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Cancer Cell Killing by Celecoxib: Reality or Just In Vitro Precipitation-Related Artifact?

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ABSTRACT

Among NSAIDs Celecoxib is one of the most efficient in triggering in vitro cancer cell death, and from this perspective has been subject of numerous studies. However, it is still controversial whether this in vitro-observed effect can also occur in vivo and contribute to the antitumor action of the drug. Moreover, besides common agreement on the involvement of COX-independent pathways, the mechanisms underlying Celecoxib toxicity are still unclear. In an attempt to shed light on these mechanisms, I found that cell death only occurs at insoluble concentrations of the drug, and follows irreversible binding and damage of the plasmamembrane by precipitates. This evidence strongly suggests that Celecoxib is devoid of true molecular toxicity. Moreover, since plasma levels reached during therapy are far below the threshold of toxic precipitation, direct cytotoxicity by Celecoxib is unlikely to occur on tumor cells in vivo. Thus the antitumor effect might be only due to COX inhibition, which requires significantly lower levels of the drug. Nonetheless, direct cytotoxicity might not be confined to an in vitro artifact, but contribute to the upper gastrointestinal side effects of Celecoxib. Overall, these findings represent an important basis for further studies on Celecoxib, where true molecular actions of the drug should be discriminated from the precipitate-dependent ones, and the relationship between in vitro and in vivo effects considered at the light of the precipitate-dependent model. Moreover, remarkably, this article indicates a model of critical analysis that can be extended to other poorly soluble drugs. J. Cell. Biochem. 114: 1434–1444, 2013.

KEY WORDS: CELECOXIB; SOLUBILITY; PRECIPITATES; MEMBRANE; CANCER; TOXICITY; CELL DEATH

The in vitro toxicity of NSAIDs has been studied in relationship with their antitumor properties [Cha and DuBois, 2007; Greenhough et al., 2009; Jendrossek, 2011; Gong et al., 2012] and gastrointestinal side effects [Tomisato et al., 2004b; Tanaka et al., 2005; Lichtenberger et al., 2006]. According to the current knowledge, COX-inhibition is a major player in the antitumor action of NSAIDs [Cha and DuBois, 2007; Greenhough et al., 2009]. However, COX-independent mechanisms might be involved as well. In fact, different NSAIDs are able to induce COX-independent growth-inhibition and death of cancer cells in vitro [Grosch et al., 2006; Schonthal, 2007; Chuang et al., 2008; Schiffmann et al., 2008], which might be reflected by growth inhibition and cancer-cell killing in vivo. Among NSAIDs, Celecoxib is one of the most efficient in triggering in vitro tumor cell death [Schonthal, 2007; Jendrossek, 2011]. Despite numerous studies aimed at the definition of the mechanisms involved in this effect, there is no final agreement on common pathways or molecular targets [Jendrossek et al., 2003; Kulp et al., 2004; Ding et al., 2005; Maier et al., 2005; Tanaka et al., 2005; Fukada et al., 2007; Ishihara et al., 2007; Pang et al., 2007; Schonthal, 2007; Schiffmann et al., 2010; Jendrossek, 2011; Reed et al., 2011]. Also the involvement of direct cytotoxicity in the anticancer effect of the drug in vivo is highly controversial [Williams et al., 2000; Maier et al., 2005; Schonthal, 2007; Jendrossek, 2011; Gong et al., 2012]. Here the in vitro toxicity of Celecoxib is demonstrated to rely on cell damage by precipitates. The implications and challenges of this unexpected finding for what concerns the antitumor effect and gastrointestinal toxicity of Celecoxib are also discussed.

MATHERIALS AND METHODS

CELL CULTURE AND REAGENTS

MDA-MB-231 and MCF7 were from ECACC before its merging into the HPA Culture Collections (2003). HCT116 cells were from the HPA Culture Collections. AGS cells were from CLS (CLS, Cell Lines Service, Eppelheim, Germany). To reduce experimental variability

Abbreviations used: NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase.
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these cell lines were all cultured in RPMI/DMEM 1:1 (RPMI 1600, DMEM 1000), 2 mM glutamine, 50 U/ml Penicillin, 50 μg/ml Streptomycin, 10% FBS. Cell culture media and reagents were from Invitrogen (Invitrogen, Carlsbad, CA), cell culture plates from BD (BD Biosciences, San Jose, CA). To eliminate unwanted particles and precipitates, FBS was centrifuged and filtered (0.2 μm) before use. Filters were from Millipore (Billerica, MA). Fifteen millimolar Hepes was added to the medium to reduce pH variations. For pH-controlled experiments Hepes-buffered medium without $NaHCO_3$ was used in an incubator with normal atmosphere. Celecoxib was from Aartidrugs (Aartidrugs, Mumbai, India), and was diluted from stocks 500× in DMSO 10–20 min before use.

