Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation

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Keywords:
Colon cancer, Hydroxytyrosol, EGFR degradation, Olive oil, Polyphenols

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Abbreviations

CHX: Cycloheximide; HT: 2-(3,4-dihydroxyphenil)ethanol or hydroxytyrosol; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; MAPK: mitogen-activated protein kinase; EEC: European Community; HIF-1α: Hypoxia inducible factor-1 alpha; mPGES-1: microsomal prostaglandin E synthase-1; PGE-2: prostaglandin E-2; ptyr: phospho tyrosine; VEGF: vascular endothelial growth factor

Abstract

Scope

We studied the effects and mechanism of 2-(3,4-dihydroxyphenil)ethanol (or hydroxytyrosol, HT), a polyphenol from extra-virgin olive oil, investigating the regulation of epidermal growth factor receptor (EGFR) expression in colon tumour cells.

Methods and results

We demonstrate that HT significantly down-regulates EGFR expression in human colorectal adenocarcinoma cells HT-29, CaCo2 and WiDr, and in HT-29 xenografts. HT accelerates EGFR degradation by reducing its half-life. Specifically, HT induces EGFR ubiquitination which is mediated by phosphorylation at pY1045, the docking site for Cbl, thereby enabling receptor ubiquitination and degradation. Pre-treatment with either the lysosomal inhibitor chloroquine, or the proteasomal inhibitor *MG132* blocks HT-induced EGFR down-regulation. In colon cancer cells, EGFR down-regulation by HT is associated with reduced cell proliferation. Tumour growth and EGFR expression levels are also decreased by HT treatment in HT-29 xenograft.

Discussion

We conclude that HT down-regulates EGFR expression via lysosomal and proteasomal degradation, activated by HT-induced EGFR phosphorylation at pY1045 and increased Cbl activity. Cbl activation induces, in turn, EGFR ubiquitination. Our results reveal a new mechanism for HT's anti-tumour effects that may be important for colon tumour prevention and treatment.

1. Introduction

Epidemiological and pre-clinical studies have suggested that polyphenols may exert protective effect against cancer. Extra virgin olive oil contains abundant phenolic compounds, such as tyrosol (Tyr), hydroxytyrosol or 2-(3, 4-dihydroxyphenil)ethanol (HT), lignans and secoiridoids and its consumption is reported to protect against cancer development, particularly colon cancer [1-4]. Indeed, treatment of human colon adenocarcinoma cells with olive oil polyphenols inhibits the initiation, promotion and metastasis of colon cancer [5-7].

Among phenolic compounds, HT has been the most investigated compound, primarily for its bioavailability [8]. Indeed, while HT levels are relatively low in olive oil, they are in the high μM concentration in colon, after the gastric hydrolysis and colonic fermentation of secoiridoids present in olive oil [9]. HT has scavenging, anti-inflammatory, and antiangiogenic properties, which counteract cancer development. Further, HT also acts through the modulation of pro- and anti-oncogenic signalling pathways, leading to apoptosis and growth arrest of tumour cell lines *in vitro* [10-12], including HL60 leukaemia cells [13], melanoma cells [14] and colon cancer cell lines [13, 15, 16]. We have previously demonstrated that HT, at high μM concentration, reduces colon tumour progression *in vitro* and *in vivo* by interfering with signalling pathways of inflammation (mPGES-1/PGE-2) and angiogenesis (HIF-1α/VEGF) [17].

In tumour epithelial cells, we and other demonstrated that epidermal growth factor receptor (EGFR) signalling controls both inflammatory and pro-angiogenic signalling pathways [18-21]. EGFR, one of the main driver of colon carcinogenesis, regulates important tumorigenic processes including proliferation, apoptosis, angiogenesis, and invasion [22-24]. Expression of the EGFR family and their ligands correlates with more aggressive disease and a poorer prognosis [25, 26]. Because EGFR is frequently overexpressed in colorectal cancer, it has been targeted with a variety of agents, including monoclonal antibodies [27-31].

EGFR is activated directly by various ligands, and indirectly it is transactivated in response to stimuli, such as tumour necrosis factor or prostaglandin E2 [18, 32, 33]. Ligand binding to EGFR leads to receptor dimerization, phosphorylation and increased tyrosine kinase activity [34], but also leads to rapid internalization and proteasomal/lysosomal degradation of the receptor. This process results in the down-regulation of both total and cell surface receptors. Degradation of EGFR is initiated by phosphorylation in tyrosine 1045, followed by binding to Cbl adaptor protein and phosphorylation of Cbl. Tyrosine phosphorylation of Cbl increases its ubiquitin ligase activity, resulting in EGFR ubiquitination on multiple sites and its internalization [35, 36]. It is well documented that EGFR is processed by lysosomal degradation [37, 38], however, proteasomes have also been proposed to regulate EGFR degradation [39].

In this study we investigated in normal and tumour colon cells, and in HT-29 cell xenograft model whether HT affected EGFR down-regulation and its underlying mechanism. We report that, in tumour cells, HT reduces EGFR level by promoting its degradation via both lysosomal and proteasomal mechanisms, while it does not affect EGFR level in normal colon cells. Further, at low concentration, HT was found to synergize with Tyr in reducing EGFR expression. These results illustrate an interesting mechanism of action for HT, which might be exploited in combination with chemotherapy for colon cancer treatment.

2. Material and methods

2.1 Cell lines

HT-29 (passages 10-20, ATCC® HTB-38™), CaCo2 (passages 12-20, ATCC® HTB-37™) and WiDr (passages 5-20, ATCC® CCL-218™), human colorectal adenocarcinoma cells, or CCD18Co (passages 2-15, ATCC® CRL-1459™) human colon fibroblast cells were obtained from the American Type Culture Collection (ATCC, LGC Standards S.r.l., Sesto San Giovanni, Italy), and certified by STRA and cultured as recommended.

2.2 Reagents

Reagents were as follows: Chloroquine, Cycloheximide (CHX), Tyr, anti-β-actin (Sigma Aldrich, Milan Italy); HT, MG132 (Cayman Chemicals, VinciBiochem, Vinci, Italy); anti-Ki67 (Merk Millipore, Vimodrone, Italy); EGF (RELIAtech, VinciBiochem, Vinci, Italy), anti-EGFR, anti-ubiquitin, anti-phospho EGFR-Y1045, anti-phospho Cbl, anti-phospho tyr, anti phospho-p44/42 mitogen-activated protein kinase (MAPK, ERK 1/2) and anti-phospho-Akt (Cell Signalling, Leiden, the Netherlands).

2.3 MTT assay

Cell proliferation was quantified by Vybrant MTT (Sigma Aldrich) cell proliferation assay as described [18]. Briefly, colon cells (3×10^3) were seeded in 96-multiwell plates in medium with 10% serum for 24 hrs and then exposed to HT (100 µM) with/without EGF (25 ng/ml) for 48 hrs. Data are reported as cell growth at 540 nm absorbance/well.

2.4 BrDU labeling assay

Cell proliferation was determined by 5-bromo-2′-deoxy-uridine (BrdU) incorporation using a colorimetric ELISA, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, USA). Briefly, 1.5×10^3 cells were seeded in 96 multiplate. After 24 hrs cells were incubated with either HT or Tyr (both at 1, 3, 10, 30 100 or 300 µM) in presence/absence of EGF (25 ng/ml) for 6 hrs. BrdU was added during the late stage (4 hrs) of incubation. Cells were washed, fixed in ethanol and incubated with a monoclonal antibody directed against BrdU, followed by an alkaline phosphatase-conjugated secondary antibody. Stained cells were randomly counted (10 fields) with a light microscope (Nikon Eclipse E400, Florence, Italy) at 20 X magnification. Data are reported as BrdU labelled cell/well.

