Resveratrol (trans-3,5,4’-trihydroxystilbene) is a phytoalexin present in a wide variety of plant species, where its synthesis is induced by stress conditions such as infection or trauma (1). Different phenolic compounds, including resveratrol, show potent antioxidant effects and may have several therapeutic applications including neoplastic diseases (2,3). The mechanisms by which resveratrol exerts its antitumor effects are still unclear but may include apoptotic cell death activation, or inhibition of cell division, of DNA polymerase, of ribonucleotide reductase, of protein kinase C, or of cyclooxygenase-2. Moreover, this agent was found to reduce markedly reactive oxygen species (ROS)-mediated carcinogenesis (4).

Several reports suggest that resveratrol suppresses proliferation of colon cancer cells by in vitro apoptosis induction (5) and cell cycle arrest (6). However, these effects cannot fully explain the underlying molecular mechanisms for the anti-cancer activity of the drug.

Telomeres are non-coding regions containing hexameric repetitive sequences (TTAGGG) placed at chromosome ends that undergo progressive cleavage upon cell duplication. Telomerase is an RNA-dependent DNA polymerase with reverse transcriptase activity, that restores telomere length by adding the repetitive sequences to telomeres in dividing cells (7). This enzymatic activity is essential to ensure chromosomal stability and to prevent ageing (8). High telomerase activity (TLMA) was found to be associated with cancer cells, and not with terminally differentiated non-dividing cells of homologous normal tissues (9). Most tumor cells, with indefinite proliferative ability, maintain telomeres through the expression of TLMA, which is always associated with the expression of the catalytic subunit of the enzyme, the human telomerase reverse transcriptase (hTERT).

A strong correlation has been observed between hTERT mRNA expression and TLMA in a variety of epithelial cancers, including colon cancer (10). Inhibition of hTERT results in telomere loss and limits the growth of tumor cells that undergo apoptosis when their telomeres reach a critically short length.

The present report illustrates the effects of resveratrol treatment on cell growth and TLMA of HT29 and WiDr, two human colon cancer cell lines. Our results confirm the growth-inhibitory effects of resveratrol on malignant cells and point out that this natural product is able to down-regulate TLMA. Therefore, it can be suggested that the anti-TLMA activity of resveratrol could contribute significantly to its antineoplastic properties.
Materials and Methods

Reagents
Trans-resveratrol (trans-3,5,4’-trihydroxystilbene) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mM (both reagent purchased by Sigma Chemical Co., St Louis Mo). The stock solution was stored at -80°C, and diluted in culture medium just before use.

Cell culture
The human colon cell lines HT-29 and WiDr, were cultured at 37°C in 5% CO2 humidified atmosphere and maintained in RPMI-1640 (Hyclone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS-Hyclone Laboratories, Logan, UK), 2 mM L-glutamine (Glu), and antibiotics (Life Technologies Ltd., Paisley, Scotland), hereinafter referred to as complete medium (CM).

Determination of cell growth
Fully confluent cells were harvested, counted and suspended in CM. Cells were seeded in 24-well tissue culture plates (Falcon) at a concentration of 1 x 10^5 cells/ml and allowed to adhere overnight. Cells were incubated with resveratrol at the final concentrations of 5, 10, 20 and 40 µg/ml (3 wells for each treatment) or DMSO alone as control. The plates were incubated at 37°C in a 5% CO₂ humidified atmosphere for 24, 48 or 72 hrs. Cell growth and viability were evaluated every 24 hrs. Trypsinized cells were manually counted using a hemocytometer and cell viability was determined by trypan blue dye exclusion assay.

Evaluation of TLMA
Samples of cells treated with resveratrol for 48 or 72 hr were harvested, counted, pelleted and processed for determining TLMA by using classical TRAP technique.

Frozen pellets were lysed directly in the wells using 80 µl of ice-cold extraction buffer (11). PCR amplification of telomerase extension products was performed as previously described (11). Briefly, amplification of the telomeric products was performed by PCR (GeneAmp PCR System 9700, PE Applied Biosystems). Forty-five out of 50 µl of the PCR were loaded on a 10% non-denaturing polyacrylamide gel. Gels were fixed and exposed to X-ray film (Kodak, Rochester, NY, U.S.A.). Bidimensional densitometry of the repetitive ladder bands was performed using a Bio-Rad (Richmond, CA, U.S.A.) scanning apparatus (Imaging densitometer, GS-670; Molecular Analyst software Software, Richmond, CA, USA). The signal intensity of each ladder was measured and the sum of the signals, expressed in optical density per mm² (OD) of the ladder products in each lane, was used for semi-quantitative analysis.

