Cerivastatin enhances the cytotoxicity of 5-fluorouracil on chemosensitive and resistant colorectal cancer cell lines

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Abstract **Cerivastatin is one of the synthetic 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors used for the treatment and prevention of hypercholesterolaemia. The observation that patients receiving this drug had a lower incidence at cancer led to our interest in using it as a putative anticancer agent. In this study, we tested the cytotoxicity of cerivastatin on a panel of 5-fluorouracil (5FU) sensitive and resistant cell lines in vitro. Cerivastatin was cytotoxic to both 5FU sensitive and resistant cells. Cerivastatin significantly augmented the cytotoxic effect of 5FU on drug sensitive (6–22-fold) and resistant (229–310-fold) cell lines. Cerivastatin and 5FU acted synergistically. Cerivastatin inhibited nuclear factor κB DNA binding activity. The enhancing effect of cerivastatin on 5FU was partially mevalonate pathway independent. Cerivastatin may allow successful 5FU therapy in chemoresistant patients.**

Key words: 3-Hydroxy-3-methylglutaryl coenzyme A; Cerivastatin; Colorectal cancer; 5-Fluorouracil; Nuclear factor κB

1. Introduction

5-Fluorouracil (5FU) has been used for several decades in the treatment of various solid tumours. 5FU interferes with DNA synthesis by blocking production of the pyrimidine nucleotide dTMP from dUMP in de novo DNA synthesis, through inhibition of thymidylate synthase, as well as through incorporation of fluoro-nucleotides into DNA and RNA [1]. Chemoresistance is still the major obstacle for successful 5FU chemotherapy. In efforts to improve the therapeutic index of 5FU, various 5FU analogues and 5FU combination therapies have been evaluated [2].

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyses the formation of mevalonate from HMG-CoA which is a key step in the biosynthesis of cholesterol and other isoprenes (i.e. mevalonate-derived products) [3]. HMG-CoA reductase inhibitors (statins) have been widely used for prevention and treatment of hypercholesterolaemia and atherosclerosis [4]. In recent years, several studies indicate that statins also inhibit cancer cell growth via a non-lipid related pathway [5]. Cerivastatin is a synthetic statin. It has been reported that cerivastatin can induce leukaemia specific apoptosis and inhibit the proliferation of several solid tumour cell lines [6–8]. More importantly, cerivastatin can improve therapeutic index as it is non-toxic to normal human bone marrow progenitors [6]. Cerivastatin also inhibits tumour invasiveness and metastasis in vitro [7]. As with other statins, cerivastatin induced cytotoxicity is mevalonate pathway dependent. Its cytotoxicity can be totally reversed by mevalonate or its derivatives [farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)] [6–8].

Several studies demonstrate that lovastatin enhances the cytotoxic effect of some anticancer drugs in vitro and in vivo [9–11]. In this study, we demonstrated that the cytotoxic effect of 5FU on 5FU sensitive and resistant colorectal cancer (CRC) cell lines was significantly enhanced by cerivastatin. The enhancing effect of cerivastatin on 5FU was partially mevalonate pathway independent.

2. Materials and methods

2.1. Cell culture and cytotoxicity analysis

Two 5FU resistant ($H630_{5FU}$ and $R10_{5FU}$) and the parent 5FU sensitive CRC cell lines ($H630_{wt}$ and $R10_{wt}$) were chosen for this study (the resistant cell lines were kindly provided by Prof. P.G. Johnston, The Queen's University of Belfast, Department of Oncology). Cell characteristics and culturing conditions have been described previously [12–14]. The cells (5000/well), cultured overnight in 96-well flat-bottomed microtitre plates, were exposed to 5FU (Sigma-Aldrich, Poole, UK), cerivastatin (Bayer, Wuppertal, Germany) or a combination of both for 96 h and then subjected to a standard 3-[4,5-dimethylthiazol-2-yl]diphenyltetrazolium bromide (MTT) assay as previously described [12,15]. For the add-back experiments, mevalonate (250 μM) was added into the culture. Each experiment was done in triplicate plates and repeated twice. The IC_{50} dose of 5FU was calculated using CalcuSyn software (Biosoft, Cambridge, UK).

