Celecoxib can induce cell death independently of cyclooxygenase-2, p53, Mdm2, c-Abl and reactive oxygen species

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Cell lines that do not overexpress functional cyclooxygenase-2 are resistant to the normal plasma levels of celecoxib achieved following oral ingestion. Cell growth inhibition was demonstrated after 24 h exposure to 80 μmol/l celecoxib while significant death was not detected at concentrations below 120 μmol/l following 24 h exposure. This growth inhibition and death induction was identified to be independent of p53 and Hdm2 in these cells, despite wild-type p53 stabilization and Hdm2 diminution in some lines. Cell death induced by celecoxib was preceded by the generation of reactive oxygen species within 4 h of drug exposure. The precise mechanism of elicitation of reactive oxygen species in these cells remains to be elucidated, although it was found to be independent of p53 and c-Abl, while *in vitro*, celecoxib enhanced superoxide radical production by xanthine oxidase. Importantly, the failure of anti-oxidants to protect from death indicates that celecoxib induces death independently of reactive oxygen species and that reactive oxygen species generation may be an insufficient trigger of death in p53-deficient cells.
***Anti-Cancer Drugs* 17:609–619 © 2006 Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2006, **17**:609–619

Keywords: c-Abl, celecoxib, colorectal cancer, Hdm2, p53, reactive oxygen species, xanthine oxidase

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Received 18 January 2006 **Revised** 14 February 2006
Accepted 15 February 2006

Introduction

The nature of celecoxib-mediated colorectal cancer (CRC) cell death is only partially defined. While celecoxib is renowned for its specific inhibition of cyclooxygenase-2 (COX-2), an enzyme that is frequently elevated in CRC cells [1], additional targets are also subject to its influence. One molecule that appears to interplay with COX-2 and is therefore likely to be affected by COX-2 inhibition is the tumor suppressor wild-type wt p53.

Wt p53 can upregulate COX-2 expression; COX-2 in turn can suppress p53 transcriptional activity [2]. In addition, the COX products prostaglandins PGA_1 and PGA_2 have the remarkable ability of preferentially accumulating wt p53 in an altered conformation in the cytoplasm [3], a step that would negate its transcriptional activity. The counterpart to these observations is that COX-2 inhibition promotes p53 activity [2] and high concentration celecoxib (100 μmol/l for 24 h, but not in response to 50 μmol/l in contrast) has been identified to promote the accumulation of p53 in the nucleus of colorectal cancer (CRC) cells [4]. While celecoxib inhibition of COX was initially considered to be responsible for this nuclear accumulation, the high concentration requirement for this effect led to the alternative suggestion that this phenomenon is COX independent [where the inhibitory concentration 50% (IC_{50}) for human COX-1 is 15 μmol/l and for COX-2 is 0.04 μmol/l [5]]. The inhibition of the AKT pathway has been offered as an alternative explanation for this observed accumulation of p53 in the nucleus in response to celecoxib [6].

In cancer cells that gain a survival advantage through the utilization of the AKT pathway [7], high concentration celecoxib-mediated inhibition of AKT phosphorylation appears to promote apoptosis in a COX-2-independent manner [8–11]. One branch of AKT activity involves 3-phoshoinositide-dependent kinase 1 (PI3K/PDK1) that phosphorylates and consequently promotes the accumulation of AKT, which in turn phosphorylates and stabilizes Hdm2, a ubiquitin ligase that is instrumental in suppressing p53. Inhibition of this pathway would then be predicted to induce the accumulation of transcriptionally competent p53 [6].

Given the respective roles that COX-2 and wt p53 appear to play in mediating the impact of celecoxib, how does celecoxib influence cells in the absence of both these targets (a scenario relevant to CRCs with mutant p53 and lacking significant COX-2)? Our study was initiated to unequivocally ascertain whether p53 and/or Hdm2, in the absence of elevated levels of functional COX-2, could influence the response of chosen CRC lines to celecoxib

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exposure. Extensive evaluation of many lines that lack significant levels of functional COX-2, from the colon, prostate and mouse embryo fibroblasts (MEFs), identified a similar degree of high concentration celecoxib sensitivity, apparently independent of the intactness of their p53 pathways and their Mdm2 status. Alternate targets that might generate a universal provocator of death were consequently considered. Rapid reactive oxygen species (ROS) production appears to be common to all the cell lines examined in this study. Importantly, however, ROS production was not found to contribute to cell death in these cells.

Materials and methods

Cell lines and materials

The human colorectal adenocarcinoma cell lines HT-29 (p53 mutant), RKO (wt p53), mouse embryo fibroblasts [MEF 35-8 ($p53^{-/-}$, $Hdm2^{+/+}$), a kind gift from Professor Gigi Lozano; MEF 174-2 ($p53^{-/-}$, $Hdm2^{-/-}$) [12]; MEF ($p53^{-/-}$, $c\text{-}Abl^{+/+}$); MEF ($p53^{-/-}$, $c\text{-}Abl^{-/-}$)] [13] and the breast cancer line MCF-7 (wt p53) were cultured in Dulbecco's modified Eagle's medium. HCT 116 lines (wt p53 and p53 null; a kind gift from Professor Bert Vogelstein) were cultured in McCoy's medium. The prostate line LnCap (wt p53) was cultured in modified Roswell Park Memorial Institute (RPMI) medium, according to the American Type Culture Collection (ATCC) protocol. All media was purchased from Sigma (St Louis, Missouri, USA) and supplemented with 10% fetal calf serum, and penicillin (100 U) and streptomycin (100 µg), purchased from Biological Industries (Beit-Haemek, Israel).

Celecoxib was a generous gift from Dexon (Hadera, Israel). All other materials were purchased from Sigma, unless otherwise stated in the text.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

For determination of the relative metabolic activity of cell lines cultured in celecoxib, cells in 40 µl of media were plated into individual wells of a 96-well plate. Cells were added at concentrations determined to be appropriate for the duration of culture: for 4 and 24 h, 18 000 cells; for 72 h, 2000 cells. The cells were incubated (37°C, 5% CO_2; incubation conditions that were used throughout the assay) for 24 h and the drug or the dimethylsulfoxide (DMSO) vehicle was then added to each well in a volume of 20 µl. The final DMSO concentration was 0.1% (v/v) in the tests and controls. Four hours before the termination time point of the experiment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to a final concentration of 0.5 mg/ml (1.2 mmol/l), incubated for an additional 4 h, when solubilization of the formazan crystals was achieved by the addition of an equi-volume of 10% (v/v) sodium dodecyl sulfate in 0.01 mol/l HCl. After incubation (24 h), the absorbance was read at 570 nm, relative to a reference wavelength of 690 nm (modified [14]).