ANALYSIS OF CELL GROWTH AND DEATH

Cell growth was measured by crystal violet staining. Briefly, after 10′ fixation with 10% formaldeide, cell were stained with 0.1% crystal violet (Sigma–Aldrich, St. Louis, MO) for 1 h, solubilized with 30% acetic acid, and read at 550 or 570 nm with a Spectra Max 190 plate reader (Molecular Devices, Silicon Valley, CA). Cell viability was assessed by Trypan blue (Invitrogen), Propidium Iodide (PI; Sigma), or Hoechst 33258 (Invitrogen) staining and manual count or flow cytometric analysis with a BD FACSAria (BD Biosciences). Cells were seeded 8–14 h before the treatments at 75,000 cells/ml, corresponding to 15,000 cells/well in 96-well/plates with 200 μl/well, or a proportional number in 48- and 24-well plates. The increase in cell number due to proliferation was ≤30%.

SOLUBILITY ASSAYS

Light scattering analysis of precipitation was performed in cuvette at 400 nm using a SPEX Fluoromax fluorimeter (Spex Industries, Edison, NJ) 20′–30′ after diluting Celecoxib [Ashok et al., 2011; D'Arca et al., 2010].

MICROSCOPY

Precipitates and cells were observed using a Leica DMIL microscope, a Leica DMRD microscope equipped with a LEICA DC500 digital camera (Leica Microsystems GmbH, Wetzlar, Germany,) or a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany). Cells were fluorescently labeled before plating with the amino-reactive dye CFSE (Invitrogen) 5 μM for 5′, or DiO (1 μM × 20′; Invitrogen). Precipitates were stained with 0.1–0.5 μM DiA as indicated (Invitrogen). Excitation of CFSE and DiO was at 488 nm with BP500-530 emission filter, excitation of DiA at 543 nm with LP560 emission filter.

STATISTICAL ANALYSIS

The data are reported as mean ± SD of at least three independent experiments, each average of 2–5 replicates. The onset of statistical significance ($P < 0.05$) is indicated (*) when useful.

RESULTS

CELECOXIB INDUCES IN VITRO CANCER CELL DEATH WITH A VERY SHARP DOSE-RESPONSE AND IN A COX-INDEPENDENT MANNER

Preliminary analysis of the in vitro effects of Celecoxib on tumor cell growth and death was performed on the human mammary carcinoma cell lines MDA-MB-231 and MCF7, and the colon carcinoma cell line HCT116. For adequate comparison with the conditions used by previous authors, toxicity was analyzed at different serum concentrations: serum-free medium, low serum (0.5%), high serum (10%). The condition of low serum was analyzed more in detail, since it well supported short-term viability and growth while reducing the variability of precipitation/toxicity caused by different protein content in different batches of serum (see later). A time point of 30 h after the addition of Celecoxib was initially selected because it allowed, at the same time, a good estimation of changes in both cell growth and viability. As expected, Celecoxib toxicity appeared inversely proportional to serum-concentration (Fig. 1A–D), and the prevalent effect of the drug was induction of cell death, which appeared at 15–20 μM in serum-free medium, 20–25 μM in low serum, 50–75 μM in high serum, only preceded by limited growth inhibition (Fig. 1A–D and not shown). Only a slight increase of the concentration (≥30 μM in low serum, ≥100 μM in high serum) was sufficient to induce massive cell death. The toxicity appeared even sharper looking at the time course of cell death and contact time needed to cause cell death. In low serum, 25 μM Celecoxib required prolonged contact and several hours to kill a minority of the cells, while higher concentrations induced fast to almost immediate cell death (Fig. 1E). Moreover, only a short contact with Celecoxib >25 μM was sufficient to irreversibly prime cells to death (≥10′ with 50 μM Celecoxib, ≥2′ with 100 μM, <30″ with 150 μM; Fig. 1E). Celecoxib toxicity was also confirmed to be COX-independent. In fact, growth inhibition and death occurred at concentrations of Celecoxib significantly higher than the COX-inhibiting range, were not prevented by the addition of exogenous PGE_2, and were independent of the levels of COX expression and prostanoid production, which are quite different between the three cell lines tested (Supplementary Fig. S1 and unpublished results).

