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Disturbing intracellular replication of *Helicobacter pylori* by sorafenib treatment *in-vitro*

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ABSTRACT

Helicobacter pylori (*H. pylori*) is a Gram-negative, spiraling, microaerophilic bacteria that normally infect the stomach of human and produces stomach inflammation which can develop to gastric ulcers of the stomach or the upper part of the small intestine. Several cellular signaling pathways such as mitogen activated protein kinases (MAPKs) and RNA activating factor 1 (RAF-1) signaling cascade may be involved in *H. Pylori* infection. MAPKs are a type of extracellular communication that consists of a chain of proteins that extends from cell receptors to nuclear DNA. MAPK signaling is usually triggered by cell receptors attaching to epidermal growth factors (EGF), also known as the growth factor pathway or extracellular signal-regulated kinase (ERK). By administering HeLa cells with Sorafenib (SOR), a systemic medication for malignant malignancies, we studied the possibility of inhibiting *H. pylori* replication. In pre-treated and infected cells, the expression of RAF-1 and autophagy related 5 (Atg5) was monitored to see if targeting these factors can disrupt H. Pylori intracellular replication. Surprisingly, the relative expression of bacterial 16s ribosomal RNA in SOR-treated cells revealed a competitive suppression of bacterial replication (16srRNA). Furthermore, SOR therapy successfully controlled the expression of the Raf-1 and Atg5 genes without causing any toxicity. In addition, SOR therapy lowered the production of tumor necrosis factor (TNF-) in a dose and time-dependent manner. These data suggest that SOR can disrupt H. Pylori replication in HeLa cells by suppressing MAPK and autophagy signaling, with minimal TNF- generation from treated cells.

Keywords: Helicobacter pylori, sorafenib, HeLa cell, MAPK signaling and autophagic process

INTRODUCTION

Helicobacter pylori usually cause a life-long infection in the stomach, where it triggers a strong immunological response with local lymphocyte and macrophage infiltration (Kusters *et al.*, 2006; Vandenplas, 2000). The processes underpinning the onset of additional *H. pylori*-implicated lymphomas have been revealed, and other pathways in adenocarcinoma induction have been described (Kim *et al.*, 2011). Although stomach ulcers are known to be caused by the *H. pylori* bacteria, scientists are still largely in the dark about this crucial health issue. *H. pylori* are usually spread from person to person through saliva or through feces-contaminated food or water. *H. pylori* prevalence occurred throughout childhood in impoverished nations due to a combination of causes including contaminated water, crowded surroundings, and poor hygiene (Brown, 2000; Kayali *et al.*, 2018).

Several cellular signaling pathways, including mitogen activated protein kinases (MAPKs), protein kinase C (PKC), and

autophagosome production, are involved in *H. pylori* infection (Devenport and Shah, 2019; Islam et al., 2018). PKC is a versatile serine/threonine kinase that regulates biological processes such cell proliferation, differentiation, and death. The activation of the PKC signaling pathway results in the creation of autophagosomes, which protect cells against death and regulates a variety of physiological activities (Black and Black, 2013).

RAS, RAF-1, Mek1/2, and ERK protein kinases are examples of MAPK protein kinases that autophosphorylate their serine and threonine residues to activate or deactivate their substrates (Soares-Silva *et al*., 2016). MAPK signaling is important because it can control a variety of cellular processes like proliferation, apoptosis, autophagy, and self-defense. In mammalian cells, three MAPK signaling pathways have been identified; ERK1/2, c-JUN N-terminal kinase 1, 2 and 3 (JNK1/2/3) (Dong *et al*., 2002). Hormones, growth factors, and proinflammatory inducers boost ERK1/2, whereas cellular and environmental conditions, as well as proinflammatory stimuli activated JNK1/2/3 and p38 MAPK (Raman *et al*., 2007).