Trypan blue dye exclusion assessment of cell viability

For determination of cell viability, cells were plated on 9-cm Petri dishes (1.6×10^6 cells per 7 ml) and incubated for 24 h. Drugs were then added in a volume of 3.5 ml and incubated for an additional 24 h. Cells in suspension and adherent were harvested together using Trypsin (0.25 w/v%) in ethylenediaminetetraacetic acid (0.02 w/v%) with phenol red (without Ca^{2+} and Mg^{2+}). Harvested cells were diluted with an equal volume of Trypan blue (0.4% w/v solution) and counted using a hemocytometer. Membrane integrity of live cells excludes dye uptake, while cells with damaged plasma membranes are permissive to the blue stain.

Western blot analysis

Cells harvested from Petri dishes using Trypsin (as described for Trypan blue dye exclusion analysis) were pelleted by centrifugation (800 *g*, 5 min) and solubilized in reducing protein sample buffer. Cells were diluted to a standard ratio of 0.5×10^6 cells per 25 µl. Cell lysates were separated using gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5–15% acrylamide) and Western-transferred. Immunoblotting was undertaken using the anti-human p53 monoclonal antibodies PAb1801 and D01 (Novocastra, Newcastle upon Tyne, UK), the anti-Hdm2 antibody Hdm2-323 (Sigma M; H7815), anti-PUMA (DB043) and anti-Bax (DB005; Delta Biolabs, Campbell, California, USA). Antibody staining was detected using the enhanced chemiluminescence detection method.

Amplex red

The Amplex Red Hydrogen Peroxide assay kit (Molecular Probes, Eugene, Oregon, USA) was adopted to establish whether the increase in MTT reduction up to 4 h after celecoxib introduction was due to ROS. Hydrogen peroxide (an oxygen radical that is formed either spontaneously from superoxide or through the activity of superoxide dismutase) is readily permeable to cell membranes and can thus be monitored outside the cell plasma membrane by its capacity to reduce the cell membrane impermeable Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) to its colored product resorufin. As a result of the nature of the reactivity of the Amplex red color reagent with phenol red and serum, and the poor solubility of celecoxib, the assay procedure employed had a number of significant modifications compared with that utilized for the MTT assay. The modified nature of the celecoxib exposure in this assay, as compared with the direct incubation of MTT in the presence of celecoxib, would be expected to introduce a degree of variation into the levels and time of ROS detection between the two assays.

Cells were plated at a ratio of 9×10^5/ml in 40 µl per well and allowed to recover for 24 h. To ensure celecoxib solution in the assay a DMSO stock was diluted into 10% fetal calf serum containing Dulbecco's modified Eagle's medium (as per the MTT assay). After the designated celecoxib exposure time (30 min, 1 h, 1 h 30 min or 2 h), the supernatant was aspirated, the cells were rinsed in phosphate-buffered saline and 20 µl of Krebs buffer was added. The reaction was initiated immediately with the addition of 100 µl of Amplex red reaction mixture in Krebs–Ringer buffer (prepared according to the manufacturer's instructions regarding measurement of hydrogen peroxide released from cells). Amplex red absorbance was measured at 570 nm at progressive time intervals and the 5.5 h time point is depicted.

Xanthine oxidase/xanthine

Superoxide was generated using the hypoxanthine/xanthine oxidase reaction in the presence of catalase on the basis of the method of Okado-Matsumoto and Fridovitch [15] and reduction of the tetrazolium salt WST-1 was measured at absorbance 450 nm [16].

Anti-oxidant studies

The cancer cell line RKO was exposed to superoxide dismutase over a range of concentrations (35, 75 and 300 U/ml) for 10 min before a coincubation with increasing concentrations of celecoxib for 4 and 20 h; subsequently, the MTT assay was performed as described above. The cell line HT-29 was treated with additional anti-oxidants including ascorbic acid (1 mmol/l, prepared in Kreb's buffer [17]) and *N*-acetyl cysteine (20 mmol/l adjusted to pH 7.4 prior to use [18]). Twenty hours after the introduction of increasing concentrations of celecoxib, the anti-oxidants and the drug were washed from the cells and subjected to the MTT assay.

Results

Status of p53 does not influence the effect of celecoxib on the metabolism of celecoxib-mediated colorectal cancer lines that do not overexpress functional cyclooxygenase-2

CRC cell lines, differing in their p53 status, that do not express high levels of functional COX-2 were used to evaluate the role of p53 in the cellular response to celecoxib. The repertoire of lines examined included three lines lacking functional COX-2: HT-29 (mutant p53 Arg273 His), HCT116 $p53^{+/+}$ (wt p53) and HCT116 $p53^{-/-}$ (null for p53) [19]; and a line with modest COX-2 levels, RKO (wt p53) [20]. Cells were exposed to increasing concentrations of celecoxib for 4, 24 and 72 h, and MTT reduction was assayed (Fig. 1). Surprisingly, all the four CRC lines, regardless of their p53 status or COX-2 levels, responded similarly to celecoxib treatment. Cells were refractory to celecoxib at low drug concentrations (i.e. 20 µmol/l, in the vicinity of physiologically measured plasma levels). In contrast, at high celecoxib concentrations after 24 h (> 80–160 µmol/l range) and 72 h (> 20–160 µmol/l range) MTT activity decreased dramatically. Notably, in all four cell lines a burst of MTT reduction was monitored within the first 4 h. Thus, at high celecoxib concentration, MTT reduction peaked in the first few hours and is followed by a complete loss by 72 h.

