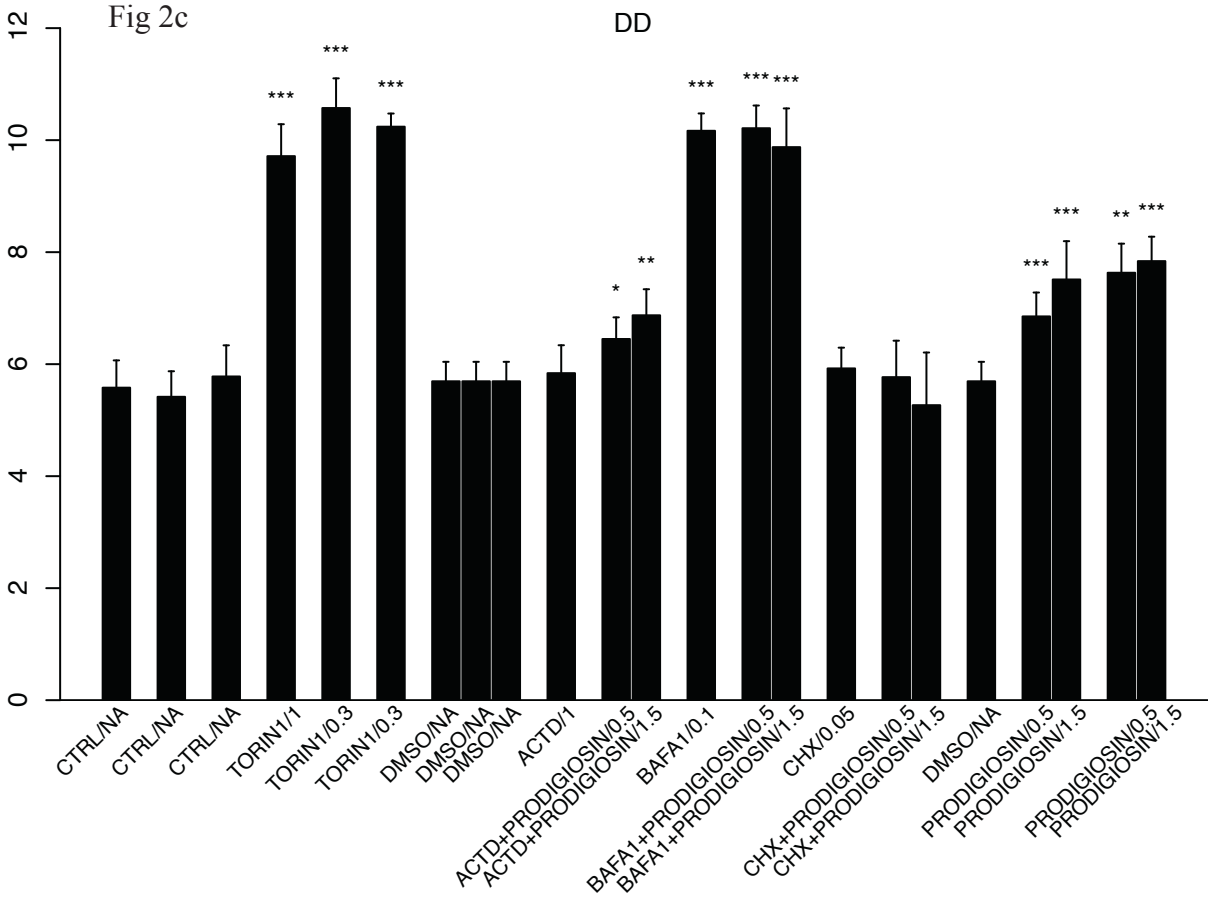
PRODIGIOSIN/1.5
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5

Fig 2c


DD
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2
4
6
8
10
12
CTRL/NA
CTRL/NA
CTRL/NA
TORIN1/1
TORIN1/0.3
TORIN1/0.3
DMSO/NA
DMSO/NA
DMSO/NA
ACTD/1
ACTD+PRODIGIOSIN/0.5
ACTD+PRODIGIOSIN/1.5
BAFA1/0.1
BAFA1+PRODIGIOSIN/0.5
BAFA1+PRODIGIOSIN/1.5
CHX/0.05
CHX+PRODIGIOSIN/0.5
CHX+PRODIGIOSIN/1.5
DMSO/NA
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5

Fig 2c

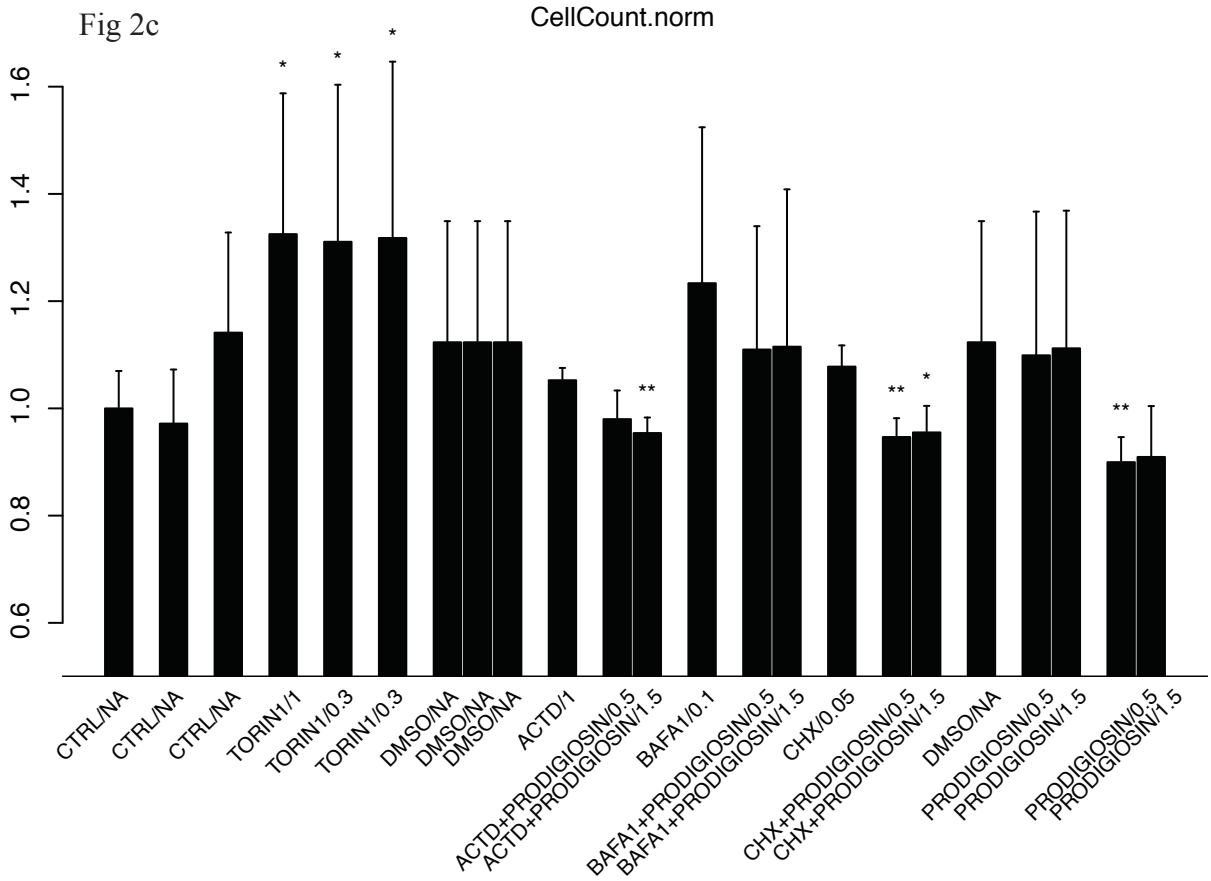

CellCount.norm
0.6
0.8
1.0
1.2
1.4
1.6
CTRL/NA
CTRL/NA
CTRL/NA
TORIN1/1
TORIN1/0.3
TORIN1/0.3
DMSO/NA
DMSO/NA
DMSO/NA
ACTD/1
ACTD+PRODIGIOSIN/0.5
ACTD+PRODIGIOSIN/1.5
BAFA1/0.1
BAFA1+PRODIGIOSIN/0.5
BAFA1+PRODIGIOSIN/1.5
CHX/0.05
CHX+PRODIGIOSIN/0.5
CHX+PRODIGIOSIN/1.5
DMSO/NA
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5
PRODIGIOSIN/0.5
PRODIGIOSIN/1.5