In 2006, the FDA authorized sorafenib (SOR) for the treatment of advanced renal cell carcinoma. SOR was licenced as a one-of-a-kind target therapy for advanced hepatocellular carcinoma (HCC) in 2007 (Zhu *et al*., 2017). SOR is a type of oral multikinase inhibitor that inhibits cancer cell growth and tumorigenesis-induced programmed cell death (PCD) (Gauthier and Ho, 2013). Autophagy, on the other hand, has been discovered as a conserving mechanism that involves mass breakdown and recycling of unneeded cytoplasmic components via lysosome delivery (Dahb Hassen *et al*., 2017; Khalil *et al*., 2019). Normally, hunger and bacterial infection induce the creation of autophagy vesicles (Abd El Maksoud *et al*., 2020; Abdelaziz *et al*., 2015). Atg5-Atg12, Atg6-Atg9, and Atg16L are autophagy proteins acquired by pre-autophagosomal vesicles (Elimam *et al*., 2020). The cytosolic Atg8 isoform (LC3-I) is then attracted to vesicles and converted into the membraned isoform, LC3-II (Khalil, 2012).

In the present study SOR was used on infected HeLa cells to study the probable inhibitions of *H. pylori* intracellular replication and find out the molecular interaction that might be involved. Monitoring the cytotoxic effect of SOR in treated cells was also considered, as was its effect on cell survival and TNF- production.

MATERIALS AND METHODS

Cells lines

The cervical cancer cell line HeLa was obtained from (VACSERA, Giza, Egypt) and cultured in RPMI medium with 4 mM L-glutamine, 4 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 10% bovine calf serum (BCS). The cultivated cells were incubated at 37°C with a 5% CO_2 atmosphere.

SOR bacterial infection and therapy

On 2 ml of RPMI media, cells were planted at a density of 200000 cells per well in a 6-well plate and incubated overnight in a CO2 incubator. The cells were then treated with either SOR (100 g/well) or 10 ul DMSO, which served as a control. Infected cells were infected with *H. pylori* (MOI of 1) two hours later and incubated overnight as previously described.

Bacterial strain and infection protocol

For five days, *H. pylori* strain P12 (wild type, USA) was grown on agar plates containing 10% horse serum in microaerophilic conditions at 37°C and 10% CO2. Then, as previously mentioned, the Brucella broth liquid medium supplemented

with 10% (v/v) heat-inactivated FBS was used to culture and grow *H. pylori* in a liquid media (Blanchard and Nedrud, 2006). At -80, different aliquots of the growing *H. pylori* in liquid medium were kept. The bacteria were extracted from culture plates in Dulbecco's phosphate-buffered saline (PBS) (pH 7.4) and the concentration of bacteria was quantified using a mass spectrophotometer by optical density at 600 nm, corresponding to 1108 colony forming units (CFU/ml) (Jung *et al*., 2015). For 48 hours, the bacterial stock was introduced to HeLa cells at various multiplicities of infection (MOI of 1). PBS was incubated with the cells for the same time periods as a control. An inverted microscope was used to monitor cells during infection (Poppe *et al*., 2007; Schneider *et al*., 2011).

Viability of cells and cytotoxic consequences

The number of living cells and representative images of cells taken with an inverted microscope were used to evaluate cell morphology and viability rate after SOR therapy. Using an LDH production kit, the production of lactate dehydrogenase (LDH) in the medium from treated cells was evaluated in a 96-well plate. According to the manufacturer's instructions, the same volume of sample and LDH buffer (primary antibody) were incubated for 2 hours, then 1 hour with LDH substrate (secondary antibody), and the relative activity of LDH was measured and computed using 470 nm absorbance values. Triton x-100-treated cells were employed as a positive control for cytotoxicity (Khalil *et al*., 2018).

Total RNA isolation and cDNA synthesis

In clean and RNase-free tubes, treated and infected cells were collected from cell culture plates. TriZol (Invitrogen, USA) chloroform techniques were used to isolate total RNA. The concentration of all samples was adjusted to a final concentration of 100 ng/ul by dissolving the isolated RNA in RNase-free water and resuspending it. Then, using a cDNA synthesis kit, 10 ul of each extracted and purified total RNA were utilised to create cDNA (Qiagen). Total RNA was incubated with reverse transcriptase and oligo (dT) primer for one hour at 45°C, followed by five minutes at 95°C, according to the manufacturer's instructions. The cDNA was then stored at -20°C until it was needed (Farghaly *et al*., 2018).