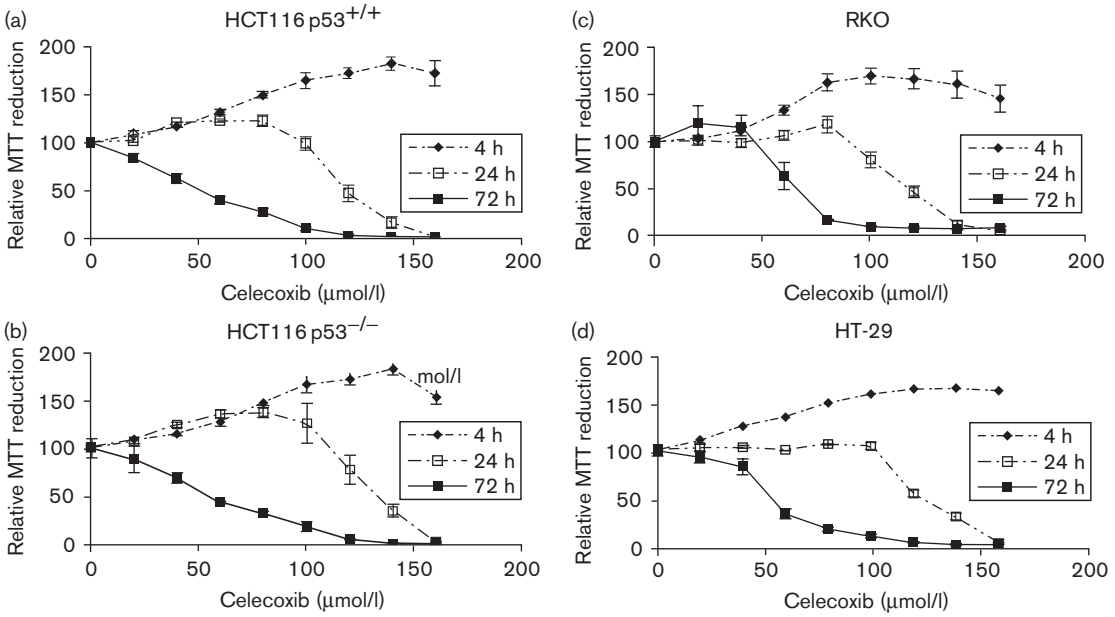
The bioreduction of tetrazolium salts (such as MTT) to formazans by viable cells is accepted as a measure of 'redox activity' [21] and is frequently interpreted as a measure of cell proliferation [22]. Our results indicate that within the first 4 h of exposure to celecoxib, MTT activity increased in cultured cells in a manner that appears to be divorced from the process of proliferation (see below). While the oxidoreductase activity of viable cells is generally understood to be responsible for MTT activity (reviewed by Tan and Berridge [16]), tetrazolium salts (MTT [23] and WST-1 [24]) have been demonstrated to be reduced by superoxide and have also been used to monitor oxidative burst (MTT [25] and WST-1 [16]). Understanding how MTT is reduced in cells in response to celecoxib during the first 4 h of exposure is potentially relevant to our understanding of the cellular impact of this drug.

p53 and Mdm2 status does not influence the effect of celecoxib on the growth of celecoxib-mediated colorectal cancer cells without overexpressed functional cyclooxygenase-2

While the MTT assay is a commonly accepted indication of cell proliferation after 24 h or more of drug exposure, it is unable to distinguish whether variations induced by treatments reflect altered numbers of cells in the sample or a change in the rate of metabolic activity; also metabolic shutdown and cell death cannot be distinguished.

To supplement the information derived from the MTT assay on the influence of celecoxib on the CRC cell lines, cell viability was determined by dye exclusion using Trypan blue, which discriminates between live and dead cells. In all four cell lines, exposure to 100 µmol/l celecoxib or higher concentrations for 24 h resulted in a significant reduction in cell numbers (Fig. 2). This growth inhibition is likely to be the result of growth arrest induced by celecoxib. Cell death, as measured by Trypan blue intake was detected at concentrations of 120 µmol/l and significantly increased at 160 µmol/l. Clearly, the dose of the drug as a function of time is a critical factor determining the success of celecoxib to halt, as opposed to curbing, the growth of tumor cells and this effect is p53 independent. It is possible that growth inhibition at lower concentrations is followed by cell death at later time points.

Fig. 1



MTT reduction was measured in four CRC cell lines after 4, 24 and 72 h of exposure to increasing celecoxib concentrations: (a) HCT116 (p53 wt +/+, undetectable COX-2); (b) HCT116 (p53 null −/−, undetectable COX-2); (c) RKO (wt p53, COX-2 levels not overexpressed) and (d) HT-29 (p53 Arg273 His; highly overexpressed non-functional COX-2). The levels of MTT reduction are expressed as percentages relative to the untreated controls, which were defined as 100% reduction levels. Each data point represents triplicate data. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CRC, celecoxib-mediated colorectal cancer; COX-2, cyclooxygenase-2; wt, wild-type.

The data from the MTT and dye exclusion assays have been analyzed to determine the IC_{50}s of celecoxib treatment of the four CRC lines. A comparison of these IC_{50}s after 24 h of celecoxib treatment identified remarkable similarity between them (Table 1 [12,13,19,26–35]). This further corroborates the notion that the response of these CRC cells to celecoxib is p53 independent. After 24 h of exposure to celecoxib at concentrations greater than 100 μmol/l, all cells were inhibited metabolically with a steep response profile.

