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Increased Susceptibility to Resveratrol of *Helicobacter pylori* Strains Isolated from Patients with Gastric Carcinoma

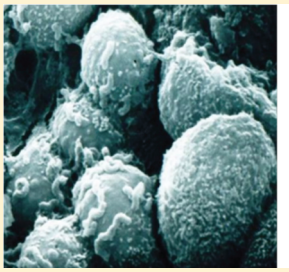
Silvia Martini,*[,†] Claudia Bonechi,[†] Claudio Rossi,[†] and Natale Figura[§]

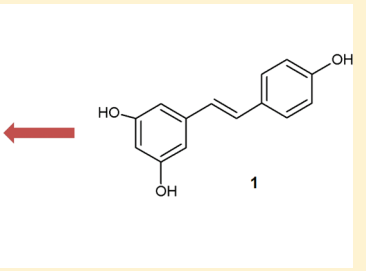
[†]Department of Pharmaceutical and Applied Chemistry, University of Siena, Via Aldo Moro, 2−53100 Siena, Italy, and Centre for Colloid and Surface Science (CSGI), University of Florence, Via della Lastruccia, 3 Sesto Fiorentino (FI), Italy

[§]Department of Internal Medicine, University of Siena, 53100 Siena, Italy

Supporting Information

ABSTRACT: The increasing therapeutic failures against *Helicobacter pylori* infection has determined the need to develop new drugs. The susceptibility to resveratrol (**1**) of twenty-six *H. pylori* strains representing nine CagA+ strains from patients with gastric carcinoma and eight CagA− and nine CagA+ strains from patients with chronic gastritis only was evaluated. Compound **1** was dissolved in DMSO and double diluted in *Brucella* broth with 10% BFS; ca. 10^6 organisms were added to each dilution. After incubation, subcultures were performed using Columbia-blood agar plates. The lowest concentration in broth at which all the organisms were killed was considered the minimum bactericidal concentration (MBC). Tests were performed in triplicate. The mean MBC of **1** for CagA positive GC strains was significantly lower than those for both CagA positive and CagA negative CG strains. An F1 ATPase of *H. pylori* showed a significant linear homology with a human ATPase considered a possible target of **1**. It was hypothesized that strains infecting patients with gastric carcinoma have a reduced expression of F-type ATPases, which normally protect the bacteria from low pH levels by maintaining a proton gradient across membranes. Such behavior can be considered as an adaptive response to decreased gastric acidity. Since the targets of resveratrol (**1**) are also the bacterial ATPases, their putative reduced expression could increase the susceptibility to this compound, so that it saturates its targets more quickly and efficiently.





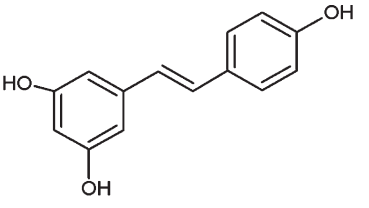
trans-Resveratrol, (3,5,4′-trihydroxy-*trans*-stilbene, **1**), a phenolic compound present in grapes, wine, peanuts, and other food products, has antioxidant properties and exerts a potent anticarcinogenic activity and protective effects against atherogenesis and cardiovascular diseases.[1−5] In recent studies, **1** has been reported to possess anti-*H. pylori* activity in vitro[6,7] and has been identified as an active compound responsible for the antibacterial activity of red wine.[8]

The outcome of *H. pylori* infection is more severe in patients infected by strains that possess the chromosomal insertion *cag*; *cag*-positive (*cag*+) clones are endowed with an increased inflammatory potential and secrete a highly immunogenic protein called CagA, which is linked to the development of premalignant and malignant histological lesions.[9−11]

The treatment of *H. pylori* infections consists of two or three antibiotics and a proton pump inhibitor, but the wide use of antimicrobials has increased the number of therapeutic failures, which strongly indicates the need to improve current therapeutic strategies and to develop new drugs, as non-antibiotic substances.[12−14]

Epidemiological studies have indicated a negative correlation between *H. pylori* seropositivity and the consumption of vegetables.[15] This observation may suggest the occurrence of an inhibitory effect on bacteria of a wide range of fruits and vegetables. The mechanism by which natural compounds inhibit *H. pylori* has been ascribed to the inhibition of the urease activity,[16] adhesion to human gastric mucus,[17] disintegration of the outer membrane,[18] inhibition of the VacA cytotoxic activity,[7,19,20] and the inhibition of bacterial ion pumps.[21]