Q-RT-PCR investigation parameters

To study the relative expression of RAF-1 as an indicator of MAPK signaling and Atg5 as an indicator of autophagic process, Q-RT-PCR was used to detect the expression levels of RAF-1 and autophagy related 5 (Atg5) following treatment. As a bacterial replication indicator, the relative gene expression of 16s ribosomal RNA was measured in treated and infected cells. The Quanti-Tect SYBR green PCR Kit (Qiagen, USA) and oligonucleotides specific for each gene were used in the Q-RT-PCR.
Raf-1-sense-5-TTTCCTGGATCATGTTCCCCT-3,
Raf-1-antisense-5-ACTTTGGTGCTACAGTGCTC A-3,
Atg5-For-5-CGTGTATGAAAGAAGCTGATGC-3,
Atg5-Rev-5-ACGAAATCCATTTTTTCTTCTGGA-3.
The following oligonucleotides were utilized for 16s ribosomal RNA:
F-5'-TCGGAATCACTGGGCGTAA-3', R-5'-TTCTATGGTTAAGCCATAGGATTTCAC-3'.
GAPDH levels were amplified using particular oligonucleotides, (sense) 5'-TGGCATTGTGGAAGGGCTCA-3' and (antisense) 5'-TGGATGCAGGGATGATGTTCT-3' which served as an internal control for normalisation. The real-time PCR reaction was carried out in a bio-system apparatus at 95°C for 5 minutes, followed by 35 cycles of 15 seconds each at 95°C, annealing at 60°C (15 seconds), and extension at 60°C (45 second). SDS 2.2 was used to do data analysis on threshold cycle (CT) results

(Farghaly *et al*., 2018; Khalil *et al*., 2016).

ELISA test

TNF- was measured in infected cells using a sandwich enzyme-linked immunosorbent assay (ELISA) kit by human ELISA kits as an indicator for the MAPK signaling cascade (Abcam, 181421). As a result, cells were seeded in a 96-well plate, then treated with SOR and infected with bacterial stock at a MOI of 1. The media was collected and the concentration of TNF- was evaluated in a time course experiment on day 1, day 2, and day 3. Standards and samples were placed into the 96-well plate as directed, and the immobilised antibody bound the given TFN- to the wells. Biotinylated antibody was added after washing, followed by HRP-conjugated streptavidin antibodies. Finally, the wells were filled with a TMB substrate solution, and the colour developed in proportion to the amount of cytokine bound. At 450 nm, the colour intensity was measured (Khalil, 2012; Khalil *et al*., 2017, 2018).

Agarose gel electrophoresis

The standard PCR products were loaded into a 1 percent agarose gel prepared by dissolving 1 g agarose in 50 ml TAE (1X) in a graduated cylinder and then transferring to an agarose flask. The top of the flask was wrapped with a paper towel and microwaved until it was fully dry. Ethidium bromide (10 ul) was added to the warm gel, which was then poured into the DNA electrophoresis cassette and allowed to cool. After putting the samples onto the gel, electrophoresis was performed for one hour at 50 volts (Dahb Hassen *et al*., 2017).

Statistical Analysis:

Microsoft Excel was used to construct the final plots and histograms for our data. The Student's two-tailed t-test was used to examine the significance of all data obtained from real-time PCR analysis. Using delta-delta Ct equations, the qRT-PCT data was analysed with SDS2.2.2 software to provide Ct values for putative gene expression (Khalil *et al*., 2016; Schmittgen *et al*., 2004).

RESULTS AND DISCUSSION

In HeLa cell lines, the Raf-1 inhibitor sorafenib (SOR) has no cytotoxic effects.