PRECIPITATION AND TOXICITY OF CELECOXIB ARE STRICTLY RELATED ON A MOLAR BASIS

At the beginning of the present study, the sharpness of the dose-response of toxicity and the appearance of sporadic crystals in cultures of cells killed by Celecoxib (Supplementary Fig. S2), suggested the existence of a correlation between precipitation and toxicity of the drug. Since the literature only reported the solubility of Celecoxib in aqueous solutions without serum ([Seedher and Bhatia, 2003] and Supplementary Table S1), and the latter could significantly affect this parameter [Seedher and Bhatia, 2006], precipitation of the drug was measured, here for the first time, in cell culture media with serum, using different experimental approaches [Pan et al., 2001; Alsenz and Kansy, 2007].

The highly sensitive light scattering method indicated a limit of solubility between 10 and 12.5 μM in water, 5 and 7.5 μM in serum-free medium (pH 7.4), 7.5 and 10 μM in 0.5% serum, 17.5 and 20 μM in 10% serum (Fig. 2A, Supplementary Fig. S3A and Table S1). Preliminary dissolution studies supported these observations, indicating slightly lower solubility: about 4 μM in serum-free medium, 5 μM in 0.5% serum, 10 μM in 10% serum. This suggested the permanence of super-saturated Celecoxib after dilution from DMSO.