2.2. Analysis of the combined effect of 5FU and cerivastatin

Overnight cultured cells (5000/well) were exposed to various concentrations of each drug or a combination of these two drugs at a constant ratio [5FU:cerivastatin = 100:1 ($R10_{wt}$ and $R10_{5FU}$), 168:1 ($H630_{5FU}$) and 50:1 ($H630_{wt}$)] with or without mevalonate (250 μM) for 96 h. The cells were then subjected to MTT analysis as described above. The combined cytotoxic effect of 5FU with cerivastatin was determined by CI-isobologram using CalcuSyn software (Biosoft) [16]. Mutually exclusive equations were used to determine the combination index (CI).

2.3. Electrophoretic mobility shift assays (EMSA)

The cells were cultured in 96-well plates in drug-free medium until 70% confluent. Cells were then grown in medium containing different combinations of 5FU (10 μM), cerivastatin (1 or 3 μM) and mevalonate (250 μM) for another 24 h. The cells were collected by trypsinisation. Nuclear protein extraction and EMSA were described previously [17]. Briefly, equal amounts (5 μg) of nuclear extract were

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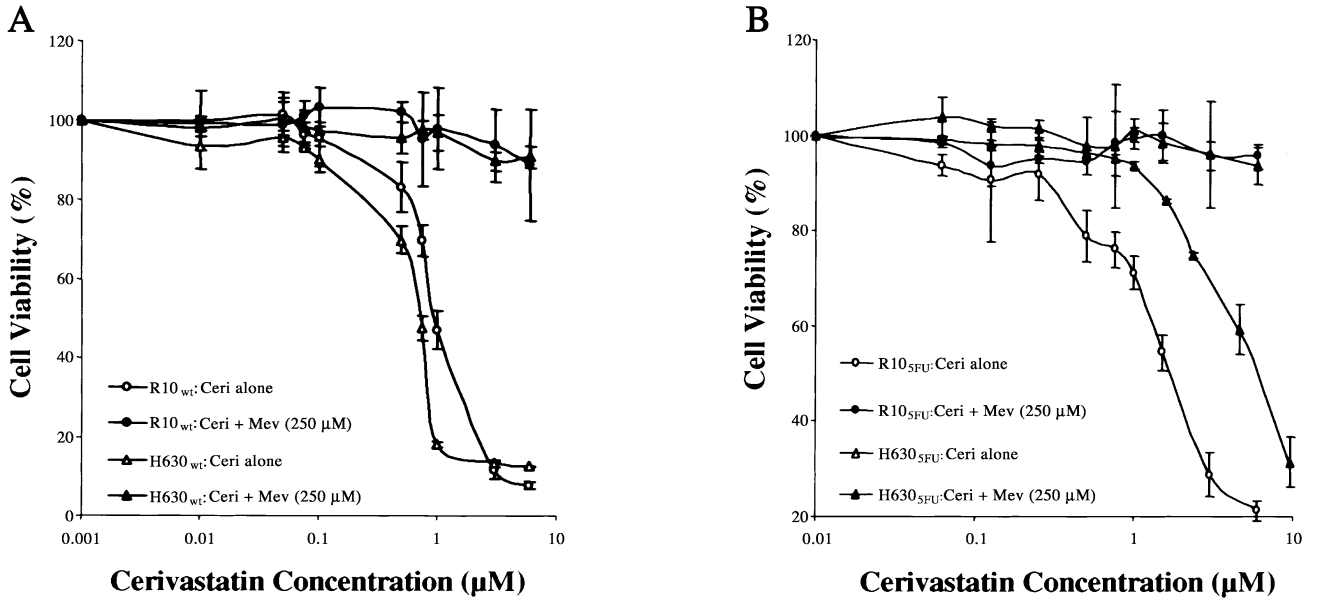

Fig. 1. Cytotoxic effect of cerivastatin with/without mevalonate on 5FU sensitive (A) and resistant (B) CRC cell lines. Cells were cultured in cerivastatin (ceri, 1–10 μM) with or without mevalonate (mev, 250 μM) for 96 h and subjected to MTT analysis.