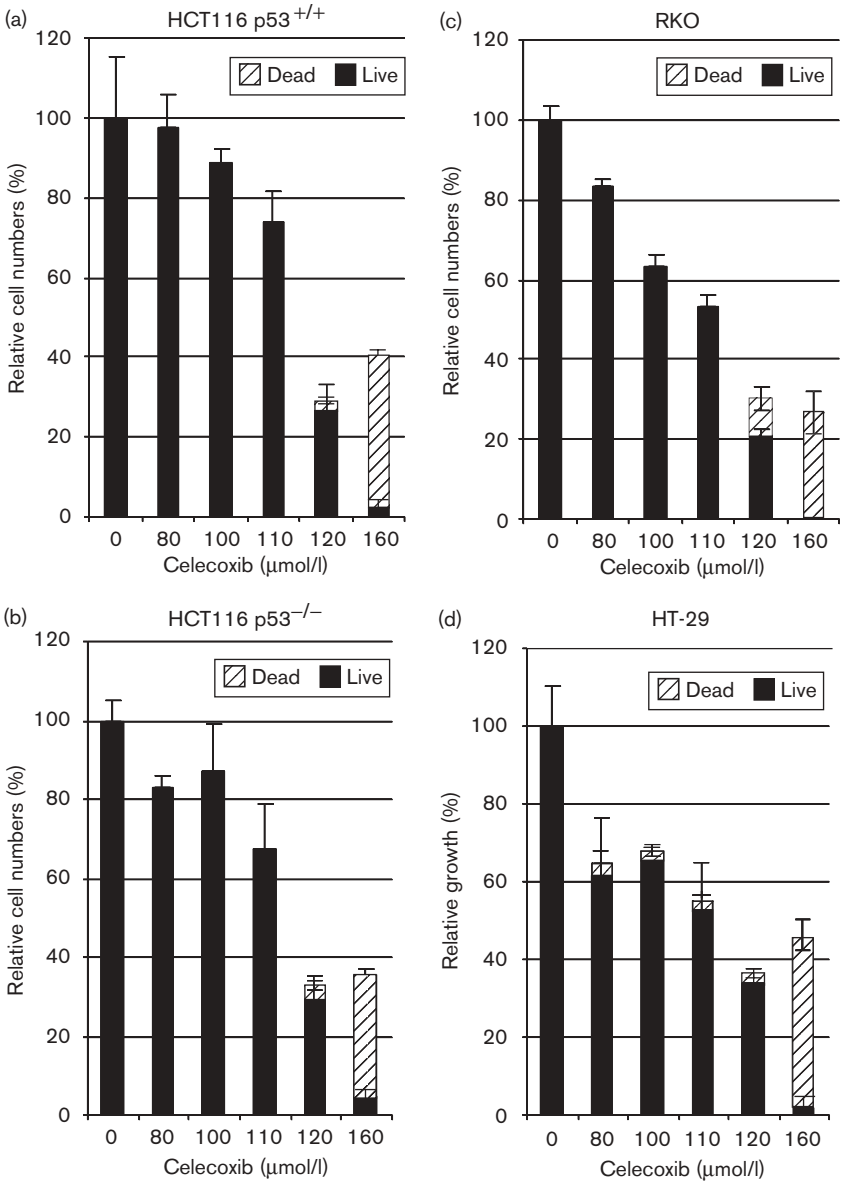
To examine whether the observed effects of celecoxib are confined to CRC lines, we extended the assays to additional cell lines from different cell types. These included the wt p53 breast cancer line MCF-7 and the prostate line LnCap. The IC_{50} values of these lines were compared with those of the four CRC lines. As outlined in Table 1, the IC_{50} values for all the lines examined were identified within a narrow window of variability.

Next, we wished to examine whether the oncogene Mdm2, which is a p53 target, but also has p53-independent functions, influences the cellular response to celecoxib. For this purpose, we compared two MEF lines lacking p53, one expressing Mdm2 (MEF 35-8; $p53^{-/-}$, $Hdm2^{+/+}$) and the other null for Mdm2 (MEF 174-2; $p53^{-/-}$, $Hdm2^{-/-}$). This comparison revealed no significant difference, suggesting that Mdm2 is not a significant determinant of the response of cells to celecoxib.

Accumulation of p53 and its target genes do not affect the cellular response to celecoxib

The two most important levels of p53 regulation are at the transcriptional activity and protein stability levels. Therefore, the effects of celecoxib on p53 protein expression, and on the expression of an important target gene Hdm2 were examined. Cells were exposed to increasing concentrations of celecoxib (80, 100 and 120 μmol/l) for 24 h, and then cell extracts were prepared and subjected to Western blot analysis using anti-p53 and anti-Hdm2 antibodies (note: Fig. 3 presents only the control and 120 μmol/l data, as lower concentrations did not provoke an affect that differed from that in the controls). Elevation in p53 expression was observed in cells expressing wt p53 $HCT116^{+/+}$ and RKO, but not in cells expressing mutant p53 (HT-29) (Fig. 3a). This result supports previous findings that wt p53 levels are elevated in response to celecoxib [4], but strongly suggests that celecoxib effects on cell growth inhibition and death in these cells are independent of p53 status, despite influencing its expression level. Further, the variable effect of celecoxib on Hdm2 expression (Fig. 3b) strongly indicates that Hdm2 is not a major determinant of celecoxib susceptibility in these cells. Additional

Fig. 2



Four CRC cell lines: (a) HCT116 (p53 wt +/+, undetectable COX-2); (b) HCT116 (p53 null −/−, undetectable COX-2); (c) RKO (wtp53, COX-2 not overexpressed); and (d) HT-29 (p53 Arg273 His; highly overexpressed non-functional COX-2), exposed for 24 h to increasing celecoxib concentrations were analyzed for total live and dead cell numbers using Trypan blue dye exclusion. Data are presented as cell numbers relative to the control. Each data point represents triplicate data. CRC, celecoxib-mediated colorectal cancer; COX-2, cyclooxygenase-2; wt, wild-type.

protein targets of p53 that were analyzed include Bax and Puma, and in these cases also no correlation was found between their expression and the cellular response to celecoxib treatment (data not shown).

High concentration celecoxib elevates cellular reactive oxygen species levels and can stimulate superoxide production by xanthine oxidase *in vitro*