2.5 EGFR kinetic studies

HT-29, CaCo2 and WiDr cells were treated with vehicle or HT (100 μM) for 4 hrs followed by CHX (100 μg/ml). Cells were then harvested at different time points (0-18 hrs). The effect of HT on EGFR half-life was assessed using immunoblot analysis with the anti-EGFR antibody.

2.6 Immunoblot analysis and immunoprecipitation

Total protein lysates were obtained as previously described [17]. Antibodies used are as follows: anti-EGFR, anti-ubiquitin, anti-phospho EGFR-tyr 1045, anti-phospho Cbl, anti-phospho-tyr, anti phospho-p44/42 mitogen-activated protein kinase (MAPK) and anti-phospho-Akt (Cell Signalling). Cells were stimulated with HT (100 μM) for the indicated times. Anti-EGFR was added to the precleared lysates. Western blotting was performed as described [17]. Images were digitalized with CHEMI DOC Quantity One programme, blots were analysed in triplicate by densitometry using NIH Image 1.60B5 software, and the arbitrary densitometric units (ADU) were normalized for β-actin (Sigma Aldrich).

***2.8 In vivo* tumour xenograft study**

Experiments have been performed in accordance with the EEC guidelines (Law No. 86/609) and National Ethical Committee. All experimental protocols were approved by University of Florence ethical committee (authorization number 518/2015-PR). Immunodeficient mice (5 week-old female athymic nude mice, Harlan, Udine, Italy, were kept in temperature- and humidity-controlled rooms, 22°C and 50%, with lights on from 7 am to 7 pm and water and food ad libitum) were s.c. inoculated in the right flank with 10^{10} HT-29 cells in 50 μl of PBS. After 4 days, when tumours reached a 70-100 mm^3 volume, animals were randomly assigned to 2 different experimental protocols. At this time i.p. HT treatment (10 mg/kg, daily, 14 mice), or vehicle (10% ethanol, 14 mice) started. Mice were treated with 200 μl volume i.p., for 14 consecutive days. I.p. administration was chosen to maximize the absorption of HT and minimize animal discomfort. Data are reported as tumour volume (mm^3). Animals were observed daily for signs of toxicity (body weight loss, failure to drink or eat, laboured respiration, hind-limb paralysis or weakness and abdominal distension) and no side effects in terms of change in mouse body weight were observed. At day 14 mice were sacrificed by CO_2 asphyxiation, and tumours collected and split in two parts. One part was immediately frozen in liquid nitrogen for Western blotting as described [17], the other part was embedded in Tissue-Tek O.C.T. (Sakura, San Marcos, USA), for histology. Six-μm-thick cryostat sections from tissue samples were processed for immunohistochemical staining. For immunostaining, sections were fixed in acetone, and aspecific binding sites were blocked by using

3% BSA. Slides were incubated with an alexafluor 488 conjugate anti-rabbit EGFR (Cell Signalling) or anti-rabbit phospho-EGFR (Y1045). Immunoreactions for phospho-EGFR was revealed by using a TRICT-conjugated anti-mouse secondary antibody. Images were analysed using Nikon Eclypse T200, and quantification of EGFR and phospho-EGFR positive cells was performed counting positive cells in 10 random field/section for slides, keeping constant the acquisition parameters gain (1.2) and time of exposure. Slides were counter-stained with DAPI.
For histopathological analysis of Ki67 antibody we used immunohistochemical staining followed by hematoxylin counterstaining. After inactivating of endogenous peroxidase activity and blocking of cross-reactivity with 3% BSA, the sections were incubated at 37 °C for 1 hr with a diluted solution of Ki67 (1:30). Location of the primary antibodies was achieved by subsequent application of a biotin-conjugated anti-primary antibody, a streptavidin-peroxidase and diaminobenzidine (Sigma Aldrich). The staining was developed using a commercial immunoperoxidase staining kit following the manufacturer's instruction (the biotin–streptavidin complex method, Merk Millipore) and visualized at 10 X magnification (Nikon 10 X/0.25). Negative controls were established by replacing the primary antibody with PBS. Specific staining for Ki67 was categorized as either positive or negative based on the presence of brown-colour staining.

2.9 Immunofluorescence

Tumour cells (5 x 10^4 cells/well on glass cover-slips placed into 24 multiwell plates) were maintained in 10 % FBS for 48 hrs. Cells were fixed in acetone for 5 min. After the blocking of unspecific bindings in 3% BSA, cells were incubated overnight at 4°C with the primary antibody, an alexafluor 488 conjugate anti-rabbit EGFR (Cell signalling) or anti-rabbit phospho-EGFR (Y1045) (Cell signalling). Cells were then incubated with secondary antibody TRITC conjugated (Sigma Aldrich) and visualized by fluorescence microscope (Eclipse TE300, Nikon) at 60 X magnification (Nikon Plan Apo Ve, 60 X 1.4 Oil) and images taken by a digital camera (Nikon digital sight u2).

2.10 Statistical analysis

Results are expressed as means ± SD. Statistical analysis was carried out using Student's t test, two-away ANOVA, or Bonferroni post-test for multiple comparison. $P<0.05$ was considered statistically significant.

3 Results

3.1 HT reduces tumour colon cell survival

First, we investigated whether HT would affect colon tumour cell proliferation measured as BrdU incorporation. HT-29, WiDr and CaCo2 cells were challenged with EGF (25 ng/ml), and co-treated with HT at increasing concentrations ranging from 1 to 300 μM. In all tumour cells, HT reduced EGF-dependent proliferation in a concentration dependent manner (Fig. 1). As shown, HT-29 were most sensitive to HT action, as a significant growth reduction was observed at HT concentration as low as 30 μM (28% of reduction, $p<0.05$; EC50 of 181.8, 281.64, 278.5 μM, for HT-29, CaCo2 and WiDr, respectively). We used 100 μM HT concentration throughout this work, as it was the minimal inhibitory concentration in all tumour cells. We also evaluated the cell survival at 48 hrs, under the challenge with HT and EGF, by the MTT assay. While HT had a negligible effect on unstimulated cells, it reduced EGF-stimulated tumour cell survival according to the following order: HT-29 > CaCo2 > WiDr (Supporting Information Figure S1A). Conversely, in normal epithelial colon cells, CCD18co, HT (100 μM) with or without EGF (25 ng/ml) did not significantly affect cell survival (Supporting Information Figure S1B).

3.2 HT reduces EGFR expression in colon cancer cells

As we observed that HT reduced EGF-dependent cell growth, we investigated the role of the polyphenol on EGF/EGFR signalling activation in the above mentioned colon tumour cell lines. HT pre-treatment (30 min) neither affected EGFR total phosphorylation, nor the phosphorylation of the downstream ERK1/2 or Akt kinases after 15 min of EGF (25 ng/ml) stimulation (Supporting Information Figure S2A, B and C). Next, we measured EGFR expression. All the cell lines expressed comparable levels of EGFR in basal condition (10 % FBS, 0 time) (Fig. 2A). Treatment with HT (once, 100 μM) reduced EGFR amount in all cell lines with maximal effect between 2 and 4 hrs, measured by western blot and immunofluorescence analysis (Fig. 2 A and B).
Conversely, HT did not affect EGFR amount in CCD18co cells (Fig. 2C), indicating that its effect is mainly on tumour cells.