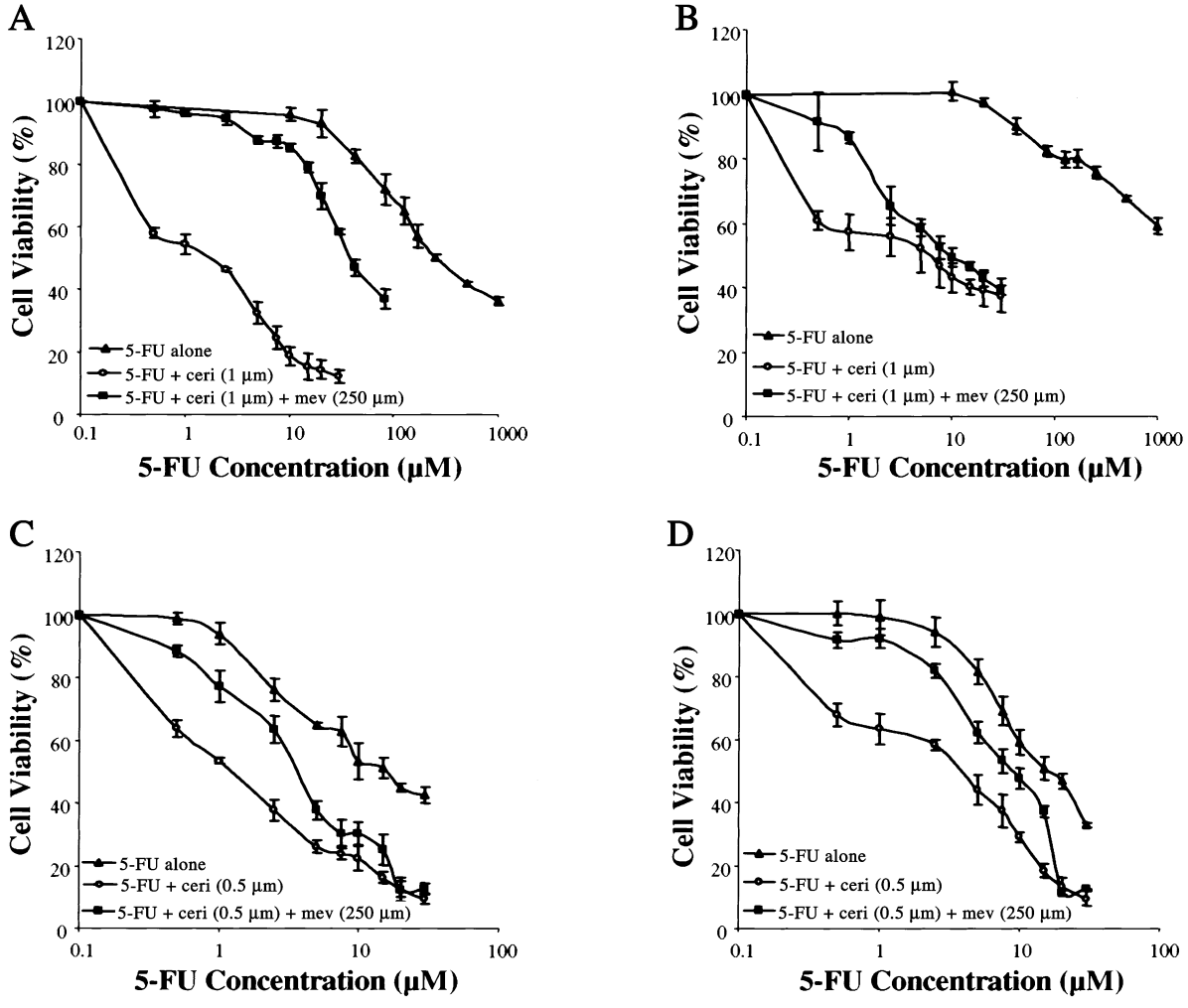

Fig. 2. Cerivastatin enhanced cytotoxic effect of 5FU on 5FU sensitive and resistant CRC cell lines. The enhancing effect of cerivastatin was partially reversed by mevalonate. Cells were cultured in medium containing 5FU (0.1–1000 μM) with or without cerivastatin (0.5 or 1 μM, as indicated) and mevalonate (250 μM) for 96 h and subjected to MTT analysis. A: $R10_{5FU}$. B: $H630_{5FU}$. C: $H630_{wt}$. D: $R10_{wt}$.

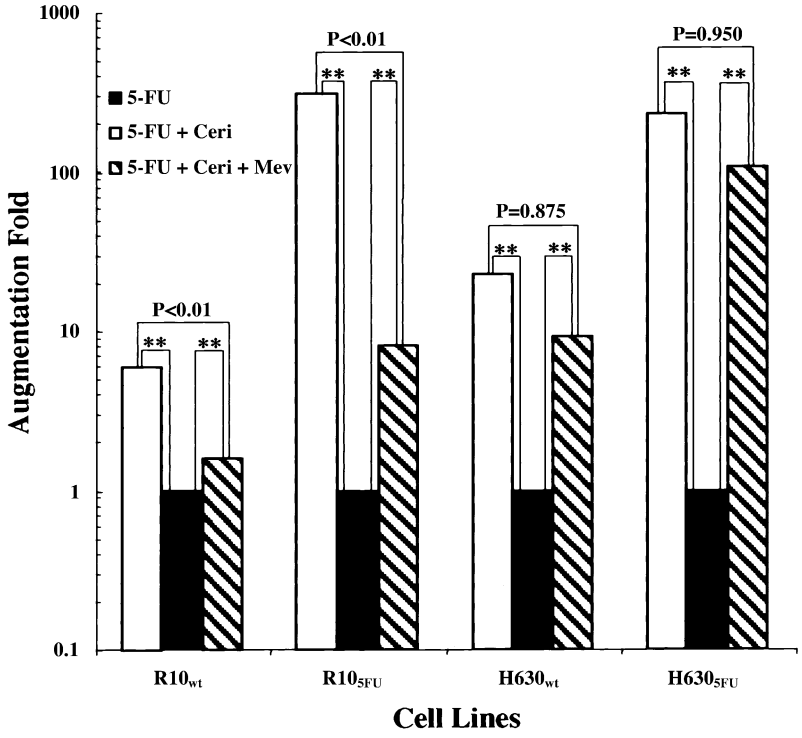

Fig. 3. The fold enhanced 5FU cytotoxicity by cerivastatin with/without mevalonate in CRC cell lines. The fold enhancement represents the ratio of IC_{50} of 5FU in combination with other drugs (as indicated)/IC_{50} of 5FU used alone. Cerivastatin (ceri): 0.5 μM for 5FU sensitive and 1.0 μM for 5FU resistant cells; mevalonate (mev): 250 μM. $^{**}P < 0.01$.

incubated with 1 μg poly(dIdC) (Sigma-Aldrich) in binding buffer [50 mM Tris (pH 7.6), 250 mM KCl, 25 mM dithiothreitol, 5 M EDTA and 25% glycerol] for 10 min at room temperature (RT). Approximately 20 000 cpm of ^{32}P-labelled 22-mer nuclear factor κB (NF-κB) DNA probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was added and incubated at RT for 20 min. To test binding specificity, 5 μg of nuclear extract mixture from four cell lines was incubated with 20× wildtype or mutant (5′-AGTTGATATTACTTTTATAGGC-3′) unlabelled NF-κB probe for 30 min before EMSA analysis. The complexes were separated on a 6% polyacrylamide gel and exposed to X-ray film for autoradiography.