Figure 2c. Bar chart. Data are expressed as means ± SD of one representative experiment and represent the GFP dots surface per cell, the GFP dots counts per cell, the DD and the cell count. (*p < 0.05, ***p < 0.0001, compared to untreated cells, Ctr DMSO).

In this condition we did not observed the fluorescence pattern induced by Prodigiosin alone. We hypothesize that the blocking of the V-ATPase by BafA1 results in impaired concentration of H^+ within the lysosome and consequently lysosomal alkalinization. In this context Prodigiosin cannot act as H^+/Cl^- symporter from the lysosome to the cytosol. Conversely, treating the cell with Prodigiosin for 3h at first and then, after changing the culture medium, with BafA1 for other 3h we observed that the florescence phenotype induced by prodigiosin seem to reverse. **Fig 2d-e**

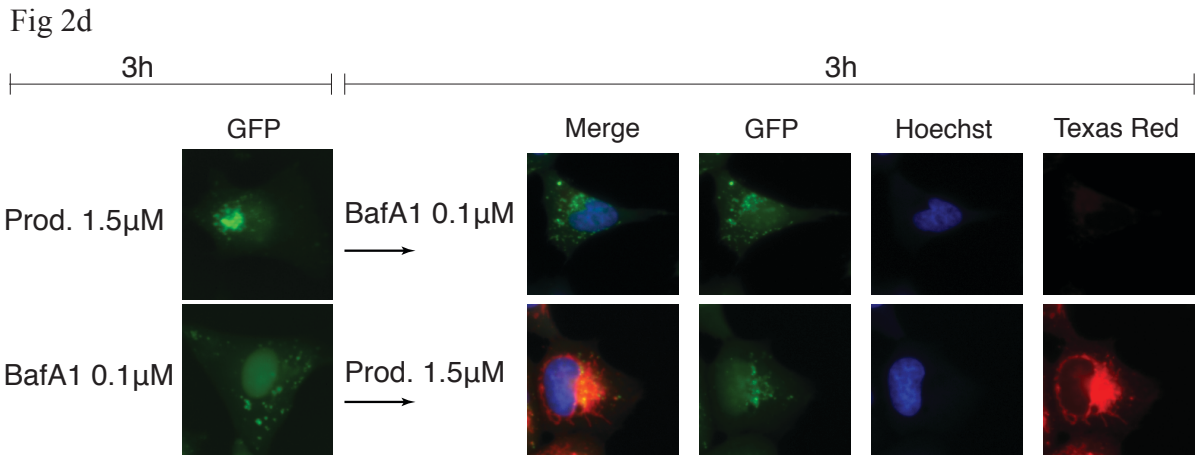
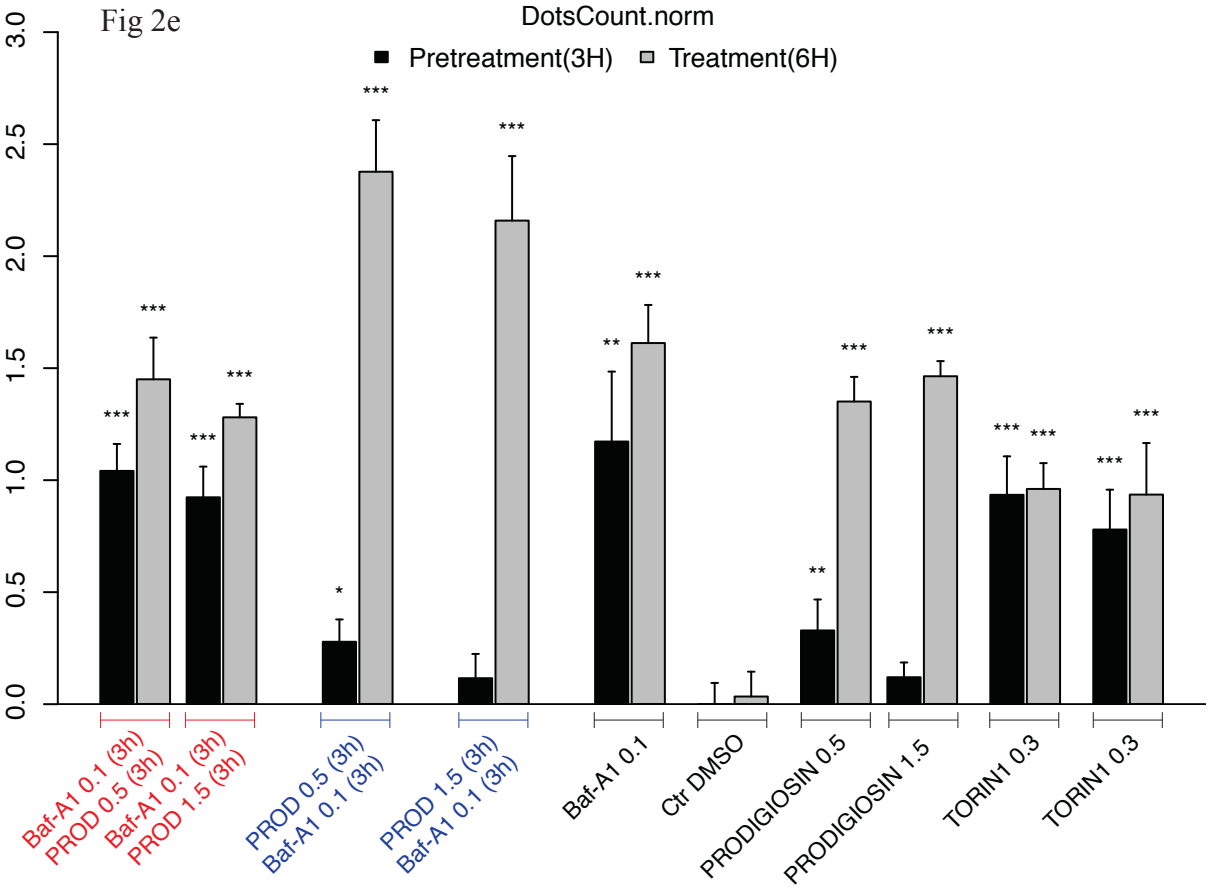


Figure 2d. Representative images of U2OS GFP cell after treatment with prodigiosin (Prod.)1.5µM and Bafilomycin (BafA1) 0.1µM for 3 hours followed by other 3 hours of switched treatment with Bafilomycin (BafA1) 0.1µM and prodigiosin (Prod.)1.5µM, respectively.