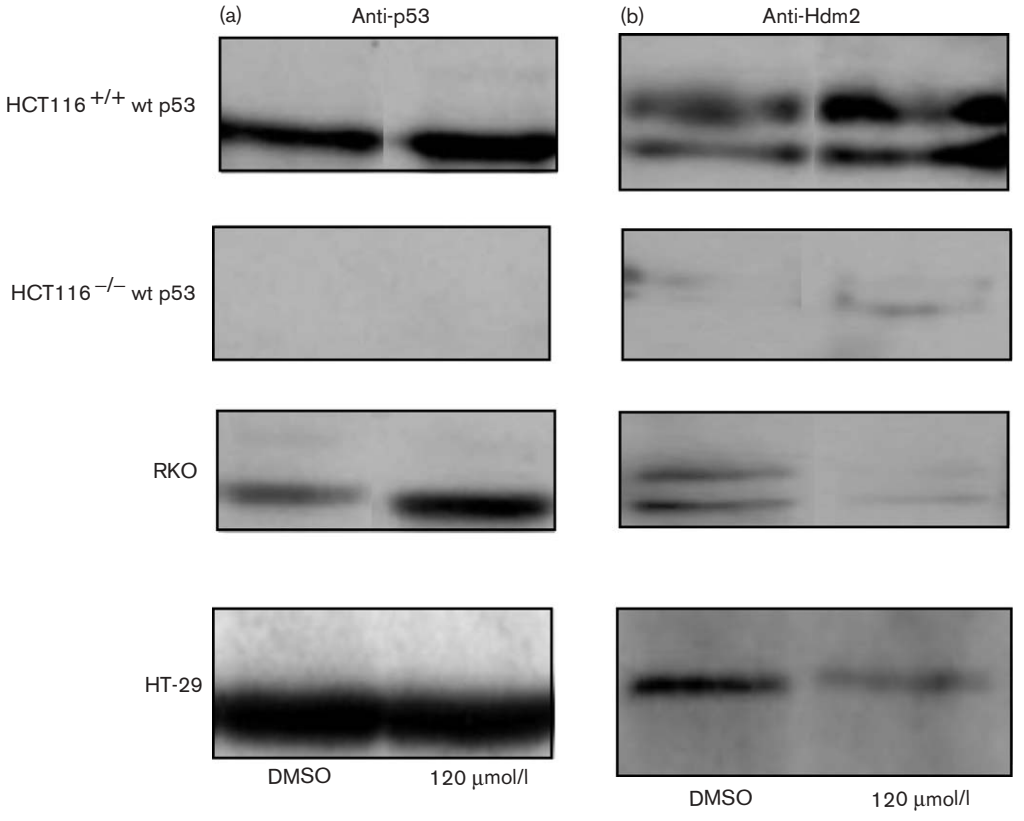
The observed burst of redox activity within the first few hours of celecoxib treatment, as measured by MTT activity (Fig. 1), raised the possibility that celecoxib may induce the production of ROS. The absorption profile of Amplex red, in response to celecoxib treatment of HT-29 cells, indicates that hydrogen peroxide is being generated in a manner dependent on the concentration and exposure time of celecoxib (Fig. 4). Celecoxib (80 μmol/l) appeared to have an extremely modest influence on ROS production under these conditions, consistent with the mild MTT reduction observed. Maximum ROS production was measured after 2 h of 110 μmol/l celecoxib and

Table 1 IC_{50} (μmol/l) of human cancer cell lines determined using Trypan blue dye exclusion after 24 h, and the MTT assay after 24 and 72 h of exposure to celecoxib

Cell line	p53 status	Hdm2 or Mdm2 status	COX-2 status	IC_{50} for celecoxib after 24 h of exposure	IC_{50} for celecoxib after 24 h of exposure	IC_{50} for celecoxib after 72 h of exposure
				Method: Trypan blue	Method: MTT	Method: MTT
HCT116	wt $p53^{+/+}$ [26]	$Hdm2^{+/+}$ [27]	lacks [19,28]	113.20 ± 1.06	134.01 ± 10.32	54.16 ± 1.89
HCT116	wt $p53^{-/-}$ [26]	$Hdm2^{+/+}$	lacks [28]	115.59 ± 2.05	134.05 ± 1.67	58.35 ± 1.00
RKO	wt p53	Hdm2 high expression [29]	not over-expressed [30]	119.0 ± 0.73	113.92 ± 8.52	62.65 ± 2.39
HT-29	p53 mutant	Hdm2 expressed [31]	non-functional; high expression [19]	112.74 ± 0.28	115.77 ± 1.23	54.03 ± 1.91
MCF-7	wt p53	Hdm2 high expression [32]	lacks [33]	ND	121.06 ± 3.76	62.76 ± 4.11
LnCAP	wt p53	high expression [34]	lacks [35]	ND	112.55 ± 0.52	ND
MEF 35-8[a]	wt $p53^{-/-}$	$Mdm2^{+/+}$ [12]		ND	91.21 ± 0.98	67.51 ± 3.91
MEF 174-2[a]	wt $p53^{-/-}$	$Mdm2^{-/-}$ [12]		ND	99.13 ± 6.87	78.67 ± 1.17
MEF	wt $p53^{-/-}$, c-$Abl^{+/+}$ [13]			ND	117.27 ± 8.89	ND
MEF	wt $p53^{-/-}$, c-$Abl^{-/-}$ [13]			ND	107.71 ± 10.90	ND

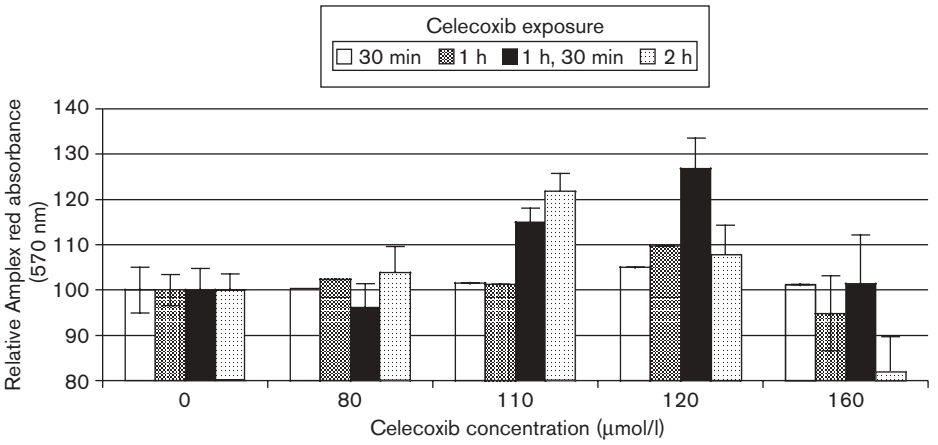
[a]Plated 9000 cells per well for 24 h, this variance in cell numbers between the other cell lines (18 000 cells per well for the 24 h assay) may explain the slight discrepancy between the MEF lines and the epithelial lines. Clearly, however, Hdm2 did not influence the response of these cells to celecoxib.
ND, not determined; COX-2, cyclooxygenase-2; wt, wild-type.