3. Results

3.1. Cytotoxicity of cerivastatin in CRC cell lines

After 96 h exposure to cerivastatin, the viability of 5FU sensitive and resistant CRC cell lines was evaluated by MTT analysis. Cerivastatin alone was toxic to the CRC cell lines. Fig. 1 demonstrates that cerivastatin was more cytotoxic to 5FU sensitive cell lines ($H630_{wt}$ $IC_{50} = 0.70$ μM, $R10_{wt}$ $IC_{50} = 1.02$ μM) than 5FU resistant cell lines ($H630_{5FU}$ $IC_{50} = 7.07$ μM, $R10_{5FU}$ $IC_{50} = 1.75$ μM). The cytotoxicity of cerivastatin was totally reversed by addition of 250 μM mevalonate which did not affect cell growth when singly used (data not shown).

3.2. Cerivastatin strongly enhanced 5FU cytotoxicity and mevalonate partially reversed the effect of cerivastatin

To test the effect of cerivastatin plus 5FU on cell viability, CRC cell lines were cultured with 5FU (0.1–1000 μM) with or without cerivastatin (0.5 μM for 5FU sensitive and 1.0 μM for 5FU resistant cell lines) for 96 h before MTT analysis. Fig. 2 and Table 1 demonstrate that the cytotoxic effect of 5FU on both 5FU sensitive and resistant cell lines was significantly enhanced ($P < 0.01$) by cerivastatin. The enhancing effect of cerivastatin on 5FU cytotoxicity was significantly higher in resistant cell lines (229-fold for $H630_{5FU}$ and 309-fold for $R10_{5FU}$) than sensitive cell lines (6.2-fold for $R10_{wt}$ and 22.7-fold for $H630_{wt}$) (Fig. 3). 5FU resistance in drug resistant cell lines was reversed by addition of cerivastatin (Table 1).

Furthermore, we tested if the enhancing effect of cerivastatin on 5FU was via the mevalonate pathway. Cells were cultured in medium containing mevalonate (250 μM) and cerivastatin (0.5 μM for drug sensitive and 1.0 μM for resistant cell line) with various concentrations of 5FU. Mevalonate attenuated but did not completely block the enhancement of 5FU cytotoxicity by cerivastatin in $R10_{wt}$ and $R10_{5FU}$ cell lines (Table 1 and Fig. 3). Even in the presence mevalonate, cerivastatin still significantly enhanced the sensitivity of the cancer cell lines to 5FU (Table 1 and Fig. 3; $P < 0.01$).

Table 1
IC_{50} of 5FU tested alone or in combination with cerivastatin and mevalonate

	$R10_{wt}$	$R10_{5FU}$	$H630_{wt}$	$H630_{5FU}$
5FU	16.04 (1.68)	477.27 (17.87)	30.85 (4.81)	1106.33 (104.66)
5FU+Ceri	2.64 (0.04)**	1.54 (0.11)**	1.36 (0.07)**	4.83 (0.19)**
5FU+Ceri+Mev	9.97 (0.58)**	58.45 (4.12)**	3.36 (1.01)**	10.39 (0.93)**

Ceri: cerivastatin; Mev: mevalonate; numbers in parentheses: standard errors. $^{**}P < 0.01$.