The number of living cells and cell representative pictures in pre-treated cells were monitored to assess the potential cytotoxic effects of SOR treatment on *H. pylori* infected HeLa cells. In order to assess cell viability rate, lactate dehydrogenase (LDH) production from pre-treated and infected cells was also measured. LDH is an enzyme that can be present in all living cells and During the Krebs cycle in mitochondria, it is responsible for the conversion of lactate to pyruvic acid. Importantly, LDH secretion denotes a systemic toxic effect on cell proliferation that results in a cell death programme. HeLa cells were planted at a concentration of 2 x 105 cells per well in a 6-well plate and incubated overnight. After that, the cells were treated with SOR at a concentration of 200g per well. Finally, *H. pylori* (MOI of 1) was inoculated into the pre-treated cells, followed by an overnight incubation with the same dosage of SOR. Surprisingly, SOR therapy had no cytotoxic effects on the treated cells, as evidenced by cell pictures. as well as the amount of live cells DMSO treatment, on the other hand, had a considerable impact on the number of live cells (Fig. 1A and B). Importantly, pre-treated cells with DMSO increased LDH production by up to fourfold, confirming DMSO's deleterious influence on cell growth during *H. Pylori* infection. Nonetheless, treatment with SOR resulted in low levels of LDH production in both treated and infected cells (Fig. 1C). These data suggest that, on the one hand, SOR

administration has no deleterious effect on HeLa cell growth and, on the other hand, SOR can protect HeLa cells from cytotoxicity caused by *H. Pylori* infection.

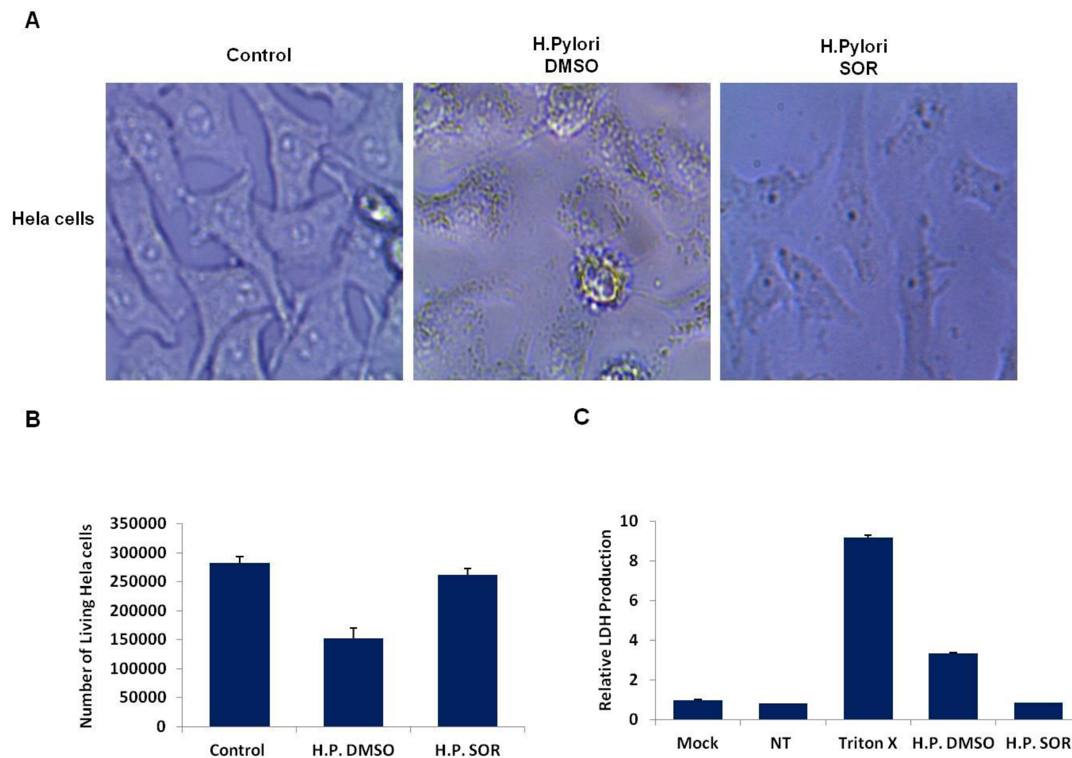

Fig. 1. SOR treatment's cytotoxic effects:

A) In comparison to DMSO-treated cells and non-treated cells, representative cell images indicate cell viability of HeLa cells that were pre-treated with Raf-1 inhibitor (NT).
B) Manually determined number of alive cells after Sorafenib treatment.
C) LDH production from treated cells vs. Triton X-100-treated cells vs. non-treated cells. The standard deviation (SD) of two independent experiments is indicated by the error bars.