Fig. 3



Western blot analysis of four CRC cell lines: HCT116 (p53 wt +/+, undetectable COX-2), HCT116 (p53 null −/−, undetectable COX-2), RKO (wt p 53, COX-2 not over-expressed) and HT-29 (p53 Arg273 His; highly overexpressed non-functional COX-2) exposed for 24 h to increasing celecoxib concentrations, and analyzed for reactivity with antibodies directed toward p53 (a) and Hdm2 (b). Shown are the controls and cells treated with 120 μmol/l celecoxib, the lowest concentration at which the response of treated cells differed from the responses of the controls. CRC, celecoxib-mediated colorectal cancer; COX-2, cyclooxygenase-2; wt, wild-type.

Fig. 4



The relative Amplex red absorbance of HT-29 cells treated for 0.5, 1, 1.5 or 2 h with celecoxib (0, 80, 110, 120 and 160 μmol/l), washed and exposed to Amplex red. Celecoxib provoked an increase in absorbance detectable at 2 h and the 5.5-h data is presented. The levels of Amplex red absorbance are expressed as percentages relative to the untreated controls, which were defined as 100% absorbance levels. Each data point represents triplicate data.

1 h 30 min of 120 μmol/l, suggesting that higher drug concentrations have a faster impact. The lower level of ROS production in response to 120 μmol/l celecoxib for 2 h presumably reflects ROS release occurring within the drug exposure time and being washed out before the addition of Amplex red. This is also likely to be the case with the cells exposed to 160 μmol/l celecoxib, where the cellular response appears to have been extremely rapid, within the first 30 min of exposure and the ROS was washed-out with the medium, before the addition of the colorimetric reagent.

Next, the possibility that a high concentration of celecoxib is capable of promoting a burst of superoxide that can be detected by tetrazolium salt reduction was evaluated *in vitro* using the xanthine oxidase/xanthine system. The reduction of WST-1 by superoxide generated in the xanthine oxidase/xanthine system [36] was confirmed and no spontaneous reduction of WST-1 was generated in the absence of either xanthine oxidase or xanthine. Celecoxib was not able to act as a substitute substrate for hypoxanthine (Fig. 5a). Remarkably, the rate of WST-1 reduction increased significantly in the presence of high concentrations of celecoxib (Fig. 5b). These findings support the notion that celecoxib may be exerting a direct effect on xanthine oxidase.

Anti-oxidants failed to protect against celecoxib-induced cell death

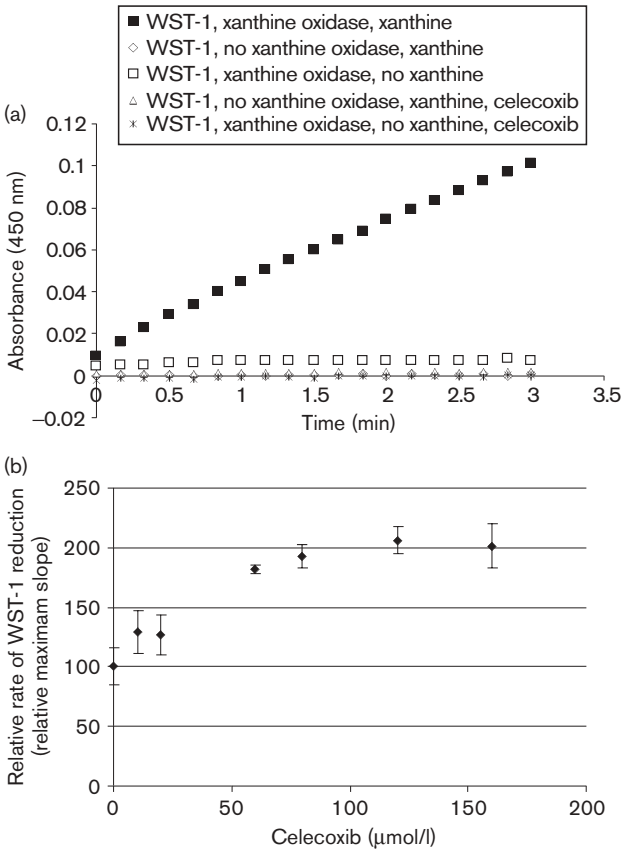
Treatment of the cancer line RKO with superoxide dismutase (150 U/ml) in the presence of celecoxib (120 μmol/l) resulted in a pronounced diminution of MTT reduction after 4 h of drug exposure (Fig. 6a; this effect was not evident at superoxide dismutase concentrations of 75 U/ml or less, and was not enhanced by 300 U/ml, data not included). Despite this 4-h impact, after 20 h, however, there was no evidence of relief from growth inhibition in the superoxide dismutase-treated samples (Fig. 6b). Expansion of the range of anti-oxidants to include 1 mmol/l ascorbic acid and 20 mmol/l *N*-acetyl-L-cysteine failed to relieve HT-29 from the 20-h growth inhibition induced by 120 μmol/l celecoxib (Fig. 6c, in which samples have been normalized to the control response).

Discussion

All cells lacking elevated levels of functional COX-2 were highly resistant to low concentrations of celecoxib and required levels far in excess of those recorded in human plasma to impede cell growth even after 72 h of exposure. The wt p53 and Hdm2, either alone or in concert, were not identified to influence the celecoxib sensitivity of cells lacking any appreciable functional COX-2. This was defined using matched pairs of cell lines with and without the relevant genes. Despite the capacity of celecoxib to elevate p53 protein levels, there was no correlation between the levels of these proteins and growth inhibition.

This observation adds an important clarification regarding the significance of wt p53 in determining cellular sensitivity to celecoxib in the absence of COX-2. In studies of murine erythroleukemia cells, the most profound celecoxib sensitivity was identified to correlate with the presence of wt p53 and high COX-2 levels. Importantly, these murine erythroleukemia cells were sensitive to growth inhibition induced by celecoxib at circulating levels (5 μmol/l). p53 mutation, with a parallel

Fig. 5



WST-1 is reduced by superoxide generated by xanthine oxidase/hypoxanthine. WST-1 reduction is not spontaneous as the presence of xanthine oxidase and its substrate xanthine are essential. Celecoxib (120 μmol/l) does not influence the rate of WST-1 reduction in the absence of either xanthine oxidase or xanthine (a). In the presence of xanthine oxidase, xanthine and increasing celecoxib concentrations, the rate of WST-1 reduction increases (b). Panels (a) and (b) present results that are representative of at least triplicate data.

reduction in COX-2 levels, correlated with an increased resistance to celecoxib [37]. This concept of interdependency between wt p53 and COX-2 levels was further demonstrated in the study by Corcoran *et al.* [2] in which an elevation in wt p53 led to an increase in COX-2. Significantly, in our study we have demonstrated a counterpart to these studies, identifying that when functional COX-2 levels are not abnormally high, the status of p53, whether wt p53 or null (as in the HCT116 lines), has no influence on celecoxib sensitivity, consistent with overexpressed levels of functional COX-2 defining low-concentration celecoxib sensitivity.