3.3. 5FU and cerivastatin had a synergistic cytotoxic effect on CRC cell lines

CI-isobologram analysis was used to determine if 5FU and cerivastatin had a synergistic cytotoxic effect on the CRC cell lines. CI > 1, = 1 and < 1 indicate antagonistic, additive and synergistic effects, respectively [16]. Fig. 4 demonstrates that 5FU and cerivastatin had a synergistic cytotoxic effect on the CRC cell lines in a wide range of cell killing fractions (0.1–0.9). Although mevalonate (250 μM) slightly increased CI values in $H630_{wt}$ and $R10_{5FU}$ cells when used in combination with 5FU and cerivastatin, 5FU and cerivastatin still showed synergistic cytotoxic effect on all four CRC cell lines (Fig. 4).

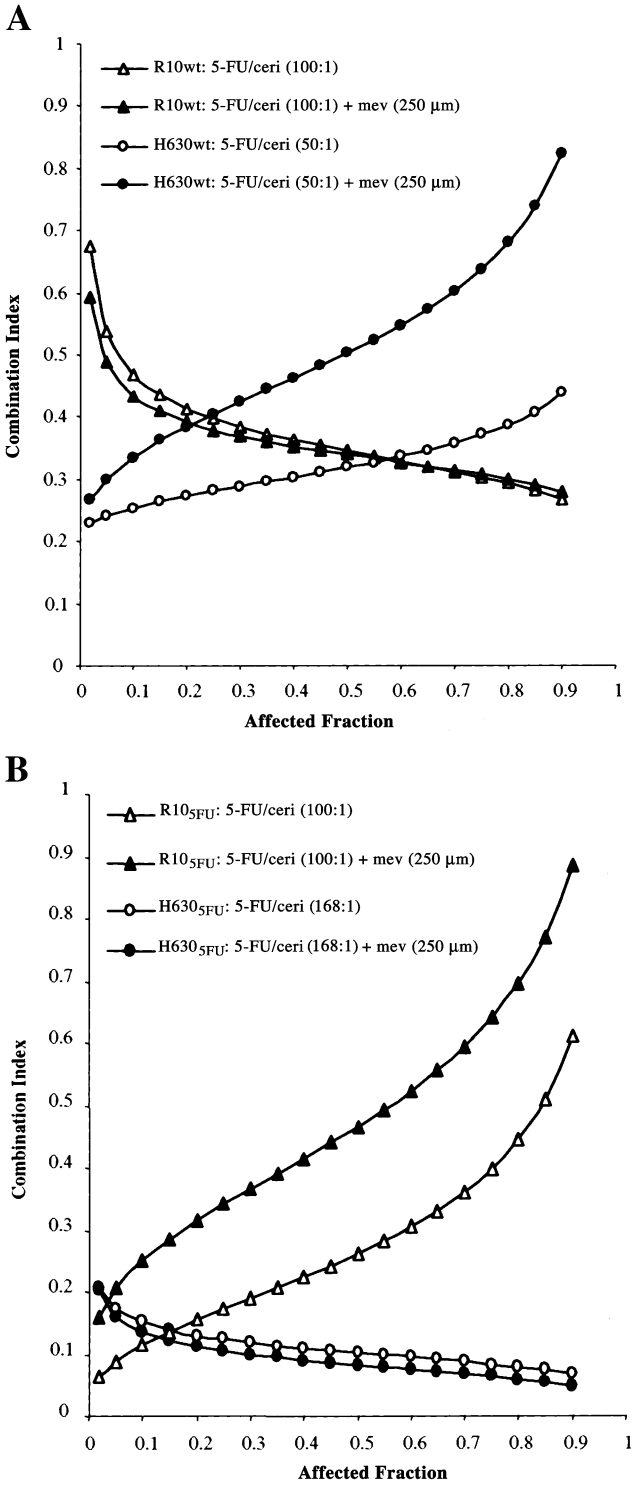

Fig. 4. CI-isobologram analysis of the combined effect of 5FU and cerivastatin with or without mevalonate. CI < 1 indicates a synergistic effect.