Our group has observed that a strain harboring *cagA* (*cagA*+) was less susceptible to polyphenols extracted from different grape cultivars and blackberry leaves with respect to a strain lacking this gene.[21,22] In order to ascertain whether resveratrol susceptibility may depend on the expression of CagA, several CagA positive (CagA+) and CagA negative (CagA−) strains were examined. Organisms were isolated from patients with gastric carcinoma (GC) and from dyspeptic patients with chronic gastritis only (CG).



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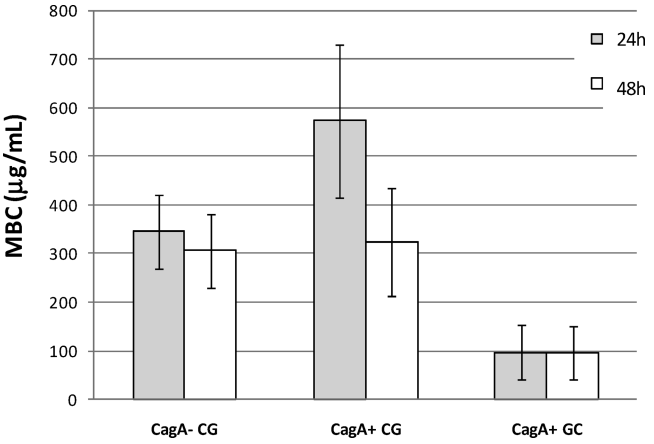

Figure 1. Average values of MBCs calculated for all strains after 24 and 48 h of exposure to resveratrol (**1**). Values of $p < 0.05$ are regarded as significant. MBC values of CagA− from CG and from CG are not statistically different, those CagA+ CG and CagA+ from GC are statistically different, while those of CagA− from CG and CagA+ from GC are statistically different.

It was found that strains isolated from GC cases showed an increased susceptibility to **1** with respect to strains isolated from patients with CG. A hypothesis for this behavior is that one target of the antibacterial action of **1** may be one or more F-type ATPases, which normally protect the bacteria from low pH levels by maintaining a proton gradient across membranes. In strains isolated from patients with gastric carcinoma, such an enzyme may be underexpressed, as an adaptive response to an environment that has lost its natural acidity. Thus, the bacterial defenses are reduced and the susceptibility to **1** is increased.

Table S1 (Supporting Information) shows the minimum bactericidal concentrations (MBC) of resveratrol (**1**) expressed in μg/mL ± SD, for the single strains, after 24 and 48 h of incubation. The mean MBCs after 24 h of exposure to **1** were 98 ± 56 for CagA+ strains from GC and 346 ± 76 for CagA− and 573 ± 158 for CagA+ strains from CG ($p < 0.05$ for CagA+ from CG and CagA+ from GC; $p < 0.05$ for CagA− from CG and CagA+ from GC) (Supporting Information). In Figure 1 the average values of MBCs calculated for all the strains after 24 and 48 h of exposure to resveratrol (**1**) are presented. The mean MBCs were 346 ± 76 for CagA− CG, 573 ± 158 for CagA+ CG, and 97 ± 56 for CagA+ GC after 24 h and 306 ± 76 for CagA− CG, 324 ± 111 for CagA+ CG, and 97 ± 55 for CagA+ GC after 48 h of incubation. In the CagA+ GC group, there was no significant difference in terms of MBC data between 24 and 48 h of incubation. For the CagA+ and CagA− CG groups, the MBC values were decreased in a time-dependent manner.