SOR treatment inhibits *H. pylori* replication in HeLa cells

HeLa cells were sown at a density of 2x105 cells per well on a 6-well plate to examine if SOR could disrupt H. Pylori replication. The cells were then treated with either 200 g/well of SOR or 20 l DMSO, the organic solvent used to prepare SOR. After two hours, all treated cells were infected with H. Pylori at a multiplicity of infection of one (MOI=1) and incubated overnight. qRT-PCR was used to measure bacterial replication in HeLa cells by quantifying steady-state mRNA of bacterial 16srRNA. As a result, using an RNA purification kit (Invitrogen, USA), total RNA was extracted and purified from treated and infected cells, followed by the synthesis of its related cDNA (Fig. 2A). In comparison to DMSO and control treated and infected cells, our findings demonstrate that the relative expression of bacterial-16srRNA in SOR treated cells was substantially disturbed (more than 80 percent reduction). This finding implies that SOR can stop H. Pylori from replicating in HeLa cells, implying that it could be employed as an anti-agent of *H. Pylori*.

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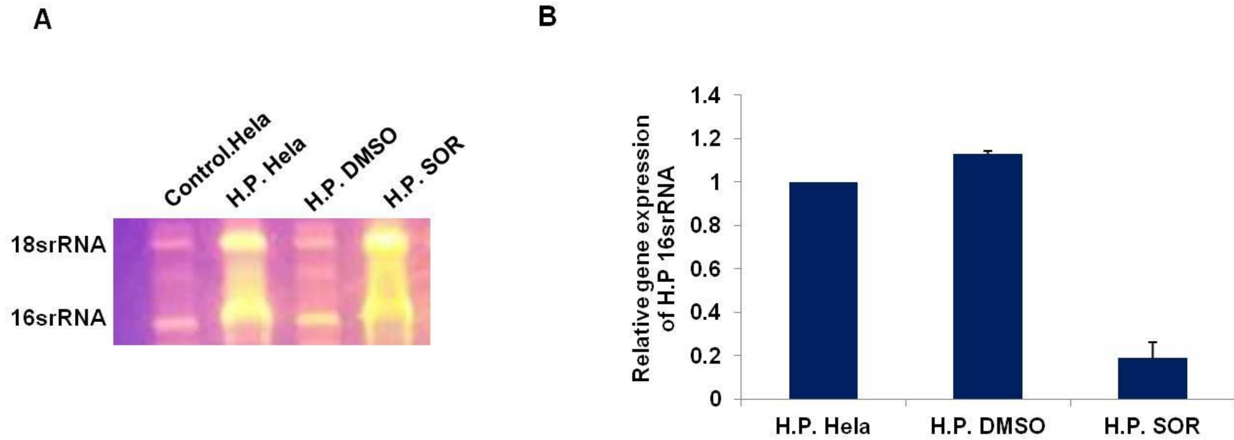

Fig. 2: Relative expression of H. Pylori indicator 16srRNA
A) Using agarose gel electrophoresis, total RNA was extracted and purified from SOR pre-treated and infected cells, as well as other control treated and infected cells.
(B) 16srRNA gene expression in HeLa cells treated with SOR vs. other control cells, exhibiting *H. Pylori* replication. The error bars show the standard deviation (SD) of two independent experiments.