Fig 2e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Pretreatment(3H)
Treatment(6H)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Baf-A1 0.1 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
Baf-A1 0.1 (3h)
Baf-A1 0.1
Ctr DMSO
PRODIGIOSIN 0.5
PRODIGIOSIN 1.5
TORIN1 0.3
TORIN1 0.3

Fig 2e

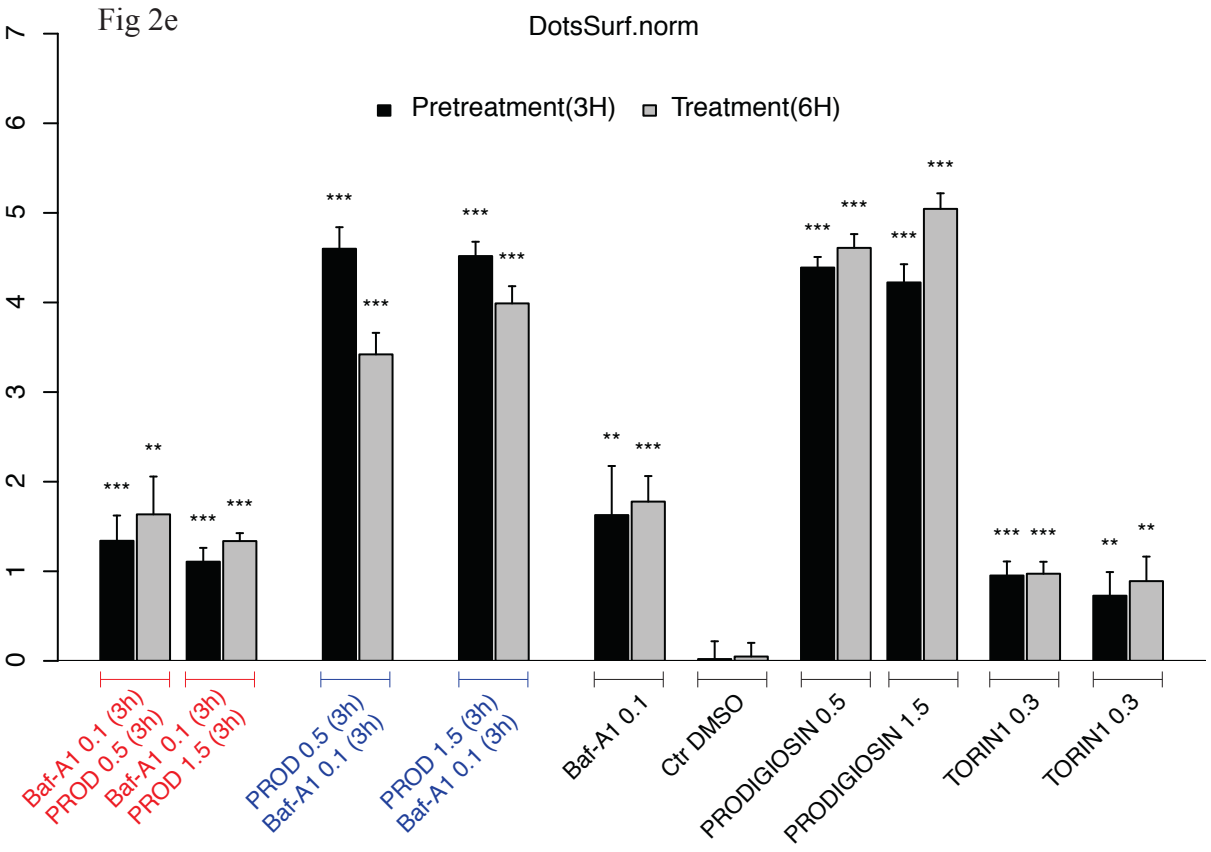
DotsSurf.norm
Pretreatment(3H)
Treatment(6H)
0
1
2
3
4
5
6
7
Baf-A1 0.1 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
Baf-A1 0.1 (3h)
Baf-A1 0.1
Ctr DMSO
PRODIGIOSIN 0.5
PRODIGIOSIN 1.5
TORIN1 0.3
TORIN1 0.3

Fig 2e

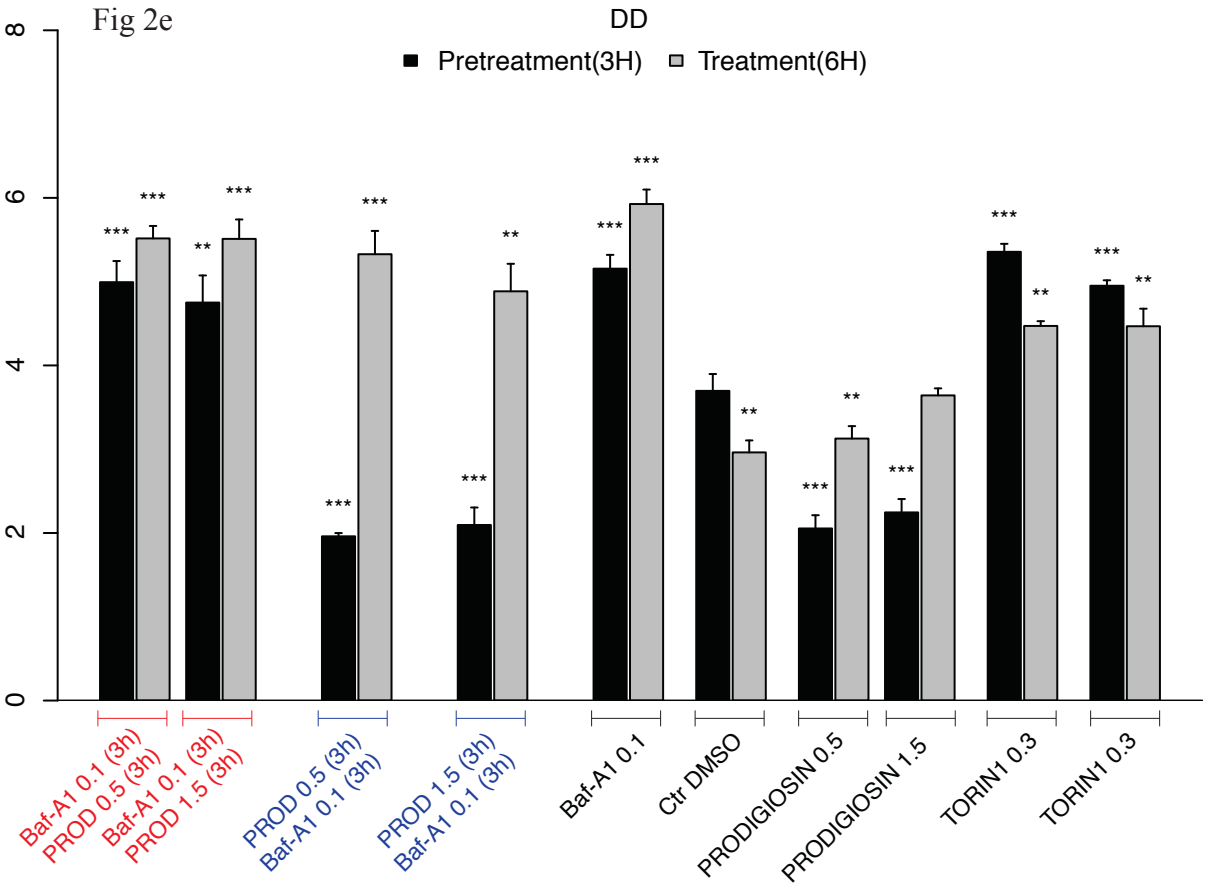
DD
Pretreatment(3H)
Treatment(6H)
0
2
4
6
8
Baf-A1 0.1 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
Baf-A1 0.1 (3h)
Baf-A1 0.1
Ctr DMSO
PRODIGIOSIN 0.5
PRODIGIOSIN 1.5
TORIN1 0.3
TORIN1 0.3