3.3 HT induces both EGFR-tyrosine 1045 and Cbl phosphorylation

The ability of HT to inhibit EGFR levels suggested that the polyphenol might increase EGFR degradation. Indeed, HT treatment provoked a marked increase (on average 11.4 fold) of EGFR phosphorylation on the tyrosine auto-phosphorylation site 1045 in all cell lines, maximal effect being at 8 hrs (Fig. 3A, B and C). Tyr1045 phosphorylation was not appreciated by using the total tyr phosphorylation antibody (see above). Moreover, since EGFR Y1045 is a binding site for Cbl,

which when phosphorylated increases its ubiquitin ligase activity for ubiquitination of EGFR [16], we measured the effect of HT on Cbl activation. HT increased Cbl phosphorylation in all colon tumour cells (Fig. 3A, B and C), indicating that it promotes EGFR down-regulation by regulating Cbl phosphorylation.

3.4 HT accelerates EGFR degradation

Given the reduced EGFR expression following HT treatment, we investigated the HT effect on EGFR stability. We performed a time-course experiment analysing EGFR levels at various time points after incubation of tumour cells with HT (100 μM) for 4 hrs, coupled with CHX (100 μg/ml) to block *de novo* protein synthesis. HT had a dramatic effect on the rate of EGFR loss, as it reduced the EGFR half-life by 50 to 75 % compared to controls, depending on the cell type (Fig. 4). T1/2 was 6, 3 and 3 hrs in HT-29, CaCo2 and WiDr respectively (Fig. 4A-C), compared to 12 hrs for controls. Furthermore, immunoprecipation using anti-EGFR antibody showed that HT increased EGFR ubiquitination in all the cell lines examined (Fig. 4D), indicating that HT ultimately promotes EGFR degradation by regulating the receptor ubiquitination.

To determine whether HT-induced EGFR degradation occurred through proteasomal and/or lysosomal degradation, tumour cells were pre-treated (1 hr) with a lysosomal inhibitor (chloroquine 10 μM) or a proteasomal inhibitor (MG132, 25 μg/ml) followed by incubation with HT (100 μM, 4 hrs) (Fig. 4E). Chloroquine, prevented HT-elicited degradation of EGFR only in CaCo2 cells, while it decreased EGFR expression in HT-29 and WiDr, probably via a non-specific mechanism (Fig. 4E). Conversely, MG132 efficiently reversed the HT-elicited degradation of EGFR in HT-29 and WiDr cells, while exerting a weak effect on CaCo2 cells (Fig. 4F). Consistently, pre-treatment of HT-29 and WiDr cells with MG132 or CaCo2 cells with chloroquine significantly interfered with HT activity on tumour cell growth (Fig. 4G). These data indicate that the inhibitory activity of HT on tumour growth is related to its effect on EGFR degradation by proteasomal/lysosomal pathway. All together it appears that EGFR degradation occurs mainly through the proteasomal pathway although, it might be, to a certain extent cell-type dependent.

3.5 HT and Tyr synergize to decrease EGFR expression

Tyr is the other phenolic compound present in olive oil (40). HT and Tyr, as free or conjugated forms, represent almost half of the total phenolic content of a virgin olive oil, and they share many biological functions (40, 41, 9). We found a synergistic interaction between HT and Tyr on tumour proliferation and EGFR expression in HT-29 tumour cells (Supporting Information Figure S3A-C). Indeed, as for HT, Tyr was unable to significantly affect HT-29 tumour cell growth in unstimulated

cells up to 300 µM (Supporting Information Figure S3A). Isobolographic analysis showed that HT (1 µM-100 µM) synergized with Tyr (1 µM-100 µM), reducing HT-29 cell growth (Supporting Information Figure S3B). This synergism was also observed on EGFR expression (Supporting Information Figure S3C), in which simultaneous treatment of HT-29 cells with Tyr 30 µM and HT 10 µM markedly reduced EGFR expression and increased EGFR Y1045 phosphorylation (Supporting Information Figure S3C, D).

3.6 HT decreases tumour growth and EGFR expression in HT-29 tumour xenograft

The HT effects on EGFR expression, observed in cultured tumour cells, were corroborated by an *in vivo* experiment in a mouse xenograft model inoculated with HT-29 colon cancer cells. We observed that sub-chronic treatment with HT (10 mg/kg for 14 days) significantly reduced tumour growth compared to control (Fig. 5A), together with a decrease in cell proliferation (Ki67 positive cells) as well as EGFR expression levels (Fig. 5B ,C and D), and conversely an increase of EGFR phosphorylated at Y1045 (Fig. 5F).

Discussion

Nutraceuticals, the bioactive food components, have numerous beneficial effects, including anticancer properties and affect multiple signalling pathways that are deregulated in cancer cells. Some nutraceuticals are currently in clinical trials, and others have already been approved for human use. Among nutraceuticals, polyphenols are the most abundant in fruits, vegetables, and plant-derived beverages.

A number of reports have delineated the anti-tumour activity of HT, a polyphenol of extra-virgin olive oil, showing that it attenuates tumour cell proliferation mainly by reducing the pro-proliferative input of chemokines [17, 42]. Here, we describe the inhibitory effect of HT treatment on the EGFR oncogenic drive, on colon tumour cells and in a mouse xenograft model.

This inhibitory effect is associated to the accelerated degradation of EGFR. In fact, HT treatment of colon tumour cells, HT-29, WiDr and CaCo2, produced a sharp decline of EGFR amount which persisted up to 8/18 hrs, depending on the cell line. The inhibitory effect of HT on EGFR amount was observed starting from 30 μM being maximal at 100 μM, a concentration of polyphenol which might be absorbed by consumption of approximately 50 ml of olive oil [40], and which has been reported to be metabolized by colon tumour cells in functional metabolites, as observed in physiological conditions [9]. Concurrently with the EGFR loss, we observed a marked rise of EGFR auto-phosphorylation at Y1045 (11 fold on average), a site associated with ubiquitin ligase activity. Indeed, the large increase of EGFR Y1045 phosphorylation (at 4 hrs on average) enhances the availability of ubiquitin ligase docking site, as indicated by the marked activation of ubiquitin ligase Cbl, and the ensuing ubiquitination of EGFR in all cells examined. Measurements of EGFR half-life provided clear evidence that HT treatment drastically curtails the receptor protein life-span by promoting its ubiquitination. Further analysis of the ubiquitin-proteasome pathway in response to HT treatment, obtained with known inhibitors of either the lysosomal or proteasome systems, namely chloroquine and MG132, suggest that EGFR processing was cell type dependent. In fact, the HT-induced degradation of EGFR was reversed in HT-29 and WiDr by MG132, while it was affected by chloroquine in CaCo2 cells. Thus, in colon tumour cells, HT specifically promotes the intracellular processing of EGFR through ubiquitin-proteasomal/lysosomal activation, while early intracellular EGFR signalling such as Akt and ERK1/2 phosphorylation was unchanged.

Importantly, HT effects on EGFR system was selectively directed at colon tumour cells. Indeed, in normal epithelial colon cells, CCD18co, HT 100 μM did not show significant effect on cell survival, nor on EGFR expression.

HT, administered sub-chronically to HT-29 tumour bearing mice, resulted in a highly significant reduction of the proliferative ability (> 70%) of malignant cells, and consequently in a marked

suppression of tumour growth. Importantly, in line with the *in vitro* results, HT treatment elicited a large increase of the EGFR Y1045, whereas the EGFR expression levels were significantly down-regulated.

Extra virgin olive oil contains simple phenolic compounds other than HT, such as tyrosol (Tyr), and HT and Tyr secoiridoid derivatives, such as oleuropein. HT and Tyr have been demonstrated to exert and to share many beneficial biological effects both *in vitro* and *in vivo*, HT, often being, more effective than Tyr [43, 44]. Here, we demonstrate that at low concentrations as 10 μM, HT synergizes with Tyr on HT-29 colon tumour cell growth and EGFR amount. Indeed, co-treatment of HT-29 cells with low concentrations of HT and Tyr, decreased cell growth and EGFR amount, while increased the receptor ubiquitination far above those obtained by administration of single compounds, suggesting that the structure similarity between HT and Tyr should be critical for these effects. The observed synergism between HT and Tyr also suggests that the natural compound mixture present in olive oil might elicit much greater functional effects than previously estimated, and sustains the relevant use of this nutrient in the Mediterranean diet to prevent colorectal tumours.