Statistical analysis
Results are expressed in terms of means (M) ± standard error (SE) of the mean. Statistical significance was determined by Student’s t test analysis.

Results
Antiproliferative effect of resveratrol
In order to test the antiproliferative effect of resveratrol, HT-29 and WiDr cancer cells were treated with graded concentrations of this agent for 72 hrs. As illustrated in Figure 1, cell growth kinetics of HT-29 (Fig 1A) and WiDr (Fig1B) was evaluated in terms of number of viable cells. In particular, the treatment with 40 µg/ml of resveratrol induced a potent cytotoxic effect in both lines, since, more than 90% of treated tumor cells were found to be dead as early as 24 hrs after treatment (data not shown). On the other hand, the growth of cells exposed to 5 µg/ml was similar to that of control cells in both lines. The exposure of cells to 10 or 20 µg/ml of resveratrol produced a significant, concentration-related, decrease of viable cell counts. Both lines showed a similar inhibition of growth kinetics when 24 or 48 hrs of culture are considered. However, when cell growth was observed after 72 hrs of culture, the treatment with 20 µg/ml produced higher inhibition in HT29 cell line respect to that found in WiDr tumor line, which showed a partial recovery of cell growth.

Effect of resveratrol on TLMA
HT-29 and WiDr cells, untreated or exposed to 2.5, 5, 10, 20 and 40 µg/ml of resveratrol, were tested for TLMA after 48 and 72 hrs of culture respectively. The results of a representative TRAP assay, confirmed by three other independent experiments, are illustrated in Figure 2. Moreover, the results, expressed as percent inhibition with respect to that of controls (DMSO alone) and calculated on the basis of densitometric analysis, as described in Materials and Methods, are illustrated in the legend of Figure 2. The results show that resveratrol at concentrations higher than 2.5 µg/ml induced a substantial and concentration-dependent down-regulation of TLMA in both
Discussion

The present study confirms that relatively high concentrations of resveratrol reduce the number of viable HT-29 and WiDr colon cancer cells, preventing their exponential growth. Furthermore, this agent has been found to reduce TLMA in the same malignant cells.
Telomerase is specifically activated in most malignant tumors that acquire "immortal" phenotype, since this enzyme complex is responsible for telomere length restoration after telomere erosion resulting from cell duplication (13, 14). Therefore, telomerase could be a novel and selective target for antitumor drug design. Actually, chemopreventive and anticancer agents able to inhibit TLMA push tumor cells into a crisis state leading to apoptosis (15, 16). Suppression of telomerase function promotes apoptosis in different types of cells, assigning to hTERT a putative antiapoptotic role, which is independent of its enzymatic and telomere-maintenance function. In particular, modulation of TLMA directly interferes at an early stage of the intrinsic apoptotic pathway upstream of mitochondrial alterations (17). It is well known that the reduced cell viability by resveratrol was due to apoptotic cell death as the percentage of cells exhibiting the sub-G(0) arrest and DNA fragmentation increased (4). The observation that apoptosis, growth delay and inhibition of TLMA were all found in a dose- and time-dependent manner, suggests a relationship between these events. There is substantial evidence showing that telomeres normally exist in a capped state but may switch to uncapped state. Uncapped chromosome ends are generally thought to be at great risk of degradation, recombination or fusion by DNA repair system. In response to damaged telomeres, cell can undergo apoptosis and die. The appropriate response to the uncapping of a telomere is activation of telomerase in order to protect the telomere from signalling into cell cycle arrest/apoptosis pathways (18). Actually, it has been confirmed that the impairment of cell growth by hTERT inhibition can be sustained by loss of the hTERT-mediated capping function of telomerase, with consequent telomere destabilization (19).

In conclusion, the interference of resveratrol on telomerase function suggests that polyphenols could play an up-to-now unexplored role in the development of novel anticancer approaches. Further studies are in progress to clarify the possible correlation between apoptosis induction and telomerase reduction observed in target tumor cells under the influence of resveratrol.

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