Fig 2e

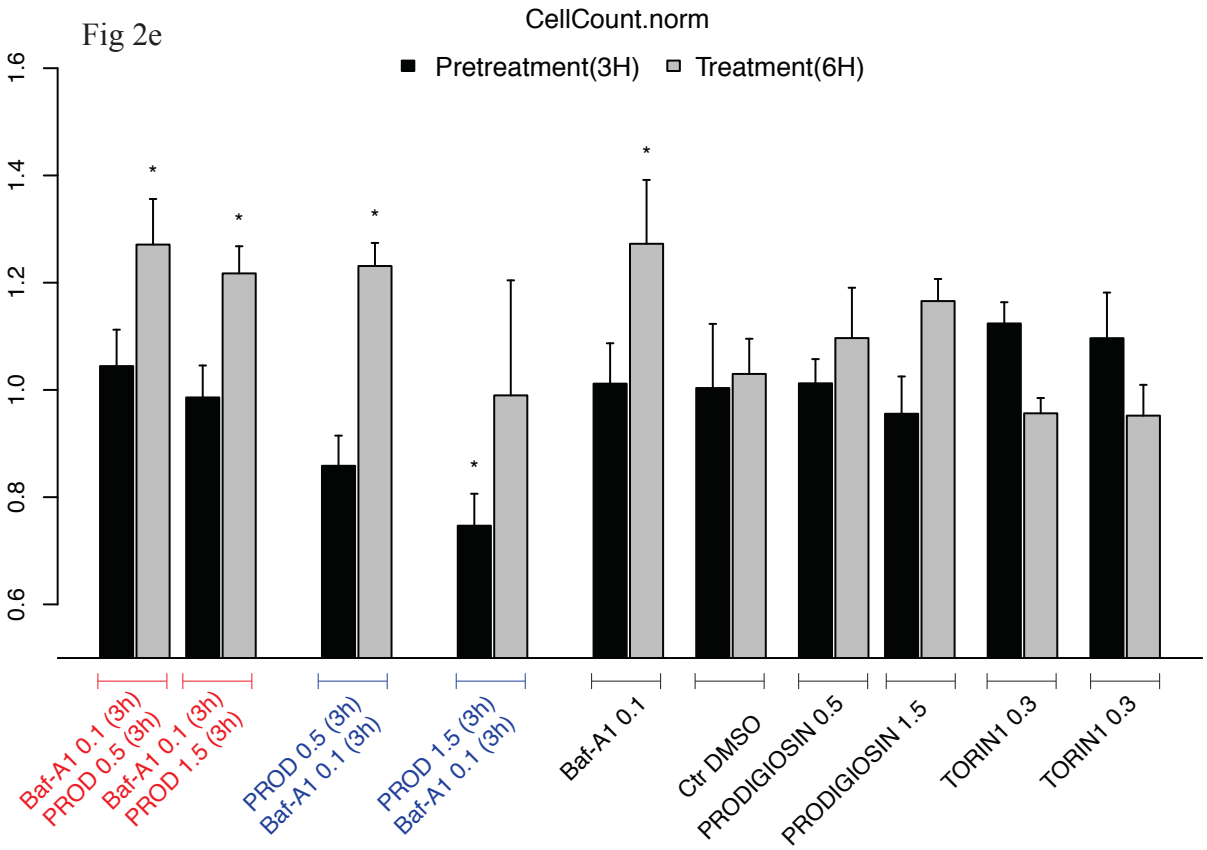
CellCount.norm
Pretreatment(3H)
Treatment(6H)
0.6
0.8
1.0
1.2
1.4
1.6
Baf-A1 0.1 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
PROD 0.5 (3h)
Baf-A1 0.1 (3h)
PROD 1.5 (3h)
Baf-A1 0.1 (3h)
Baf-A1 0.1
Ctr DMSO
PRODIGIOSIN 0.5
PRODIGIOSIN 1.5
TORIN1 0.3
TORIN1 0.3

Figure 2e. Bar chart. Data are expressed as means ± SD of one representative experiment and represent the GFP dots surface per cell, the GFP dots counts per cell, the DD and the cell count. (*p < 0.05, ***p < 0.0001, compared to untreated cells, Ctr DMSO)

Through the PC12 GFP-Q74 model we confirmed that our selected compound, the prodigiosin, seems to disrupt the autophagic machinery rather instead to boost it. In PC12 neuronal cells expressing a doxycycline-inducible autophagic cargo tagged with GFP (more in detail a GFP-tagged pathogenic huntingtin protein holding 74 glutamine repeats, Q74) prodigiosin, contrasting with the activity of positive controls (torin1 and rapamycin), increased the green florescence thus suggesting an accumulation of the autophagic substrate. **Fig 3a-c**

This evidence is consistent with an impairment of the autophagic flux.

Fig 3a

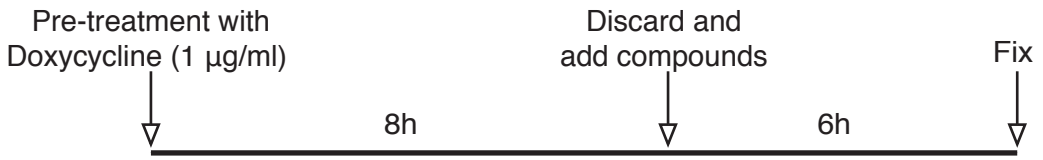

Figure 3a. Design of the experiment

Fig 3b

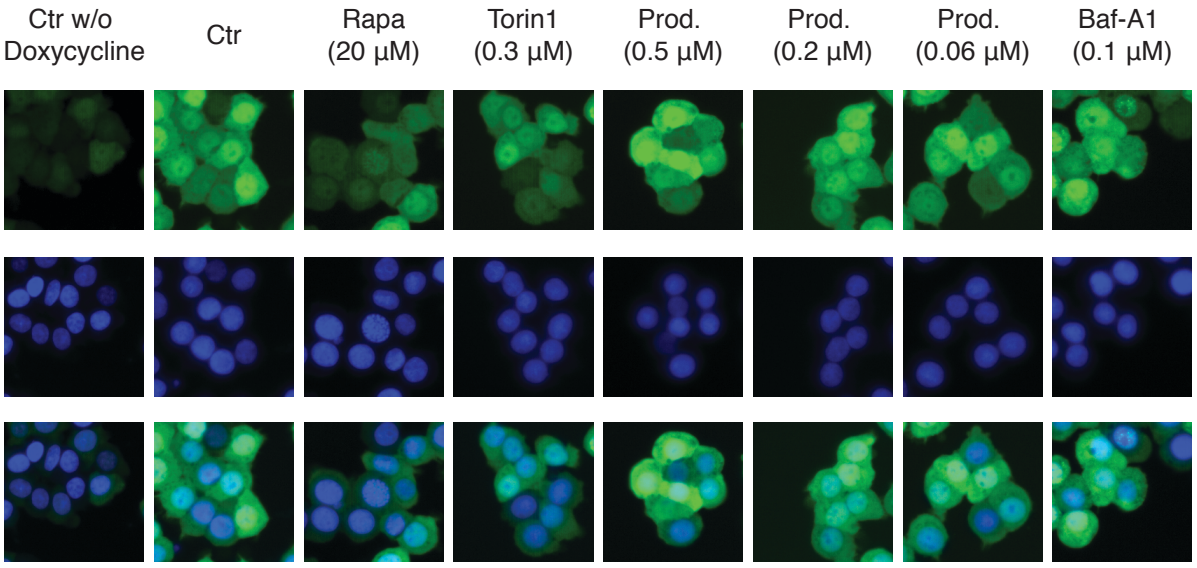

Figure 3b. Representative images of PC12 GFP-Q74 cell after 6 hours treatment with prodigiosin (0.06, 0.2 and 0.5µM), rapamycin (Rapa, 20µM) and torin1 (Torin) 0.3µM.