While most studies on the antitumor activity of HT focused on its effects on signals originating from the inflammatory tumour microenvironment (e.g. cytokines) [17, 42], this work illustrates the influence of HT on the main intrinsic oncogenic driver of colon tumorigenesis, i.e. the EGF/EGFR system. Whether this novel mechanism prevails in determining the overall chemo-preventing effect of HT remains to be explored. It is plausible that the above mechanisms might work in concert, likely to be controlled, in a context-dependent manner, by an upstream master regulator, as speculated in a report on the HT effects on ER stress-dependent signalling pathways [16]. Since HT elicits a broad range of effects, it is a conceivable candidate as master regulator. A drawback of the HT-induced activation of ubiquitin ligase Cbl is that it appears to be specific for the EGFR, as mutated EGFRs, frequently found in human tumours, consistently elude the ubiquitin processing [45]. However, a recent report describes the feasibility of overcoming the typical down-regulation of a specific ubiquitin ligase RNF125 in melanoma, thus avoiding the incipient resistance to TKs inhibitors [46].

All together these findings provide clear evidence that HT-induced activation of the ubiquitin-proteasome/lysosomal axis reduces the oncogenic EGF/EGFR drive and attenuates colon tumour development.

Author contributions
ET designed, performed the research, analysed the data and wrote the paper
SD designed the research study, analysed the data and wrote the paper
AG wrote the paper
MZ designed the research study and wrote the paper

Acknowledgments

This work was supported by Associazione Italiana sul Cancro, IG10731 (MZ), Consorzio per la tutela dell'Olio Extravergine di Oliva Toscano IGP, and Regione Toscana, bando Nutraceutica 2014, OlivaPlus Project (SD). ET is a Fondazione Italiana per la Ricerca sul cancro fellow.

The authors declare no conflict of interest.

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Figure legends

Figure 1: HT reduces colorectal cancer cell survival. HT-29 (blue line), CaCo2 (black line) and WiDr (red line) cells were exposed to different concentrations of HT in presence of EGF (25 ng/ml) for 6 hrs and growth was evaluated by BrdU incorporation. Data are reported as BrdU labelled cells/well. Numbers represent mean ± SEM of three experiments run in triplicate. *** P <0.001, ** P < 0.01, * P < 0.05 vs. EGF-treated cells. ## P <0.01 vs. untreated cells.

Figure 2. HT decreases EGFR expression. (A) Western blot analysis and quantification (arbitrary density unit, A.D.U.) of EGFR expression in HT-29, CaCo2 and WiDr cells exposed to 10% FBS and HT for the indicated times. β-actin has been used to normalize loading. N=3. Numbers are the mean ± SD. *** P <0.001, ** P < 0.01, * P < 0.05 vs. untreated cells. (B) Images of immunostaining for EGFR (green), and DAPI (blue) and in tumour cells from 10 % FBS (a), HT 100 μM (b). Scale bars=100 μm. (C) Western blot analysis and quantification (A.D.U.) of EGFR expression in CCD18co cells exposed to 10% FBS and HT for the indicated times. β-actin has been used to normalize loading. N=3. Numbers are the mean ± SD.

Figure 3. HT induces EGFR Y1045 and Cbl phosphorylation. Western blot analysis and quantification (arbitrary density unit, A.D.U.) of EGFR phospho-Y1045 and phospho-Cbl expression in HT-29 (A), CaCo2 (B) and WiDr (C) cells exposed to 10% serum with or without HT 100 μM for the indicated times. β-actin has been used to normalize loading. N=3. Numbers are the mean ± SD. *** P <0.001, ** P <0.01 vs. untreated cells.

Figure 4. Effects of HT on EGFR stability. Representative blots of EGFR in HT-29 (A), CaCo2 (B) and WiDr (C) cells exposed to HT (100 μM) for 4 hrs, followed by CHX (100 μg/ml) for the indicated times are shown. The level of EGFR was assessed at multiple time points using immunoblot analysis, and half-life was calculated. (D) EGFR and co-immunoprecipitated ubiquitin have been analysed by Western blot analysis. EGFR has been used to normalize loading. A.D.U. was reported. N=3. Numbers are the mean ± SD. *** P <0.001 vs. untreated cells. (E-F) Western blot analysis and quantification (arbitrary density unit, A.D.U.) of EGFR expression in HT-29, CaCo2 and WiDr cells exposed to 10% FBS and HT with or without the lysosomal inhibitor chloroquine (10 μM) or the proteasomal inhibitor MG132 (25 μg/ml) for the indicated times. β-actin has been used to normalize loading. N=3. Numbers are the mean ± SD. *** P <0.001 vs. untreated cells, ### P <0.001, # P < 0.05 vs. HT-treated cells. (G) Cell survival induced by 10% FBS in the presence or absence of HT (100 μM) with or without MG132 (25 μg/ml) or chloroquine (10 μM) for 48 hrs in HT-29 (a), CaCo2 (b) and WiDr (c) cells. Data reported as absorbance (540 nm)/well for MTT assay are the means ± SD of 4 experiments run in triplicate. *** P <0.001, ** P < 0.01 vs. untreated cells; ## P < 0.01; # P < 0.05 vs. HT-treated cells.

Figure 5. HT exhibits an anti-tumour effect on the xenograft model. Nude mice bearing HT-29 xenografts were treated with HT (10 mg/kg/day). (A) Tumour volume was measured at 14 day post HT treatment. (B) Tumour tissues were fixed and prepared for immunohistochemistry of Ki67. Brown nuclei represent Ki67 positive staining. (C) Quantification of human Ki67 was performed counting ten random field/section for slide; each slide has five sections. Data represent % of positive Ki67 cells counted for section. *** P<0.001 vs vehicle treated mice. (D) Representative gel (out of three) of EGFR in tumours from control (v, vehicle, white bar) and HT (h, 10 mg/ml, black bar) treated mice determined by western blotting. β-actin has been used to normalize loading. N=3. Numbers are the mean ± SD. *** P <0.001 vs. vehicle treated mice. (E) Images of immunostaining for EGFR (green) and DAPI (blue) in tumour sections from vehicle (a) or HT group (b). Scale bars=100 μm. Images obtained with fluorescence microscope Eclipse TE300 at 60 X magnification. c Quantification of human EGFR was performed counting ten random field/section for slide; each slide has five sections. Data represent % of positive EGFR cells counted for section. *** P<0.001 vs vehicle treated mice. (F) Images of immunostaining for phospho-EGFR Y1045 (red) and DAPI (blue) in tumour sections from vehicle (a) or HT group (b). Scale bars=100 μm. Images obtained with fluorescence microscope Eclipse TE300 at 60 X magnification. c Quantification of human phospho-EGFR was performed counting ten random field/section for slide; each slide has five sections. Data represent % of positive phospho-EGFR cells counted for section. *** P<0.001 vs vehicle treated mice.