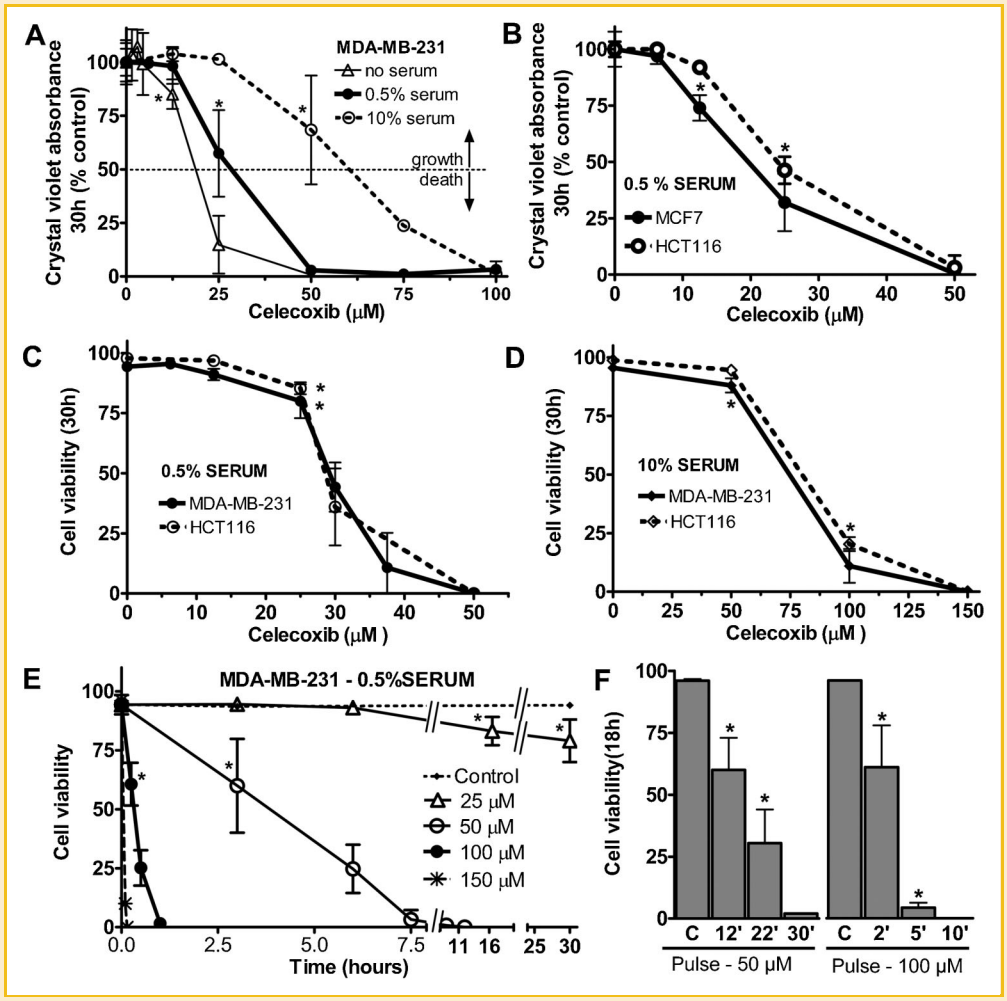

Fig. 1. Effect of Celecoxib on the growth and viability of cancer cells. A: Crystal violet staining of MDA-MB-231 cells, 30 h after the addition of Celecoxib at different serum levels, expressed as % of the control at 30 h, allowed preliminary determination of both cell growth and death. The arrows approximately indicate the prevalence of growth or death, based on the evidence that 30 h corresponds to one doubling of MDA-MB-231 (low serum), that is, time zero is 50% of the control at 30 h. B: Crystal violet staining of HCT116 and MCF7 cells indicates reproducible and cell type-independent toxicity; (C) viability of MDA-MB-231 cells treated with Celecoxib in low and high serum; (D) viability of HCT116 cells; (E) time course of cell death (MDA-MB-231 cells) in low serum; (F) cell death of MDA-MB-231 after pulses (2′–30′) of 50 and 100 μM Celecoxib in low serum, measured at 18 h (plateau of cell death).

Precipitation was also analyzed by microscopy. The visualization of small precipitates was made possible by the lipophilic red-fluorescent tracer DiA. This technique appeared less sensitive than light scattering. Visible precipitates appeared from 15 μM Celecoxib in serum-free medium, 18–20 μM in 0.5% serum, 40–50 μM in 10% serum, and increased quite sharply with Celecoxib concentration (Fig. 2B–E; Supplementary Figs. S3B,C, S4, and not shown), while sporadic, well-structured crystals, were detectable at ≥25 μM in serum-free medium, ≥30 μM in 0.5% serum, ≥100 μM in 10% serum (Supplementary Figs. S2 and S3E), if analyzed within the first 40–48 h.

Altogether, these approaches identified the existence of two levels of precipitation: an absolute limit of solubility, detected by sensitive techniques, and a threshold of gross precipitation (Supplementary Figs. S3E and S3 legend). Notably, precipitation (and toxicity) overlapped in different cell culture media and solutions (Supplementary Table S1), while serum was the main variable affecting not only the solubility of Celecoxib but also composition and shape of the precipitates, which contained 4–8% proteins in high serum (see also Supplementary Figs. S2 and S3, and relative legends).

Remarkably, when plotted against the toxicity curves, these data confirmed the existence of a correlation between solubility and toxicity of Celecoxib (Supplementary Fig. S5), and a quite striking correspondence between gross/visible precipitation and fast induction of cell death (Fig. 2D–E).

PRECIPITATES HAVE A CAUSAL ROLE IN CELECOXIB TOXICITY

For precipitate-dependent toxicity to occur, precipitates should not only seed onto the cells but interact with the plasmamembrane, leading to damage of the latter or to cellular reactions that activate death-signaling pathways. In alternative precipitates can be