Fig 3c

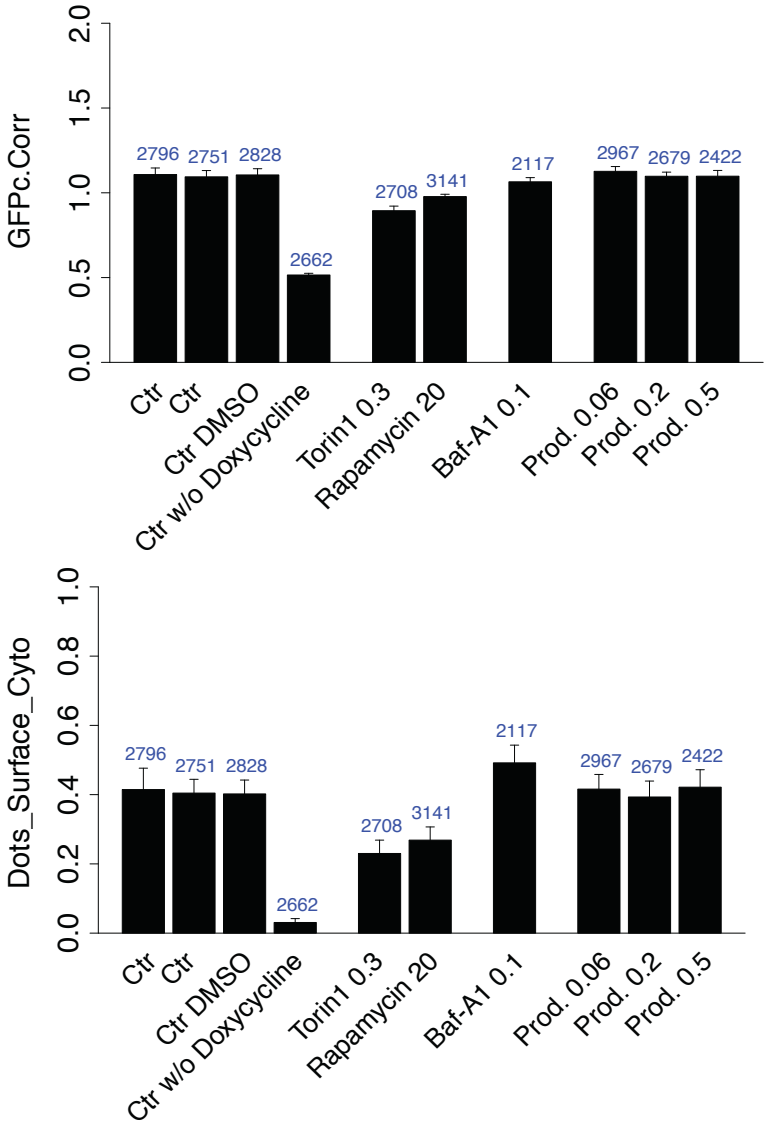

Figure 3c. Bar chart. Data are expressed as means ± SD of one representative experiment and represent the global GFP fluorescent intensity and the cytosolic dots surface per cell.

2.3.2 Prodigiosin induces a dose-depended increase of LC3-II and sequestosome 1

Prodigiosin induced a dose-dependent increase in LC3 lipidation (detectable as an intensification of the LC3-II electrophoretic band). Concurrently an increase in the abundance of Sequestosome 1 (SQSTM1 or p62) protein level has been revealed. **Fig 4a and 4b**

Fig 4a

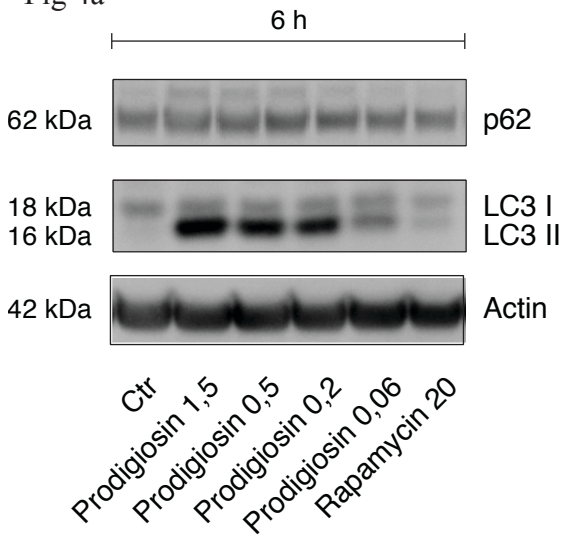

Fig 4b

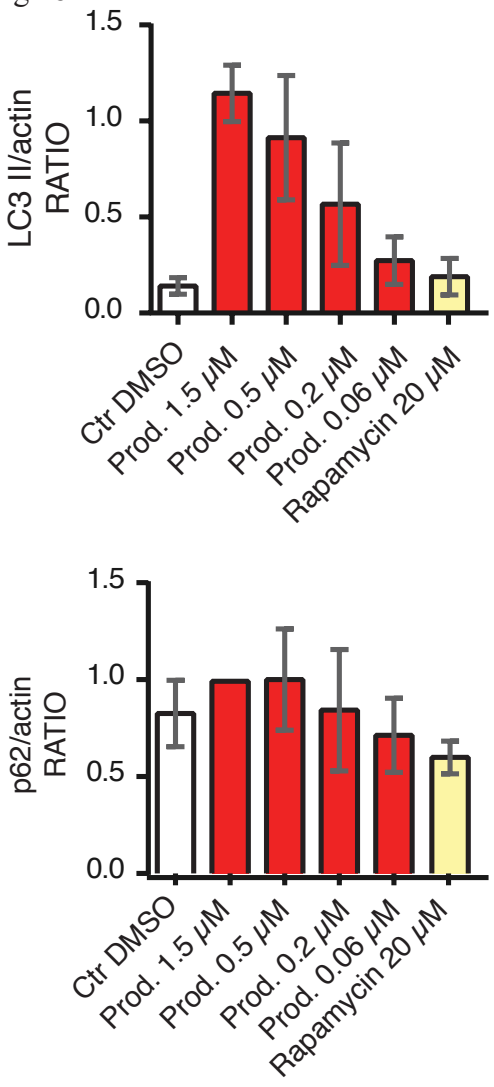

Figure 4 a-b. U2OS wild-type (WT) cells were treated with Prodigiosin (0.06, 0.2, 0.5 and 1.5μM) and torin (0.3 μM) for 6h. SDS–PAGE and immunoblots were conducted as described before. **b.** Immunoblot analysis of LC3 and p62 protein expression levels after 6-hour treatments. Band intensities of LC3-II, p62 and actin were assessed, and their ratios (LC3-II/actin) and (p62/actin) were calculated. Data are means +/- SEM of three replicates.