Figure 1

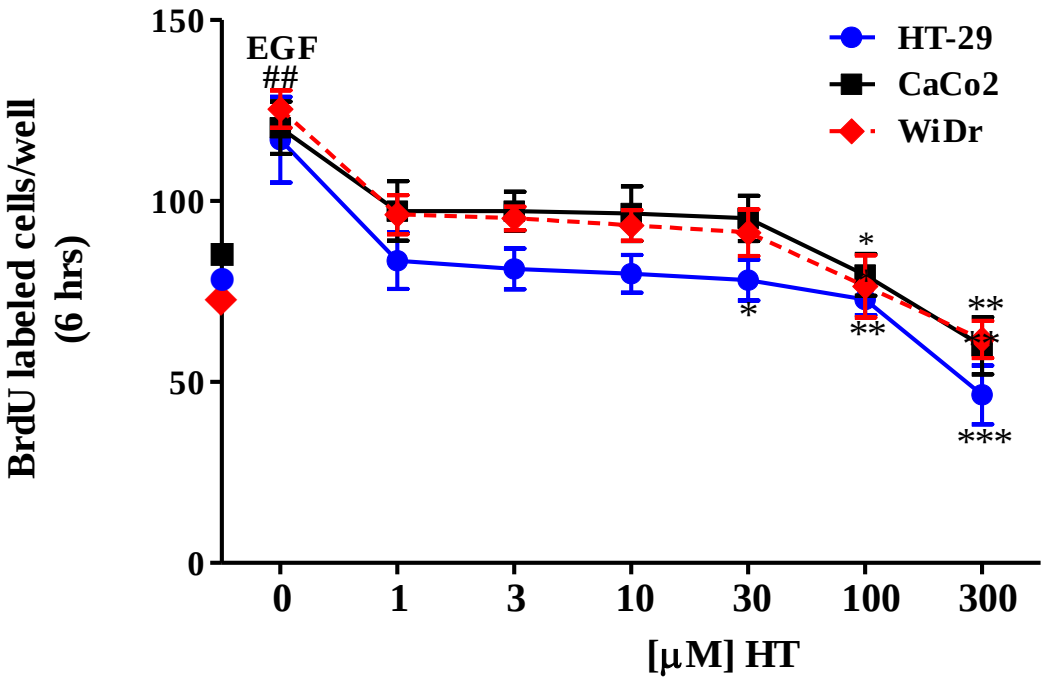

150
100
50
0
BrdU labeled cells/well
(6 hrs)
EGF
##
HT-29
CaCo2
WiDr
*
**
*
**
**
***
0
1
3
10
30
100
300
[µM] HT

Figure 2

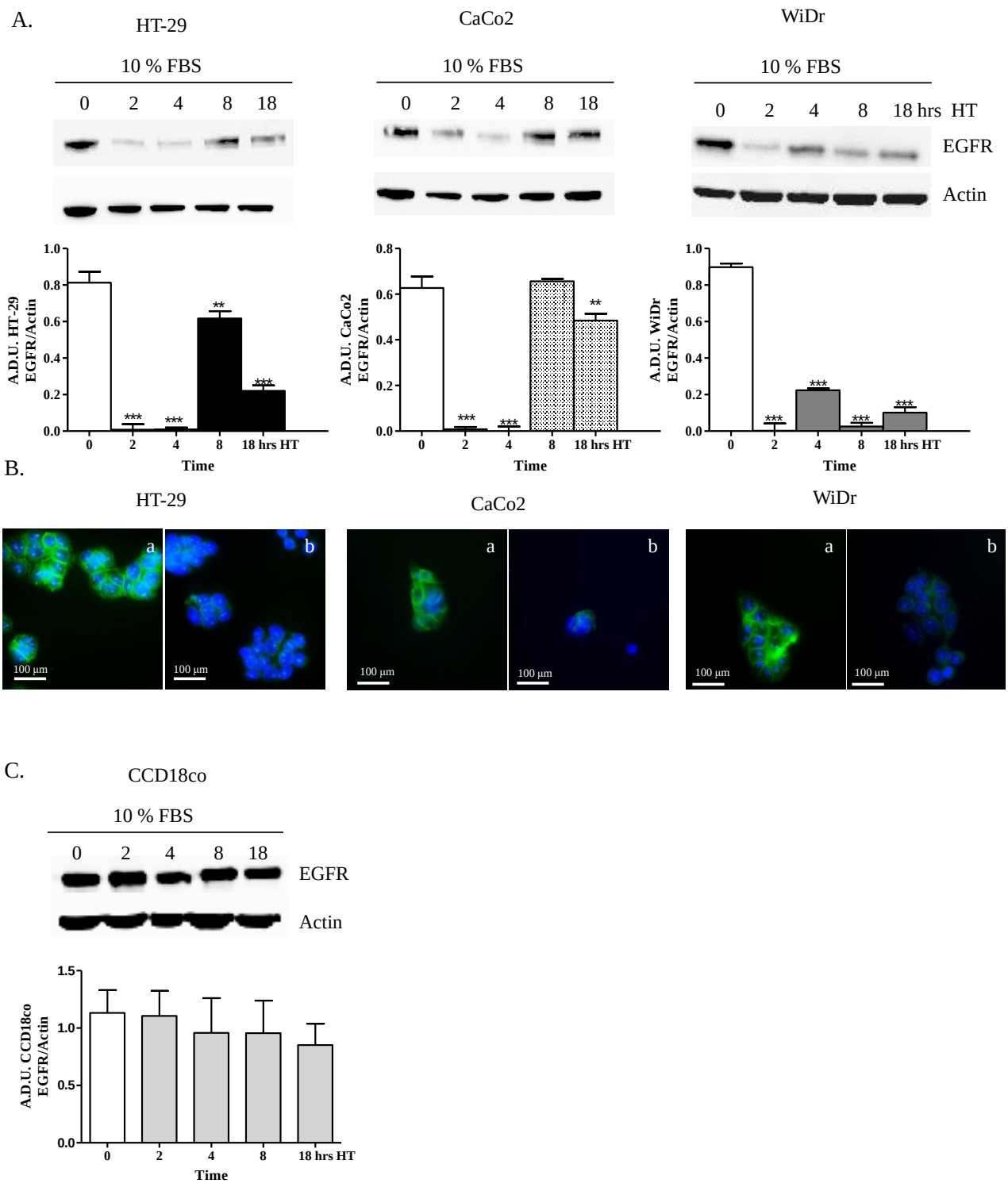
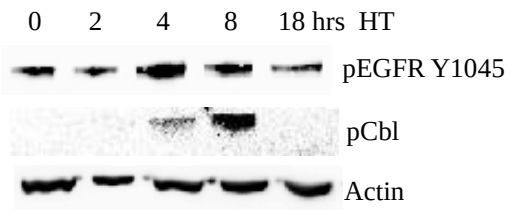
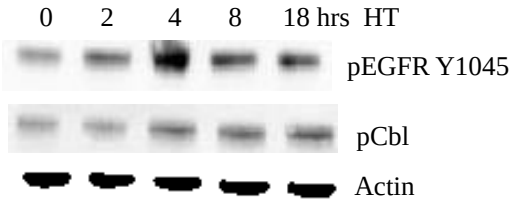

A.
HT-29
CaCo2
WiDr
10 % FBS
0 2 4 8 18
0 2 4 8 18 hrs HT
EGFR
Actin
A.D.U. HT-29 EGFR/Actin
A.D.U. CaCo2 EGFR/Actin
A.D.U. WiDr EGFR/Actin
Time
B.
HT-29
CaCo2
WiDr
a
b
100 µm
C.
CCD18co
10 % FBS
0 2 4 8 18
EGFR
Actin
A.D.U. CCD18co EGFR/Actin
Time

Figure 3

A.


0 2 4 8 18 hrs HT
pEGFR Y1045
pCbl
Actin

B.


0 2 4 8 18 hrs HT
pEGFR Y1045
pCbl
Actin

C.