3.4. Cerivastatin inhibited NF-κB DNA binding activity

Furthermore, we tested if cerivastatin affected the DNA binding activity of NF-κB, an anti-apoptotic transcription factor. High NF-κB DNA binding activity was detected from all but $H630_{wt}$ cells (Fig. 5). 5FU (10 μM) induced the activity in 5FU sensitive cells further. The high NF-κB activity in the cells was markedly suppressed by addition of cerivastatin (1 and 3 μM). The inhibiting effect of cerivastatin on NF-κB DNA binding activity was totally reversed by mevalonate (250 μM).

4. Discussion

5FU is the first line drug for chemotherapy of CRC which is the third leading cancer killer in developed nations [18]. Chemoresistance is the major barrier for successful 5FU chemotherapy. Use of combination therapies is one of the main strategies currently being evaluated to overcome chemoresistance [2]. Statins are drugs currently used for hypercholesterolaemia prevention and treatment. These drugs block the mevalonate pathway and thereby cholesterol biosynthesis by inhibiting HMG-CoA reductase. It was reported that cancer incidence in the patients receiving HMG-CoA reductase inhibitor treatment was lower than that in a placebo group [19]. Statins inhibit the proliferation of some leukaemia and solid tumour cell lines in vitro and spare normal haemopoietic precursors [6–8,20]. These results underline the feasibility of using statins as a group of drugs for cancer chemotherapy. In this study, the cytotoxic effect of cerivastatin, a synthetic HMG-CoA reductase inhibitor, on a panel of 5FU sensitive and resistant CRC cell lines was tested. Cerivastatin was cytotoxic to 5FU sensitive and resistant CRC cell lines in vitro. In comparison with sensitive cells, 5FU resistant cell lines were 1.7 ($R10_{5FU}$) and 10.1 times ($H630_{5FU}$) less sensitive to cerivastatin-induced cytotoxicity. The cytotoxic effect of cerivastatin was completely reversed by mevalonate indicating the effect was induced by HMG-CoA reductase inhibition.

It has been reported that lovastatin augments the cytotoxic effect of cytosine arabinoside, cisplatin, BCNU and 5FU on different human leukaemia and solid cancer cell lines in vitro and in vivo [9–11]. Furthermore, we tested the effect of 5FU plus cerivastatin on CRC cell lines. The cytotoxic effect of 5FU on CRC cell lines was significantly enhanced by addition of cerivastatin at a low concentration ($< IC_{30}$ dose). The chemoresistance of 5FU resistant cell lines was also reversed by cerivastatin.

Mevalonate derivatives, FPP and GGPP, are responsible for Ras and Rho isoprenylation which is a key step in their membrane translocation and cell signalling [7,21]. Some statins induce the expression of cyclin dependent kinase inhibitors p21 or p27 which block the cell cycle at the G1 checkpoint [22]. A number of studies suggest that blockage of Ras and Rho isoprenylation and induction of p21/p27 by inhibition of the mevalonate pathway may play a main role in the anticancer effect of HMG-CoA reductase inhibitors [7,20–22]. In our study, mevalonate (250 μM, Fig. 2), GGPP (2 μM) or