2.3.4 Implication of TFEB in prodigiosin-induced autophagy modifications

We described the activity of prodigiosin in inducing the transcription factor EB (TFEB) translocation in the nucleus using U2OS cells stably expressing GFP fused to TFEB. TFEB is known to promote the lysosomal biogenesis [56]

Interestingly, we found that this compound was able to induce the translocation of GFP-TFEB from the cytoplasm to the nucleus. **Fig 4c-d**

Probably this event may be attributable to the activity of prodigiosin analogues in inhibiting mTORC1 and mTORC2 complexes. [286] The inhibition of mTORC1 has been already described as a trigger of TFEB activation. [59]

We interpreted this evidence as a prodigiosin-mediated stimulus to the lysosomal genesis while standing a subsequent block to the autophagic flux. Furthermore, this could be speculatively consistent with the scope of S. marcescens to create intracellular niches where housing.

Fig 4c

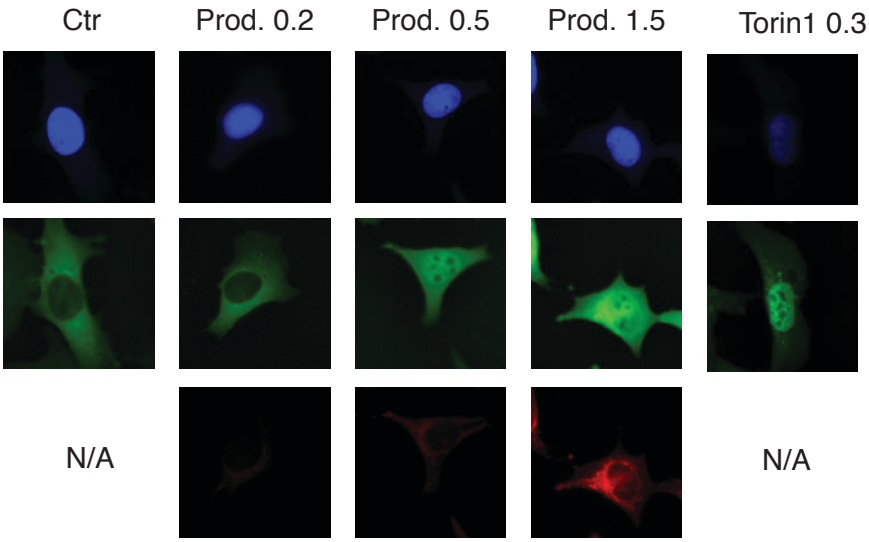

Fig 4d

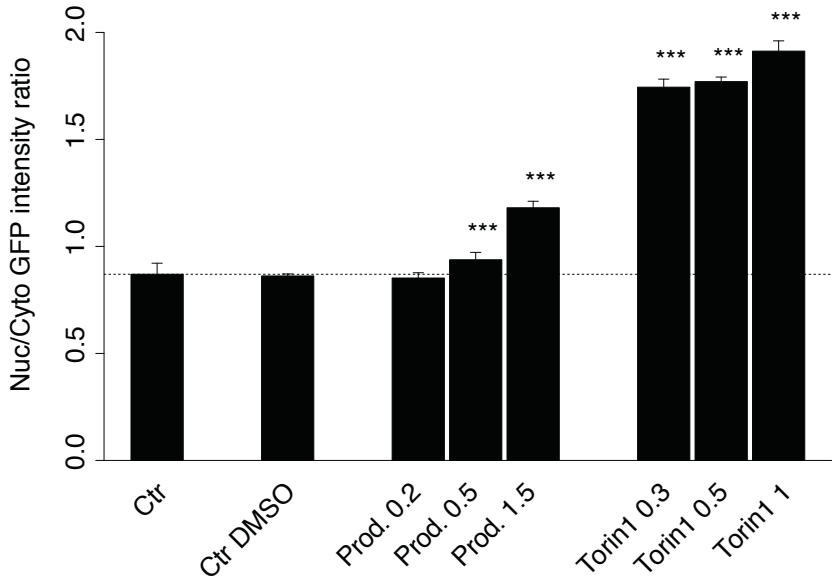

Figure 4 c-d. Prodigiosin induces TFEB translocation in the nucleus: static images and quantification. **c.** Representative images of U2OS GFP-TFEB treated with Prodigiosin (0.2, 0.5 and 1.5μM) and Torin (0.3, 0.5 and 1μM) as controls for 6h. **d.** The average ratio between GFP-TFEB florescence intensity in the nucleus versus the cytoplasm is reported in the bar chart. Data are expressed as means ± SEM of at least three independent wells from one of three independent experiment (*p < 0.05, **p <0.01, ***p < 0.0001, compared to untreated cells, Ctr).

2.3.5 Colocalization of prodigiosin with lysosomes and other organelles

We took advantage of the prodigiosin autofluorescence to find where it localizes inside the cells by matching its red fluorescence with the green fluorescence of the biosensor cell lines or fluorescent antibody staining**.** As showed by the merged images the green (GFP) and red (Prodigiosin) fluorescence remarkably seem to overlap in the Golgi apparatus and in the lysosomes, predominantly. **Fig 5a and Fig 5b**

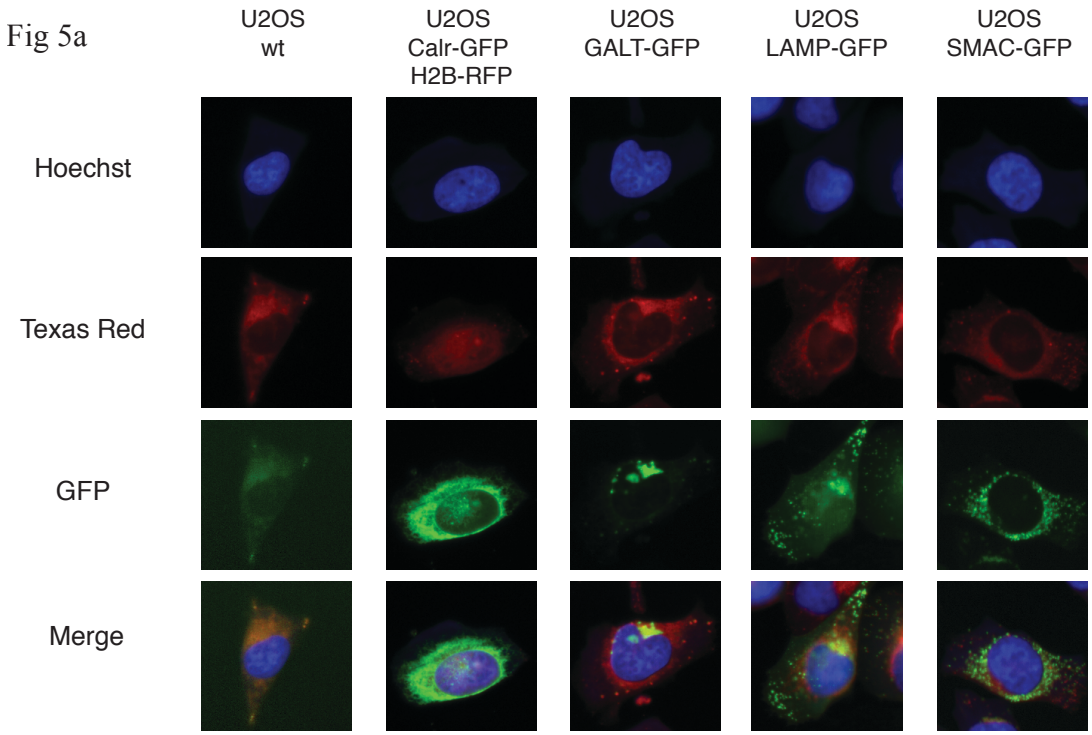

Figure 5a. Colocalization of prodigiosin with lysosomes and Golgi apparatus: static images.