SOR regulates of *H. pylori* replication via blocking RAF-1 and Atg5 expression

In cancer illnesses, a range of extracellular and intracellular signaling pathways are implicated, with protein kinase C (PKC) playing a key role in several signal transduction pathways. PKC signaling involves a number of downstream signaling pathways, including the activation of RAS/RAF/MEK/Erk oncoproteins via mitogen-activated protein kinase (MAPK) (Bhalla *et al*., 2002; Griner and Kazanietz, 2007; Koivunen *et al*., 2006). The relative expression of RAF-1hse was measured by qRT-PCR in SOR-treated cells and control treated cells to investigate the putative role of the MAPK signaling pathway in H. Pylori infection. In addition, the relative expression of the autophagy-related gene Atg5 in pre-treated and infected cells was examined. As a result of SRO treatment in infected HeLa cells, both the relative expressions of Ra-1 and Atg5 were drastically lowered in a dose-dependent manner (Fig. 3A and B). SOR therapy resulted in a competitive inhibition of H. Pylori replication in HeLa cells, as well as a significant reduction in Raf-1 and Atg5 gene expression. As a result, both MAPK and autophagy signaling are implicated in *H. Pylori* replication, and their modulation can disrupt *H. Pylori* replication in infected cells.

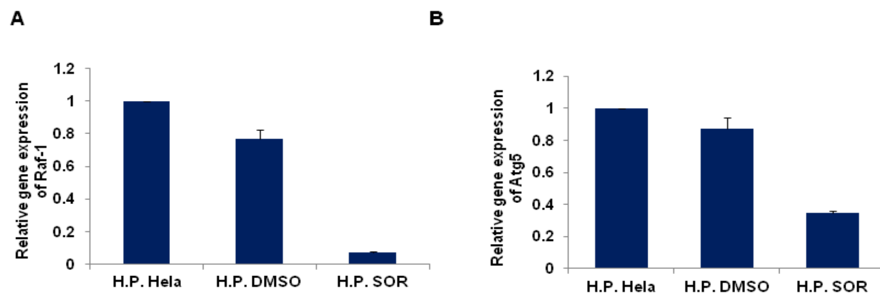

Fig. 3. Relative expression of RAF-1 and Atg5 in pre-treated and infected cells

A) Raf-1 gene expression relative to GAPDH gene expression in HeLa cells that were pre-treated with SOR compared to other control infected cells. B) In SOR-treated and infected cells, relative gene expression of Atg5 as an indication of autophagy.

The error bars represent the standard deviation of two independent experiments.

SOR regulates production of TNF-α in infected cells

Activation of p38 and c-Jun N-terminal kinase (JNK) has been linked to the production of pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-) and interleukin-6 (IL-6) as well as anti-inflammatory cytokines like IL-10, according to several studies (Chi *et al*., 2006; Park *et al.,* 2010). In addition, the RAF-1 signaling pathway is involved in the replication of H. Pylori, the hepatitis C virus, and the influenza A virus (Khalil, 2017; Peyssonnaux and Eychène, 2001; Pleschka, 2008). To investigate the impact of SOR as a potential regulator of TNF-, we measured TNF- production levels during H. Pylori replication in SOR pre-treated cells. The lowest concentration TNF- is connected with SOR treatment throughout day 1, day 2, and day 3 of infection, according to ELISA results of indicated HeLa cells alternatively, the highest levels of TNF- were found in H. Pylori infected cells (control infection) and infected cells treated with DMSO (Fig. 4). These findings show that controlling MAPK signaling controls pro-inflammatory cytokine release, which in turn inhibits *H. Pylori* multiplication in HeLa cells.

Fig. 4. Levels of produced TNF-α in SOR-treated and infected cells

TNF- concentration (pm/ml) in the fluid medium of infected HeLa cells treated with 200 g/ml SOR for the indicated time periods versus DMSO-treated cells.