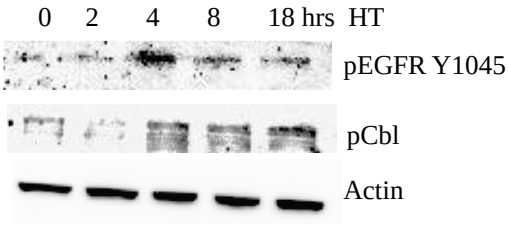
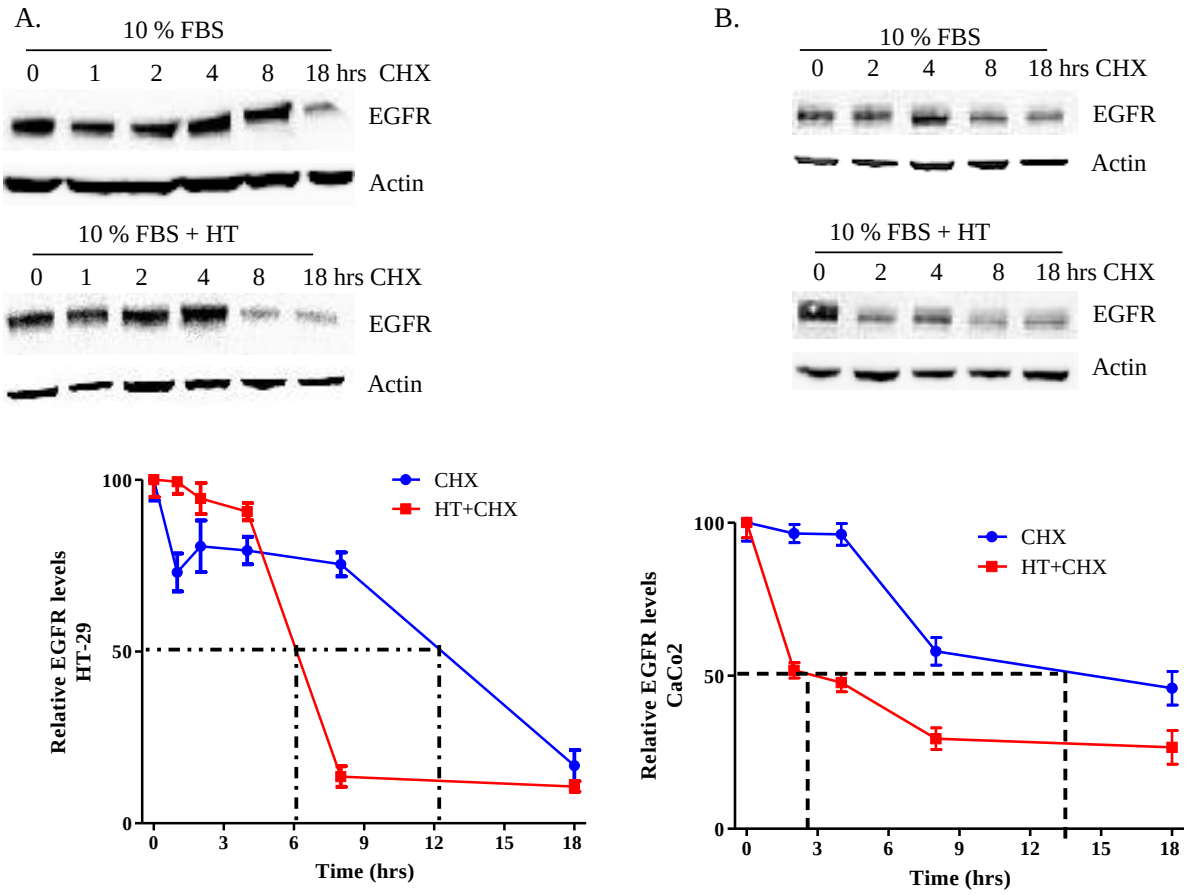
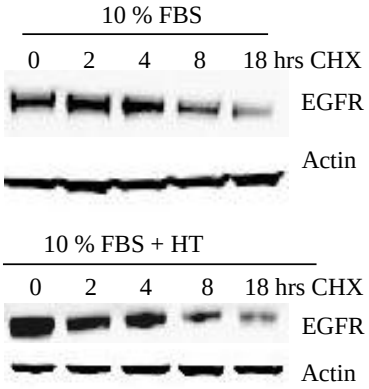
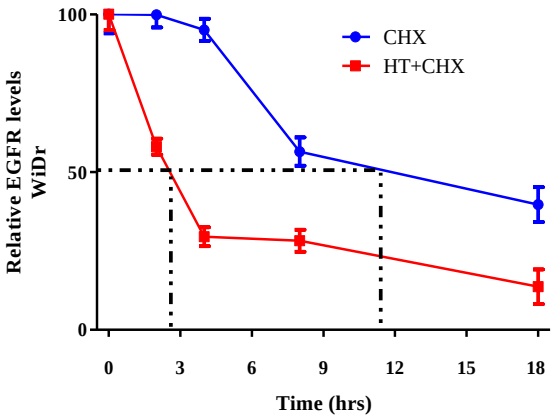

0 2 4 8 18 hrs HT
pEGFR Y1045
pCbl
Actin

Figure 4

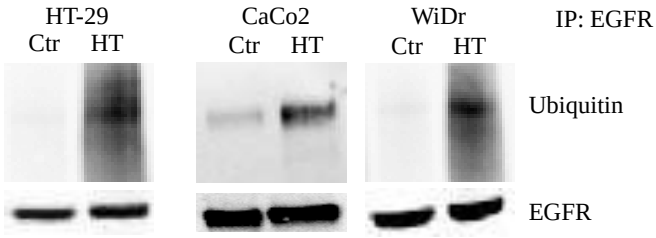

A.
10 % FBS
0 1 2 4 8 18 hrs CHX
EGFR
Actin
10 % FBS + HT
0 1 2 4 8 18 hrs CHX
EGFR
Actin
B.
10 % FBS
0 2 4 8 18 hrs CHX
EGFR
Actin
10 % FBS + HT
0 2 4 8 18 hrs CHX
EGFR
Actin
Relative EGFR levels HT-29
CHX
HT+CHX
100
50
0
0 3 6 9 12 15 18
Time (hrs)
Relative EGFR levels CaCo2
CHX
HT+CHX
100
50
0
0 3 6 9 12 15 18
Time (hrs)

C.



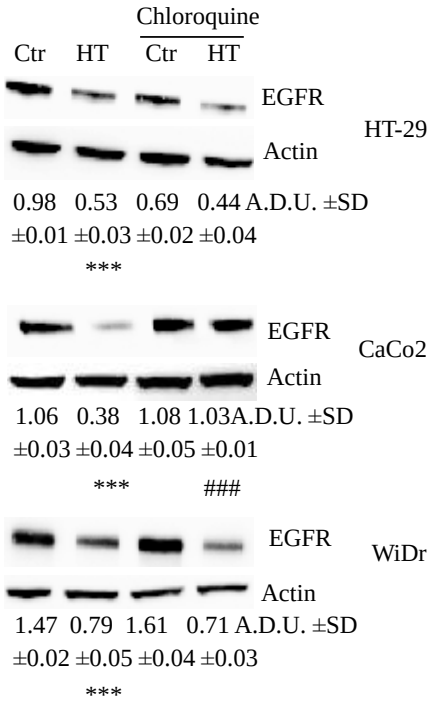


D.