Fig 5b

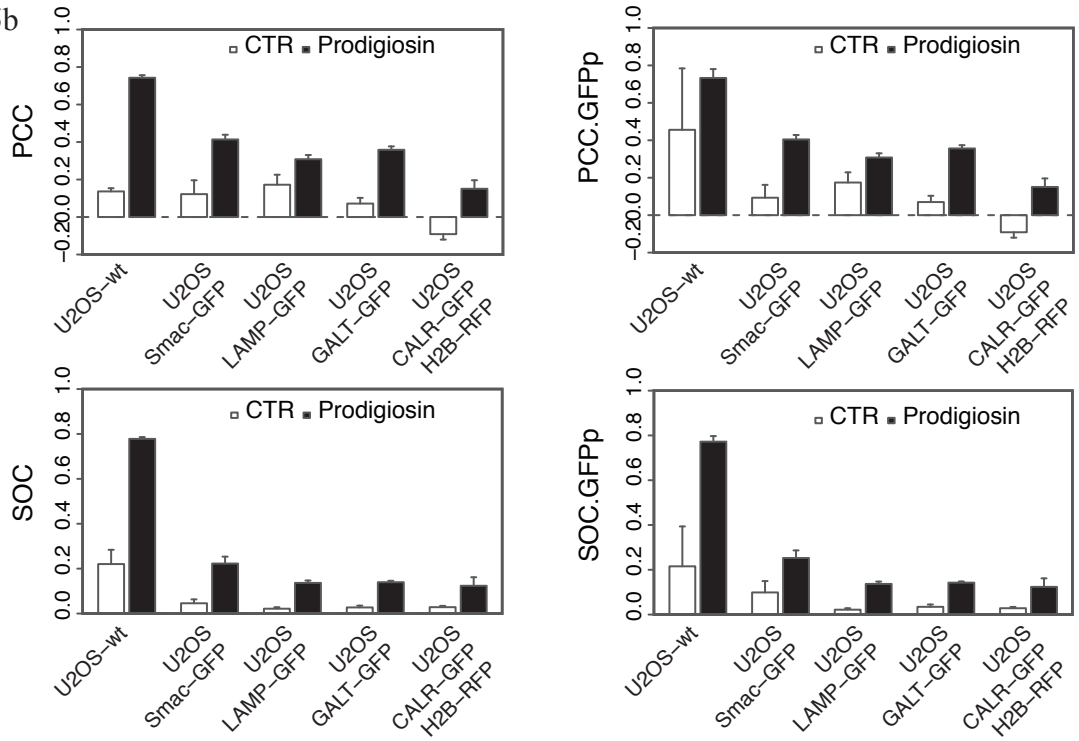

Figure 5b. Colocalization of prodigiosin with lysosomes and Golgi apparatus: quantification for all organelles.

2.3.6 Prodigiosin seems to impair the efficacy of anticancer immunotherapy.

In light of its potential and previous literature on the immunosuppressive activity of Prodigiosines we evaluated the activity of this compound in vivo to address its potential in modifying the tumor growth and the response to anti-PD-1 ICB. BALC/c female were treated according the scheme reported in **Figure 6a** with isotype control, clone 2A3, plus prodigiosin vehicle (controls); anti-PD-1 mAb (250µg/mouse; clone RMP1-14) plus vehicle; Prodigiosin plus isotype control; Prodigiosin plus anti-PD-1 mAb; anti-PD1 plus anti-CTLA-4 (100µg of anti-CTLA-4 mAb (clone 9D9). We observed that prodigiosin seems to promote the tumor growth (statistical significance not reached). Furthermore, the tumor growth was significantly higher in the group treated with prodigiosin plus anti-PD-1 as compared to the group treated with anti-PD-1 alone (mean tumor size: 45.2 vs 28.6 mm^2, respectively; $p<0.05$ by Anova statistics with Bonferroni post hoc test). **Fig. 6b**

We point out that only one experiment has been performed so far.