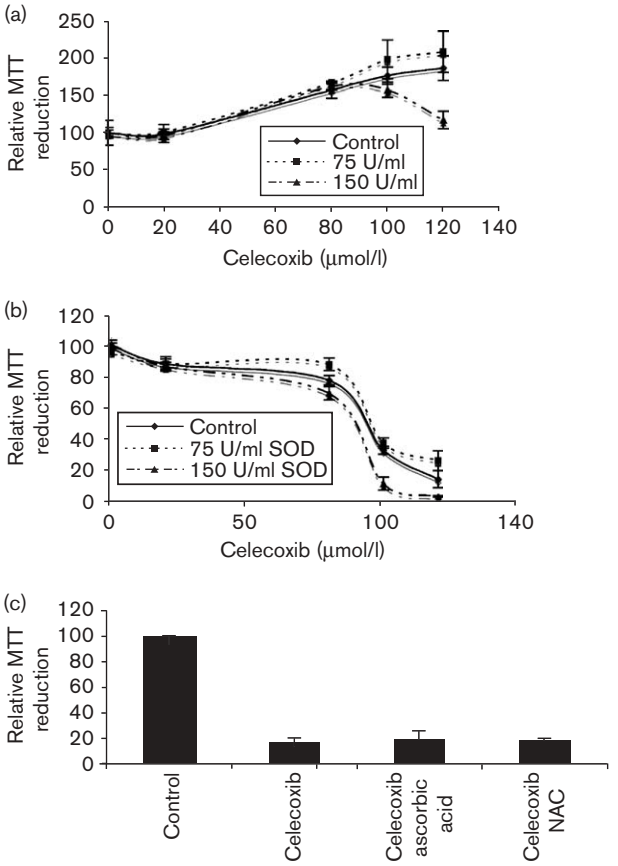
How could p53 stabilization, and the diminution of Mdm2 in response to celecoxib, occurring in only certain cell types in our study be explained? In contrast to many cancer cells with an activated AKT pathway (modeled in Fig. 7a [38]), the addition of celecoxib inhibits AKT-mediated phosphorylation of Mdm2; this is thought to render Mdm2 incapable of promoting p53 degradation, and the observed elevation in p53 levels in the relevant genetic context (Fig. 3) (HCT116 $p53^{+/+}$ and RKO), together with the activation of its targets such as p21 and p27, could be explained (modeled in Fig. 7b [8,9,37,39–42]). The absence of such a phenomenon in mutant p53 (Fig. 3) (HT29) and p53 null cells (Fig. 3) (HCT116 $p53^{-/-}$) as we observed would be expected. As no dependency on these molecules was observed with regard to death induction, the influence that celecoxib wields over these molecules would not appear to be a crucial trigger of the cell death induction process. An alternative target is clearly involved.

The universal elevation of MTT reduction in the initial 4 h of exposure to celecoxib was pronounced and unexpected. Strikingly, the celecoxib concentrations that induced increased MTT reduction in the first 4 h of exposure corresponded to the concentrations responsible for cell growth inhibition after 72 h of exposure. The questions of interest arising from our observations then relate to the (i) nature and (ii) origin of these increased MTT levels and, (iii) whether elevated levels of MTT are instrumental in mediating the subsequent cell death.

Tetrazolium salts, including MTT, are adopted for a range of applications in addition to monitoring cell viability and growth, including the measurement of oxidoreductase activity and the detection of superoxide radicals. An unequivocal definition of the effector(s) of MTT bioreduction, however, remains to be established (reviewed by Bernas and Dobrucki [43]). Confirmation that celecoxib promotes ROS (an indirect measure of superoxide) in an exposure time and concentration dependent manner was established with the Amplex red assay. A general assay for ROS was adopted in preference to a more specific assessment of superoxide on the assumption that, either spontaneously or under enzymatic catalysis, hydrogen peroxide will be generated from superoxide.

Intriguingly, in neutrophils, high-concentration celecoxib (100 μmol/l) was demonstrated to stimulate ROS through the induction of a respiratory burst response that involved G-protein signaling pathways and calcium release [44]. ROS production in the context of neutrophils reflects activation for the intention of killing microorganisms. Two recent reports are even more pertinent to our work, however; the first describes the detection of a modest elevation in ROS levels in osteosarcoma cells in response to high-dose celecoxib treatment (100 μmol/l, 4 h; detected as a faint enhanced fluorescence in 2′,7′-dichlorodihydrofluorescein diacetate-treated cells) [45]. The second study reported that 75 μmol/l celecoxib stimulated release of ROS generation in the human lung cancer

Fig. 6



MTT reduction was measured in the RKO CRC cell line after exposure to superoxide dismutase (75 and 150 U/ml) and increasing concentrations of celecoxib for 4 (a) and 20 h (b). The addition of 1 mM ascorbic acid or *N*-acetyl-cysteine (NAC) did not relieve HT-29 cells from the growth inhibition induced by 120 μmol/l celecoxib after 20 h of drug exposure (c). The levels of MTT reduction are expressed as percentages relative to the untreated controls, which were defined as 100% reduction levels. Each data point represents triplicate data. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

cell line A549 (as demonstrated using flow activated cell sorter profiling of 2′,7′-dichlorofluorescein oxidation) [46].