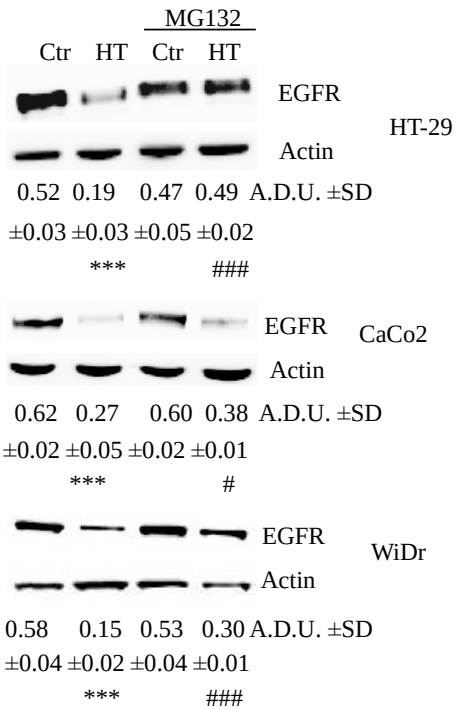
	HT-29		CaCo2		WiDr		
	Ctr	HT	Ctr	HT	Ctr	HT	
Ratio	0.05 ±0.01	0.45*** ±0.03	0.08 ±0.01	0.49*** ±0.05	0.05 ±0.01	0.76*** ±0.07	A.D.U. ±SD

E.

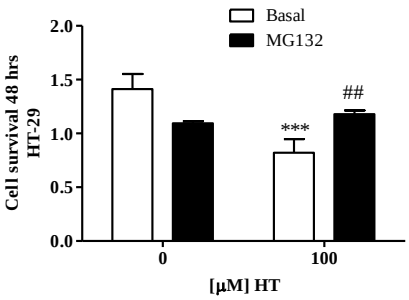


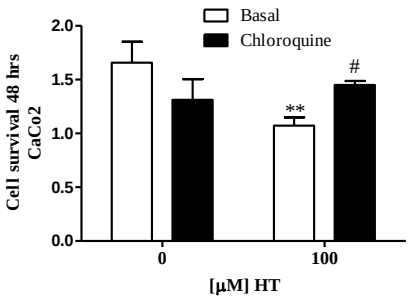
	Ctr	HT	Ctr	HT	
HT-29 Ratio	0.98 ±0.01	0.53 ±0.03 ***	0.69 ±0.02	0.44 ±0.04	A.D.U. ±SD
CaCo2 Ratio	1.06 ±0.03	0.38 ±0.04 ***	1.08 ±0.05	1.03 ±0.01 ###	A.D.U. ±SD
WiDr Ratio	1.47 ±0.02	0.79 ±0.05 ***	1.61 ±0.04	0.71 ±0.03	A.D.U. ±SD

F.



	Ctr	HT	Ctr	HT	
HT-29 Ratio	0.52 ±0.03	0.19 ±0.03 ***	0.47 ±0.05	0.49 ±0.02 ###	A.D.U. ±SD
CaCo2 Ratio	0.62 ±0.02	0.27 ±0.05 ***	0.60 ±0.02	0.38 ±0.01 #	A.D.U. ±SD
WiDr Ratio	0.58 ±0.04	0.15 ±0.02 ***	0.53 ±0.04	0.30 ±0.01 ###	A.D.U. ±SD


Basal
MG132
Cell survival 48 hrs
HT-29
2.0
1.5
1.0
0.5
0.0
***
##
0
100
[μM] HT


Basal
Chloroquine
Cell survival 48 hrs
CaCo2
2.0
1.5
1.0
0.5
0.0
**
#
0
100
[μM] HT

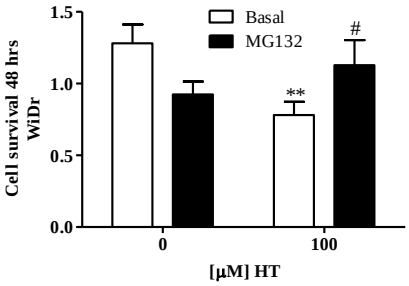
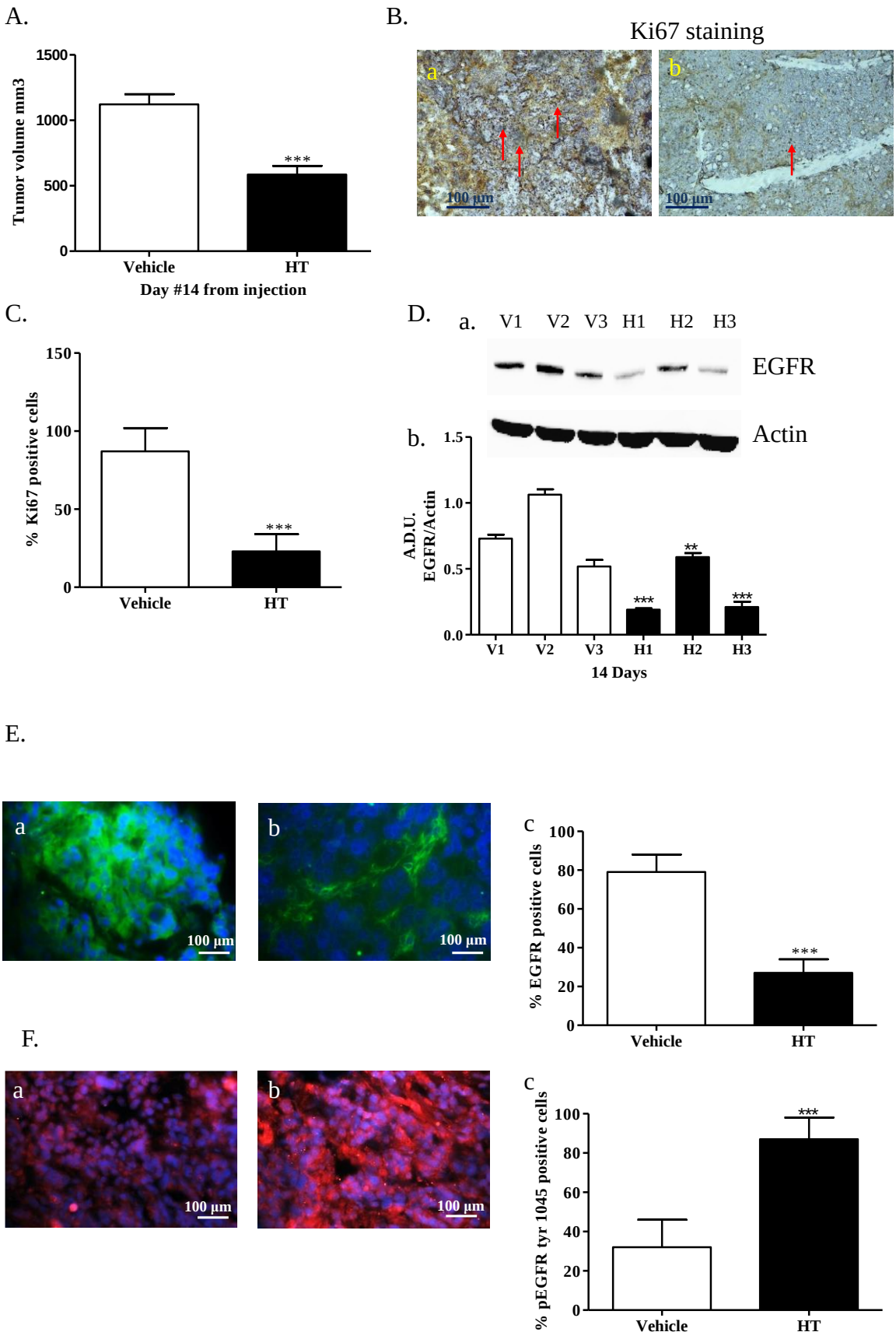