Fig 6a

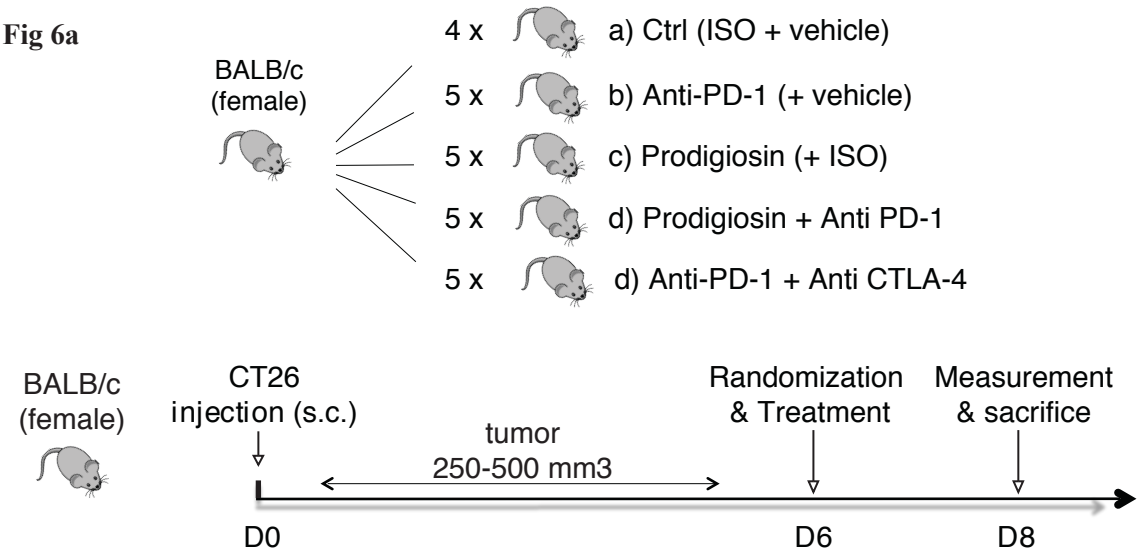

Fig 6b

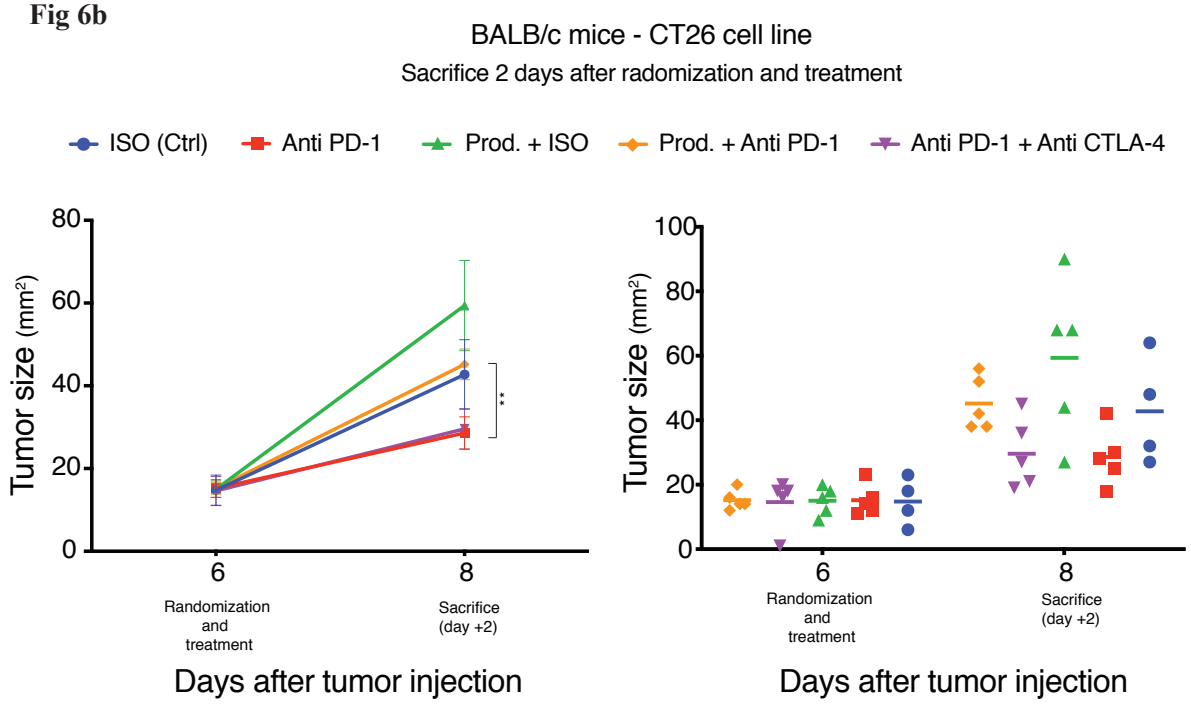

Figure 6 a-b. Prodigiosin seems to impair the efficacy of anticancer immunotherapy. **a** design of the experiment. **b**. variation in tumor volume after 2 days from the treatment administration. Tumor size differences were calculated either using two-way analysis of variance (ANOVA) and post hoc T-test with Bonferroni Correction (to compare the group treated with Prodigiosin plus Anti-PD-1 mAb versus Anti-PD-1 mAb alone: *p < 0.05, **p <0.01, ***p < 0.0001).

To date only one experiment has been performed.

2.4 Discussion and future perspectives

Autophagy plays a cardinal and double-edge role during tumorigenesis. On the one hand, this catabolic mechanism acts as a tumor suppressor by removing oncogenic components. On the other hand, autophagy helps cancer cells survive by supporting their metabolic needs in a nutrient-deprived and hypoxic microenvironment. [287] The role of certain compounds in modulating anti-cancer immunosurveillance by targeting autophagy has recently emerged. [288] However, the pleiotropic activity of certain systemic-administered molecules could deserve a double look, particularly regarding potential bystander effects on immune cells. [289]

Pharmacological autophagy inducers, as well as some lysosomotropic agents, have been shown to enhance anti-cancer immunosurveillance, potentiate immune checkpoint blockade treatment, and perhaps overcome chemoresistance, according to recent findings. [69] [290] [291] [292]

From our screen on bacterial and host metabolites, we selected prodigiosin for its prominent activity in inducing LC3 dots, even at low concentrations. This compound is already known for its potential to induce apoptosis of leukemic and other malignant cells as well as for its immunosuppressive properties.

In the present study, we further report that prodigiosin, a red metabolite produced by some strains of *S. marcescens* and other Gram-negative bacteria, is a potential disruptor of the autophagic machinery and probably hinders the efficacy of anti-PD-1 mAbs in the CT26-BALB/c mouse model. This evidence, if confirmed by replicative experiments in vivo and retrospective clinical evidence, could open a scenario on the plausible activity of this bacterial metabolite as a disruptor of immunotherapy efficacy.

In light of these results, two main perspectives remain open and could deserve attention.

The first, mainly clinical, is the investigation of the effect of immunotherapies in patients with concomitant opportunistic infection sustained by prodigiosin-producing bacteria. A research article published on NEJM in 2003 shed light on the relevance of *S. Marcescens* contamination in bronchoscopes. [197] Other previous studies depicted this bacterium as frequently involved in nosocomial infections. In light of the immunomodulatory activities of prodigiosin (especially the effect on cytotoxic CD8+ T lymphocytes) and the preliminary results of our experiment in vivo (**Fig 6a** – it requires confirmation) further investigation could be justified.

A starting point could be the assessment of the efficacy of immunotherapy in a retrospective series of patients with lung or urothelial cancer and concomitant opportunistic infections sustained by *S. Marcescens*.

The second, which mainly relies on the results of the experiment in combination with Bafilomycin (**Fig 2 b-e**) and on a previous research published on Chem [72], concerns the evaluation of the lysosome deacidification (and of the concomitant cytosolic acidification) as a potential player in reversing the canonical trafficking of autophago-lysosomes from endoplasmic reticulum to the cellular membrane.

Some previous reports have shown how the cell precisely regulates the intracellular pH. As an example, histone deacetylases can induce histone deacetylation in a condition of decreased pH. [293] This phenomenon results in the release of acetate anions which in turn were co-exported coupled with protons out of the cell at the hand of monocarboxylate transporters (MCTs). [293] Viceversa in presence of increased pH, histones are acetylated. [293] Now, the role of protein deacetylation in inducing autophagy is clear and mentioned above.

On account of that and other pieces of evidence, the intracellular-pH decrease could indirectly act as autophagy inducers. This hypothesis could open a new interesting scenario on the role of the intracellular pH decrease as an independent inducer of autophagy.

Furthermore, evidence of defective autophagy in cancer cells is already known. Conversely,

from the tumor microenvironment, which exhibits an acidic pH, the intracellular pH of malignant cells is increased. [294] [295] This evidence is consistent with the anabolic status of malignant cells, and with the observation of an increased pH in replicating cells. The pH is "de facto" one master regulator of autophagy.

In such a context, the role of anion transporters, as our hit, prodigiosin, can gain attention when viewed from a different perspective. Worthy of note is the evidence that the protonophore FCCP, a mitochondrial uncoupler, is able to induce mitophagy at the concentration of 10μm through cytosol acidification rather than mitochondrial depolarization. [39]

As a result of our experiment and previous literature, we could state that prodigiosin probably induces autophagy (possibly inducing a decrease in cytosolic pH) [296] as suggested by the TFEB translocation into the nucleus, but in the meantime hinders the autophagic flux (by inducing the alkalinization of lysosomes).

In conclusion, prodigiosin acts primarily as an uncoupler of the pH gradient between the lysosomes and the cytosol. [72] This latter evidence could be further confirmed by visualizing the intracellular proton flux. [297] Furthermore, this compound is able of inducing death by apoptosis of the malignant cell [202] [204], but its effect on the autophagic flux and its activity as an immunosuppressive agent could result in impaired efficacy of immunotherapy.

2.5 Key words and Abbreviations

Key words: bacterial metabolites; prodigiosin; immune modulators; immune checkpoint blockers; immunogenic cell death, intracellular pH.

List of abbreviations:

ActD actinomycin D

anti-PD-1 mAb anti-PD-1 monoclonal antibody

anti-CTLA-4 anti-CTLA-4 monoclonal antibody

BafA1 bafilomycin A1

CHX cycloheximide

ER Endoplasmic reticulum

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

IC50 inhibitory concentration 50

LC3 light chain 3

PIns-3P Phosphatidylinositol 3-phosphate

Prod. prodigiosin

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