Another report that is consistent with celecoxib inducing ROS is the nearly 400-fold increase in heme oxygenase (decycling) 1 (HO-1), which was identified using an array analysis of breast epithelial cells exposed for 6 h to 50 μmol/l celecoxib [39]. Furthermore, celecoxib has been demonstrated in glomerular mesangial cells to increase HO-1 levels [47]. HO-1 induction appears to be a general response to oxidative stress (including hydrogen peroxide) in mammalian cells [48]. A role for HO-1 in protecting cancer cells from oxidative damage has also been supported by the ability of an HO-1 inhibitor to enhance the sensitivity of cancer cells to oxidative stress-inducing drugs [49]. Another interesting twist in the system is added by the ability of the HO system to regulate COX; specifically, when HO activity increases, COX activity decreases [50].

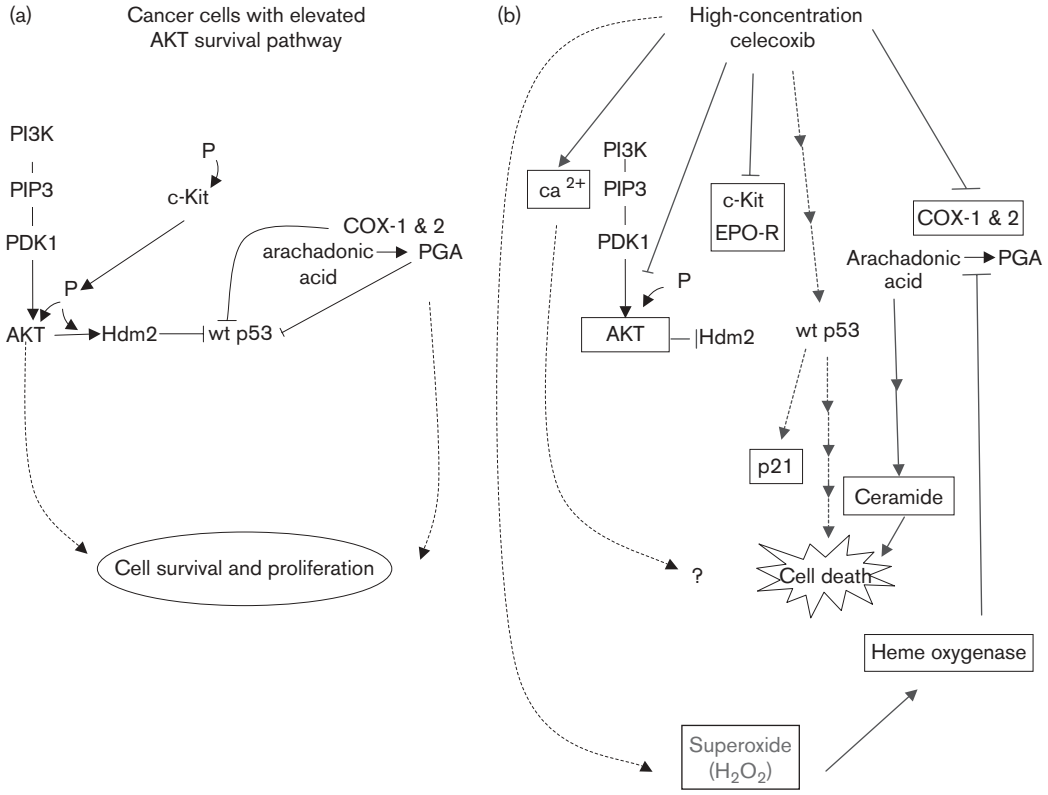
How celecoxib could impact ROS production is a central question? One possible source of excessive ROS formation may involve the overstimulation of xanthine oxidase, a cellular component that is normally tightly regulated (reviewed by Droge [51]). Intriguingly, we have demonstrated that the *in vitro* xanthine oxidase-mediated production of superoxide, as monitored by WST-1 reduction [16], was enhanced in the presence of celecoxib. Celecoxib was not able to act as a direct substrate for enzymatic reduction, as demonstrated for a number of anti-cancer compounds such as the anthracyclines (doxorubicin, daunomycin and marcellomycin) that are metabolized by xanthine oxidase to their active metabolites (reviewed by Pritsos [52]) and that most pertinently also provoke ROS in a p53-independent manner [53]. Whether this impact of celecoxib on xanthine oxidase is of relevance to ROS production in CRC cells was not addressed in these studies.

A player frequently implicated in ROS-mediated cell death is c-Abl. c-Abl is a non-receptor tyrosine kinase that is mobilized from the cytoplasm to the mitochondria under the influence of hydrogen peroxide [54] and mediates a loss in membrane potential, culminating in apoptosis. c-Abl was not identified in these studies to influence celecoxib-mediated cell death (in MEF cells, data not shown).

Despite this stimulation of ROS, however, anti-oxidants were inadequate to protect against celecoxib-provoked death, suggesting that this morbidity is independent of ROS production. A recent study on the influence of celecoxib on a lung cancer cell line also independently drew this conclusion. Specifically, they demonstrated that while overnight pre-treatment with 100 μmol/l α-tocopherol inhibited celecoxib-stimulated Ros production, it did not rescue the cells from growth suppression [46]. Thus, these findings add an important aspect to the debate on the potential of ROS stimulation in cancer therapy [55]. Our studies suggest that, in p53-deficient cells, ROS stimulation alone may be an insufficient mediator of death.

The major provocateur of celecoxib-induced death in these cells remains to be defined. One potential contender is calcium, a known participant in apoptosis onset [56]. Cellular calcium levels may be perturbed in response to celecoxib exposure [40,57] (Fig. 7b). Further studies are required to definitively elaborate how celecoxib induces death and to determine the significance of celecoxib-stimulated elevated ROS levels.

Fig. 7



(a) Interplay between the AKT pathway and cyclooxygenase enzymes in colorectal cancer (references within the text and [38]). (b) Integrated scheme of the known targets of celecoxib (Ca^{2+} [40]; AKT [8]; PDK1 [9]; c-kit, EPO-R [37]; p21 [41]; ceramide [42]; heme oxygenase [39]); where reported targets of celecoxib are framed and speculated pathways are indicated by broken lines. CRC, celecoxib-mediated colorectal cancer; COX, cyclooxygenase.

Thus, two pathways appear relevant to celecoxib death induction: one that is dependent on p53/high COX-2 expression and activated at low celecoxib concentrations, and another that is independent of p53 and high levels of functional COX-2, and activated at high celecoxib concentrations. The absence of both wt p53 and high levels of functional COX-2 leads to significant celecoxib resistance in tumor cells.

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