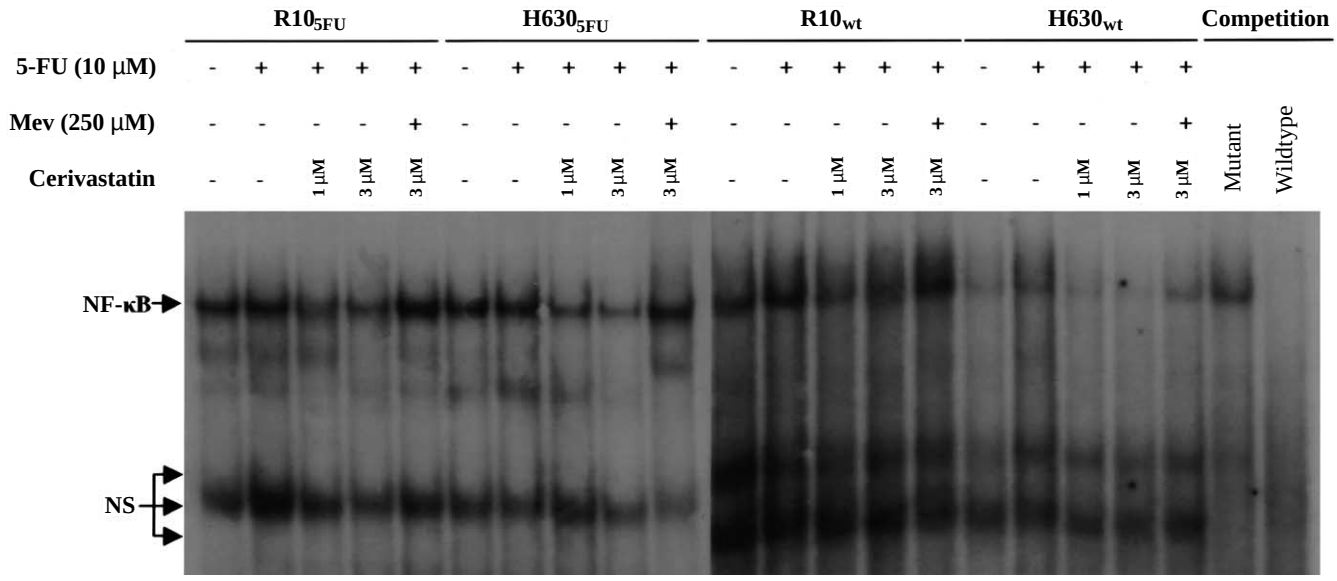

Fig. 5. The influence of 5FU (10 μM), cerivastatin (1 or 3 μM) and mevalonate (250 μM) on NF-κB nuclear DNA binding activity in CRC cell lines. NS: non-specific bands.

FPP (2 μM) (data not shown) can only partially block the augmentation of 5FU cytotoxicity by cerivastatin. Hence although the cytotoxic effect of cerivastatin on CRC cell lines is mevalonate pathway dependent, the enhancing effect of cerivastatin on 5FU cytotoxicity only partially relies on the mevalonate pathway. Other molecular mechanisms involved in the augmentation of cerivastatin to 5FU remain to be elucidated.

NF-κB is a transcription factor which antagonises the cytotoxicity of anticancer drugs by inducing the expression of anti-apoptotic genes (e.g. *c-IAPs*, *IXAP*, *A1/Bfl-1* and *IEX-IL*) [23–24]. Human cancer cells with induced NF-κB nuclear activity have demonstrated resistance to apoptosis induced by chemotherapy or radiotherapy [25–27]. CRC cell lines resistant to the thymidylate synthase inhibitors, 5FU and raltitrexed, demonstrate NF-κB mRNA and protein overexpression and high NF-κB nuclear activity. Inhibition of NF-κB activity in CRC cell lines by disulfiram enhances 5FU cytotoxicity and reverses 5FU resistance [28]. Here, we demonstrated that cerivastatin also inhibited the constitutive and inducible NF-κB nuclear DNA binding activity. Inhibition of NF-κB binding activity may be partially responsible for the anticancer effect of cerivastatin. The NF-κB inhibitory effect of cerivastatin was mevalonate pathway dependent and was abolished by addition of mevalonate.

For anti-hypercholesterolaemia purposes, cerivastatin requires to be long-term administered which has caused some severe side effects [29]. Our study indicates that for anticancer purposes, cerivastatin may be used in combination with a 5FU containing chemotherapeutic regimen, especially for CRC patients poorly responsive to 5FU, to increase tumour cell killing, rather than being used over prolonged periods as for anti-hypercholesterolaemia treatment. This would minimise the adverse effects related to long-term use of cerivastatin.

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