Several subunits of the human F1 ATP synthase aligned significantly with ATPases of the different *H. pylori* strains have been examined. The alpha subunit showed a high linear homology with different domains of the alpha and beta subunits and the flagellum-specific ATP synthase of *H. pylori* strain KX_438_AG0C1 (taxid, 499171) (a strain originally isolated from atrophic gastric mucosa). The most significant structural homology was with a segment of the beta subunit of 271 amino acids ($E = 5 \times 10^{-115}$). The two segments are 71% identical, and the percentage of amino acids that shared the same behavior is 65%. The lack of gaps (intervals lacking linear homology between two homologue sequences) suggests that the homology between the two polypeptides is very strict (Table S2, Supporting Information). In a recent publication, the amino acid sequence of the human gastric proton pump with *H. pylori* proteins has been compared, in order to determine the putative target of the antimicrobial polyphenol constituents of *Rubus ulmifolius*.[21] Data from the literature, in fact, indicate that polyphenols may inhibit the gastric acidity by reacting with this proton pump. It was found that a significant linear homology ($E = 4 \times 10^{-10}$) occurs with a copper-transporting P-type ATPase of *H. pylori*. The observations that **1** can react with human F-type ATPases[23] and can inhibit the ATPase activity of purified F1 and membrane-bound F-type ATP synthase of *Escherichia coli*[24] prompted us to compare, in the present study, the amino acid sequences of human ATPase type F with *H. pylori* polypeptides. The homology found with a bacterial F0F1 ATP synthase subunit beta ($E = 5 \times 10^{-115}$) was more than 10-fold higher than that between the human gastric proton pump and the bacterial copper transporting ATPase, suggesting that ionic pump type F may be the major target of the antimicrobial activity of **1** against *H. pylori*. Both the P-type (such as the human H^+,K^+-ATPase) and the F-type ATPases reside on cytoplasmic membranes of eukaryotic cells and bacteria; the latter can also reside in mitochondria. As far as the exact mechanism that may account for the bactericidal activity of **1** against *H. pylori* strains is concerned, the findings that resveratrol inhibits either ATP hydrolysis or ATP synthesis of a human F-type ATPase[25] and that the compound can bind and inhibit F0F1-ATP synthase of *E. coli*[24] suggest that this mechanism may reside in the prevention and/or synthesis of ATP. As ATPases are a fundamental way for cellular energy production in animals, plants, and almost all microorganisms, it is likely that resveratrol (**1**) kills *H. pylori* by precluding energy production or utilization.

In the present study, it was discovered that CagA+ strains obtained from patients with CG were less susceptible to the action of **1** than CagA− strains isolated from patients with the same disease condition. Nevertheless, all the CagA+ strains from GC cases exhibited much higher susceptibility to **1** than either the CagA+ and CagA− strain isolated from patients with CG. *H. pylori* strains isolated from neoplastic tissue, or even from normal mucosa surrounding the neoplasia, are characterized by the presence of numerous nucleotide repetitions in the *cagA* variable region.[26] Strains with many nucleotide repetitions showed a reduced tolerance to low pH levels.[27] Such phenomena may represent the consequence of an adaptation of *H. pylori* organism to reduced gastric acidity, a frequent outcome of the gastric mucosa atrophic alterations that usually precede and accompany the development of gastric carcinoma. In the acidic gastric environment, *H. pylori* expresses an adaptive response to low pH levels by means of the urease enzyme and several ionic pumps, one of which, an F-type ATPase, is of vital importance for the maintenance of a proton gradient across membranes.[28,29] Our hypothesis is that the *H. pylori* organism colonizing the gastric mucosa of patients with stomach cancer may undergo a reduced production of one or more ATPases that enable this bacterium to resist acidic conditions, namely, F-type ionic pumps. A possible consequence of a reduced synthesis of F-type ATPases could consist in an augmented susceptibility to resveratrol (**1**), which may react with and inactivate the barely expressed ionic pumps more quickly and efficiently. The results of the amino acid alignments reinforced this hypothesis, as they show that human F-type ATPase, which some researchers have demonstrated as representing a target of **1**,[23,30] has a strong homology with the bacterial F-type ATPase. The hypothesis can be supported by the observation that the

levels of susceptibility to compound **1** increased after 48 h of incubation only with strains from patients with CG, strains that may colonize an acidic gastric environment and therefore express high levels of F-type ATPases. This finding suggests that **1** may require a longer incubation to saturate all the bacterial target sites.

In conclusion, the degree of susceptibility of *H. pylori* to resveratrol (**1**) may differ, depending on characteristics such as the pH levels of the gastric environment where the bacteria reside. Such findings require confirmation with a larger number of strains. In addition, should this behavior be observed also with other polyphenols and antioxidant substances, the source of isolation of the *H. pylori* organism should be taken into consideration when performing susceptibility tests.