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We used qRT-PCR to look into the molecular effect of SOR on *H. pylori* replication, as shown by the relative expression of the bacterial 16srRNA gene. In addition, the cytotoxic potential of SOR on HeLa cells was evaluated based on cell viability and cellular morphology, as well as the amount of TNF- generated by pre-treated and infected cells. Surprisingly, our findings revealed that SOR therapy inhibited bacterial replication significantly In SOR-treated and infected cells, relative expression of both RAF-1 and Atg5 was significantly reduced, indicating that RAF-1 and Atg5 are potential targets of SOR and are important factors in *H. pylori* replication. When compared to DMSO-treated cells, the level of generated TNF- was lower in SOR-treated and infected cells, demonstrating that SOR can modulate the tumorigenesis activity of *H. pylori* infection. Infection with *H. Pylori* causes a range of cellular singling that either suppresses or ensures bacterial multiplication. MAPKs, autophagy, the PI3K/AKT pathway, and proinflammatory cytokines are among the critical cellular signaling pathways triggered by bacterial infection. Autophagy is a process characterized by the formation of double-membraned cytoplasmic vacuoles that regulate breakdown events and cellular recycling by transferring cytoplasmic materials to lysosomes. All animal cells contain lysosomes, which are membrane-bound vacuoles (Abdelaziz *et al*.,2015; Khalil *et al.*, 2016). They are spherical vesicles that carry a hydrolytic enzyme that, when combined with an autophagosome, may break down nearly any unwanted molecule. Simply put, a lysosome is a vesicle with a unique protein composition in both its membrane and lumen. The pH of the lumen (4.5-5.0) is ideal for hydrolysis enzymes, similar to the activity of the stomach (Luzio *et al*., 2007). Lysosomes are engaged in a variety of cell functions, including secretion, plasma membrane repair, cellular signaling, and cellular metabolism, in addition to polymer degradation (Bonam *et al*., 2019; Boya, 2012). TNF- is a pro-inflammatory cytokine that is activated by binding to the tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2) receptors (TNFR2). TNF-exact signaling molecular function, as well as the role of its receptors TNFR1 and TNFR2, in the inflammatory response, are unknown. TNF- has a role in DEP-induced pulmonary inflammation, according to new research, and TNFR2 is the most important receptor in mediating these inflammatory processes (Kumar *et al*., 2017). Likely, following infection of HeLa cells, the pro-inflammatory cytokines TNF- were dramatically reduced in response to SOR administration in a time-dependent manner. Our findings show that SOR's modulation of autophagy and inhibition of MAPK signaling is enough to disrupt *H. pylori* infection in vitro.

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اضطراب تكاثر بكتريا الهيليكوباكتر بيلوري في المختبر باستخدام السورافينيب

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المستخلص

تعتبر الهيليكوباكتر بيلوري بكتريا سالبة حلزونية ودقيقه ومحبه للهواء , غالبا ما تصيب معدة الانسان. تسبب عدوي الهيليكوباكتر التهاب المعدة وقد تؤدي الي قرحه المعدة أو الجزء العلوي من الامعاء الدقيقة . ينضم لعدوي الهيليكوباكتر العديد من الاشارات الخلوية مثل مسار MAPKS والذي يشمل RAF-1 ويعد MAPKS تواصل خارجي يحتوي علي سلاسل معينه من البروتين تبدأ من مستقبلات الخلايا حتي تصل الي DNA النواه. تنشط اشارات MAPKS عن طريق الربط بين مستقبلات الخلايا و EGF , لذا يسمي Growth Factor Epidemal أو يسمي Extra cellular signal regulated kinase (ERK) . تم فى هذه الدراسة تقييم امكانية تثبيط تكاثر بكتريا *H. pylori* عن طريق كلا من خلايا الهيلا HeLa cells وخلايا سرطانية من داخل القولون تسمي (CaCo-2) Colon Cancer cells باستخدام دواء سورافينيب Sorafenib (SOR) وهو دواء لعلاج الاورام ، و تم قياس التعبير عن (RAF-1) و (ATG5) للخلايا قبل وبعد اصابة الخلايا بالبكتريا. أظهرت النتائج تثبيط قوي لتكاثر البكتريا في وجود (SOR)عن طريق قياس التعبير عن (16srRNA). ويتضح ان (SOR) ناجح في تنظيم التعبير عن جينات RAF-1 و ATG5 بدون تأثير سام . بالاضافة الي أن معامل التورم TNF– α يقل عقب العلاج ب جرعه ال SOR مع قياس الزمن . كل هذا يشير الي ان دواء SOR يستطيع تشتيت تكاثر الهيليكوباكترعن طريق غلق اشارات MAPK& Autophagy في كلا من HeLa و CaCo-2 مع انتاج كميه ضئيلة من TNF- α في هذه الخلايا.