Basal
MG132
Cell survival 48 hrs
WiDr
1.5
1.0
0.5
0.0
**
#
0
100
[μM] HT

Figure 5


A.
Tumor volume mm3
1500
1000
500
0
***
Vehicle
HT
Day #14 from injection
B.
Ki67 staining
a
b
100 µm
100 µm
C.
% Ki67 positive cells
150
100
50
0
***
Vehicle
HT
D.
a.
V1 V2 V3 H1 H2 H3
EGFR
b.
Actin
A.D.U.
EGFR/Actin
1.5
1.0
0.5
0.0
**
***
***
V1 V2 V3 H1 H2 H3
14 Days
E.
a
b
100 µm
100 µm
C
% EGFR positive cells
100
80
60
40
20
0
***
Vehicle
HT
F.
a
b
100 µm
100 µm
C
% pEGFR tyr 1045 positive cells
100
80
60
40
20
0
***
Vehicle
HT

Supplementary Figures

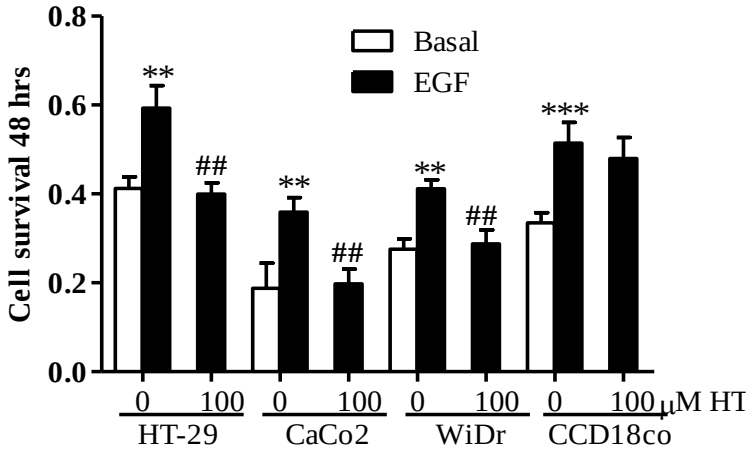
Supplementary Figure 1: HT reduces tumour colon cell survival. (A) Cell survival induced by EGF (25 ng/ml) in the presence or absence of HT (100 μM) for 48 hrs in HT-29, CaCo2, WiDr colon tumour cells and in CCD18co colon fibroblast cells. Data reported as absorbance (540 nm)/well for MTT assay are the means ± SD of 4 experiments run in triplicate. *** P <0.001, ** P < 0.01, vs. untreated cells; ## P 0.01 vs. EGF-treated cells.

Supplementary Figure 2: Role of HT on EGF/EGFR signalling activation. Western blot analysis and quantification (arbitrary density unit, A.D.U.) of phospho-tyr, -ERK 1/2 and -Akt expression in HT-29 (A), CaCo2 (B) and WiDr (C) cells exposed to EGF(25 ng/ml) with or without HT (100 μM) for 15 min. β-actin or EGFR have been used to normalize loading. N=3. Numbers are the mean ± SD. *** P <0.001 vs. untreated cells.

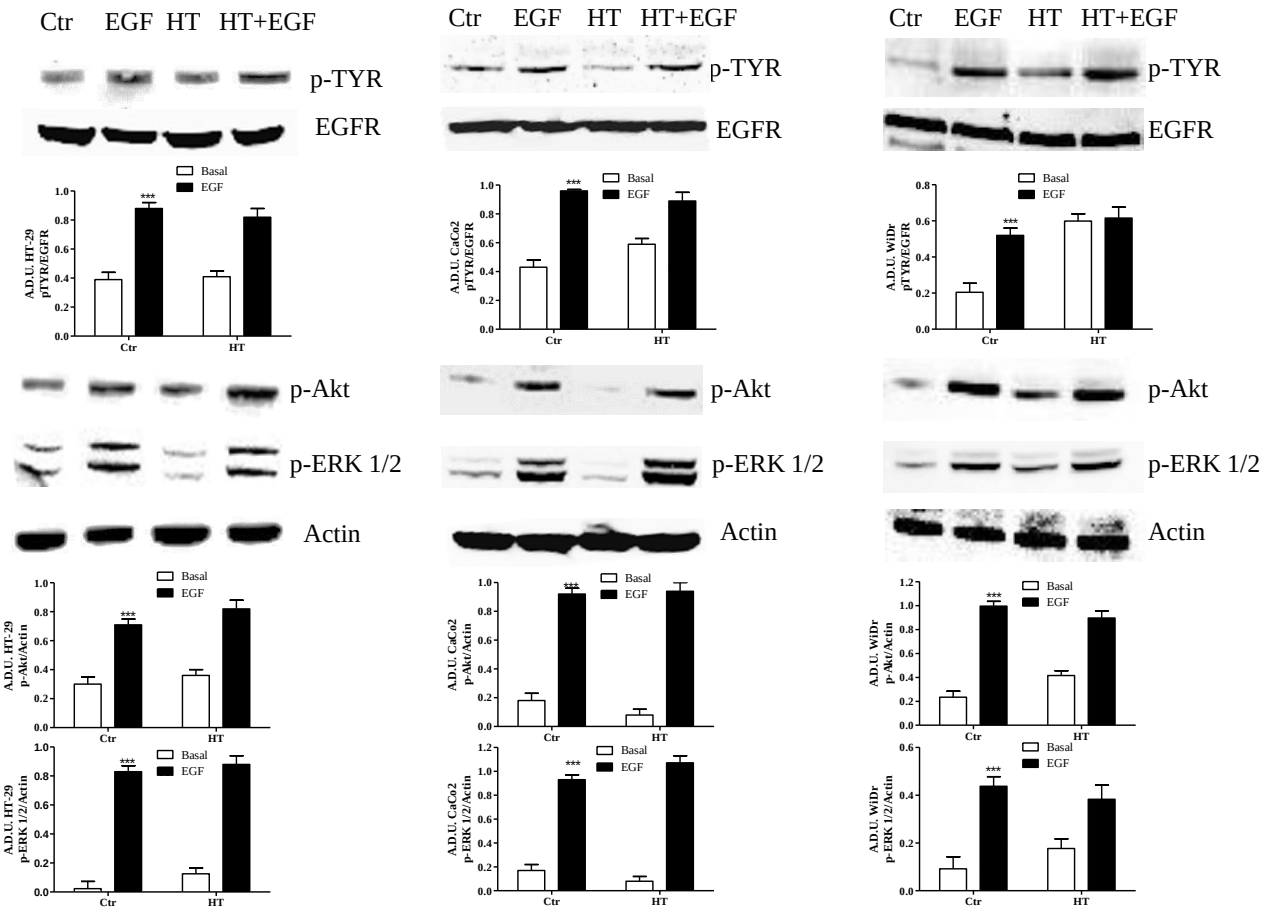
Supplementary Figure 3: HT synergizes with Tyr. (A) HT-29 cells were exposed to different concentration of HT or Tyr (1, 3, 10, 30, 100 and 300 μM) in presence of 5% of FBS for 6 hrs and growth was evaluated by BrdU incorporation. Data are reported as BrdU labelled cells/well. Numbers represent mean ± SEM of three experiments run in triplicate. *** P <0.001, ** P < 0.01, * P < 0.05 vs. untreated cells. (B) Isobolographic analysis of interaction between HT and Tyr on HT-29 growth. (C) Western blot analysis of EGFR expression in HT-29 cells exposed to 10% FBS and HT (10 μM) or Tyr (30 μM) or HT+ Tyr for the 4 hrs. β-actin has been used to normalize loading. N=3. (D) Western blot of EGFR phospho-Y1045 expression in HT-29 cells exposed to 10% FBS and HT (10 μM) or Tyr (30 μM) or HT+ Tyr for 4 hrs. β-actin has been used to normalize loading. N=3.

Supplementary Figure 1

A.



Supplementary Figure 2



Supplementary Figure 3