EXPERIMENTAL SECTION

General Experimental Procedures. *trans*-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, **1**) was purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland), purity 99%.

Isolation of *H. pylori* Strains, Characterization of the *cagA* Genotype, and Determination of Minimum Bactericidal Concentration. *H. pylori* strains were isolated by streaking mucosa samples onto selective plates provided by bioMérieux Italia s.p.a. (Florence, Italy). After 5−7 days of incubation in a microaerobic environment, suspected colonies were identified by Gram staining and by urease, catalase, and oxidase tests. Bacteria were subcultured two times to increase the number of organisms, suspended in Columbia broth with 20% of glycerol, and stored at −80 °C until susceptibility tests were performed. The presence of the *cagA* gene was determined by a polymerase chain reaction using primers specific for the constant *cagA* region.

The antibacterial activity of resveratrol (**1**) was tested against the following *H. pylori* strains: eight CagA− strains, isolated from dyspeptic patients with chronic gastritis only (G50, G21b, G104, Ba142, NS23, G12, G204, and 328 KM), with the latter strain a *cagA*− isogenic mutant of strain 328); nine CagA+ strains, isolated from patients with a similar pathology (CCUG 17874, MDO21, MD6, 328, G27, Ma01, SI10, G39, and G20); nine CagA+ strains, isolated from patients with gastric carcinoma (10K, 18K7, 10Kb, 17C7, 18C7, 4Cb, 3Cb, 1C1, and 2Ca).

Resveratrol (**1**) was dissolved in water containing 4% DMSO and sterilized by filtration. The solution was double diluted in *Brucella* broth with 10% BFS (bovine fetal serum). Tests were carried out at a final volume of 100 μL using microtiter plates. *H. pylori* suspensions were prepared starting from cultures on *Brucella* agar-BFS incubated in a microaerobic environment for 48 h. The bacterial suspensions were then added to each microwell at a final concentration of approximately 10^6 colony forming units per mL. After both 24 and 48 h of incubation in microaerobic conditions at 37 °C, 3 μL of each dilution was deposited on Columbia-blood agar plates, which were incubated for 3−5 days. The lowest concentration in broth, for which the subculture on agar showed complete absence of growth, was considered the MBC.

Alignment of F_0F_1-ATPase Amino Acid Sequence with the F-Type-ATPase of *H. pylori*. The purpose of this test was to determine the possible target of the antimicrobial activity of resveratrol (**1**). Polypeptides with similar amino acid sequences are supposed to share the same behavior when matched with substances capable of reacting with them. Since it is known that **1** interacts with a human cardiac F1-ATPase,[23,30] an alignment was performed by "blasting" the N-acid sequences of the five different subunits (alpha, beta, gamma, delta, and epsilon) that compose the ATP synthase, H^+ transporting, mitochondrial F1 complex of the human cardiac muscle, with the sequences of ATPases encoded by several strains of *H. pylori*, for which the nucleotide sequences are available at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein&cmd=search&term=): strain J99 (taxid 85963); strain B128 (taxid 544406); strain 26695 (taxid 85962); and strain HPKX_438_AG0C1 (taxid 499171). Sequences longer than five amino acids were also included, even if the alignment was interrupted by one or two nonmatching amino acids. When two proteins are made up by at least five identical (or with the same chemical properties) amino acids in sequence, they are considered a homologue (Supporting Information).

Statistical Analysis. Each sample was tested in triplicate for all experiments. The mean MBCs obtained with CagA+ strains from GC cases were compared with those of the CagA+ and CagA− strains isolated from CG patients by the Mann−Whitney test. The results of all experiments were expressed as mean ± SD. In all tests, values of $p < 0.05$ were regarded as significant.

ASSOCIATED CONTENT

Supporting Information. MBC values of resveratrol (**1**) obtained for *H. pylori* strains with different virulence and isolated from different pathologies. Designation, origin, CagA status, and MBC values of the *H. pylori* strains after 28 and 48 h of exposure to resveratrol (**1**). The most significant alignment between human F0F1 ATP synthase and *H. pylori*. This information is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +390577234372. Fax: +390577234177. E-mail: silvia.martini@unisi.it.

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