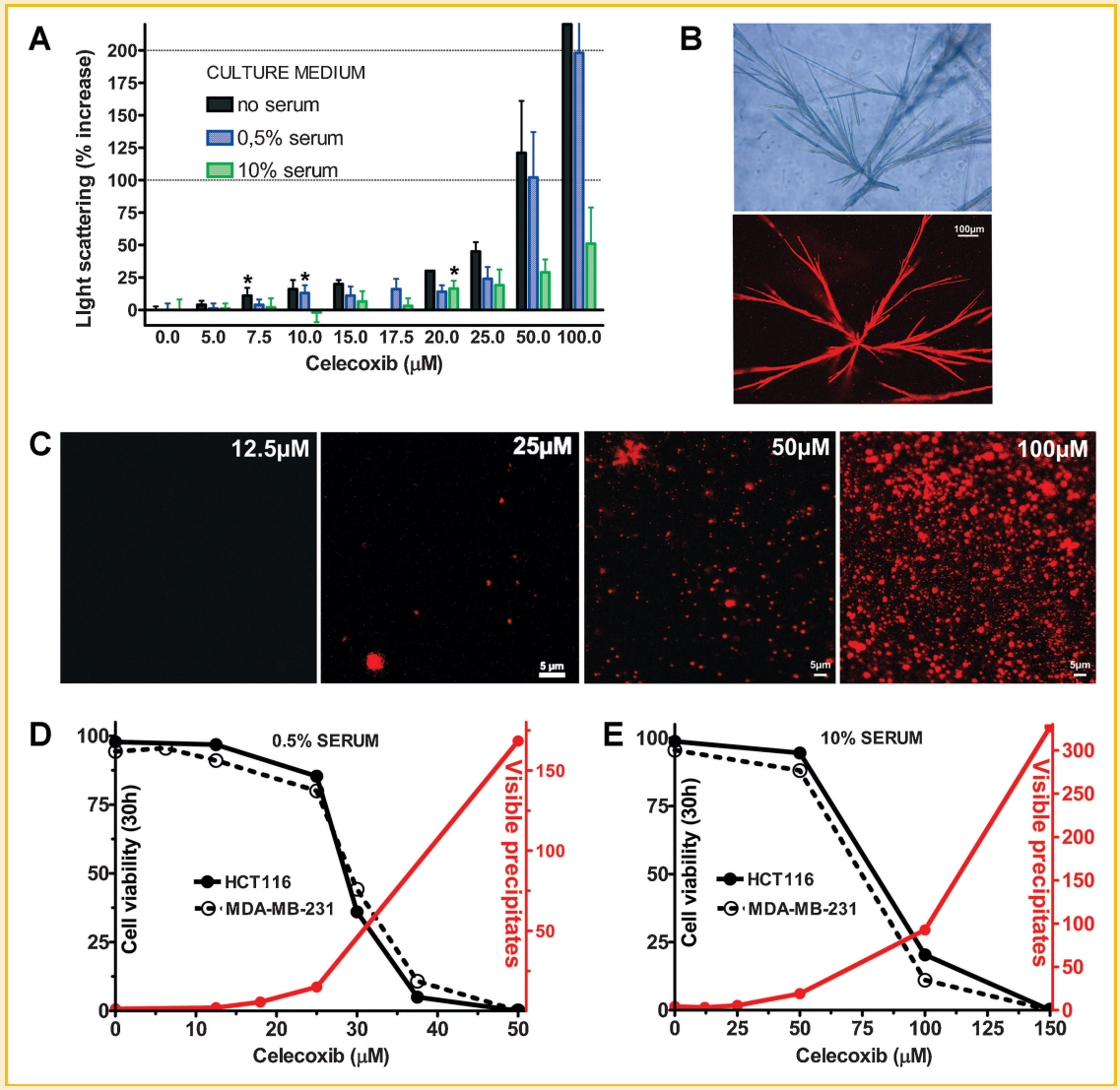

Fig. 2. Analysis of the solubility of Celecoxib and of the correlation precipitation–toxicity. A: Light scattering analysis of solubility in culture medium at different serum concentrations; (B) Celecoxib crystals (100 μM, 16 h) observed by normal light and fluorescent microscopy using the red-fluorescent lipophilic tracer DiA (0.5 μM); (C) fluorescent visualization of Celecoxib precipitates left to seed onto glass coverslips for 2 h, with the red lipophilic tracer DiA. DiA, dimly fluorescent in aqueous solutions, strongly fluoresces upon insertion into lipophilic structures. At the concentrations used, DiA did not interfere with precipitation and toxicity of Celecoxib (not shown); (D,E) correlation between induction of cell death and average number of visible precipitates counted in low (D) and high (E) serum measured in 100 × 100 μM squares indicates a striking correspondence between visible precipitation and toxicity (see also Supplementary Figs. S3B,C and S4)

internalized and damage intracellular structures [Ewence et al., 2008; Pedraza et al., 2008]. The interaction precipitates-cells was analyzed with a confocal microscope, using red-labeled precipitates (DiA) and green labeled cells (CFSE or DiO). Confocal analysis showed that Celecoxib precipitates, consistently with their lipophilic nature, were able to quickly and strongly stick to the cells, and were not significantly detached by washing (Fig. 3A–C, Supplementary Fig. S6). This demonstrated the presence of spots of irreversible alteration/damage of the plasmamembrane, which well fitted with the evidence that even a short exposition to Celecoxib can irreversibly prime cells to death (Fig. 1E,F). The capacity of Celecoxib precipitates to damage cellular membranes was particularly evident in the images obtained with 150 μM Celecoxib, showing brutal disruption of the cellular architecture (Fig. 3C). The latter well fitted with the almost immediate loss of membrane integrity observed at this concentration of the drug (Fig. 1E and F legend). In 10% serum a similar interaction precipitates-cells was observed, at the corresponding concentrations. Overall, these pictures strongly pointed to the association between Celecoxib toxicity and membrane damage by precipitates.

The causal relationship precipitation-toxicity was further demonstrated through additional and independent approaches. Filtration through 0.2 or 0.45 μm filters, which retained most of the visible precipitates, almost totally prevented cell death (Fig. 4A, Supplementary Fig. S7A). Similarly, the toxicity was progressively lost during the permanence of Celecoxib solutions in plastic tubes or cell culture plates, following precipitate binding to the plastic or to the cells (Supplementary Fig. S7B), which were

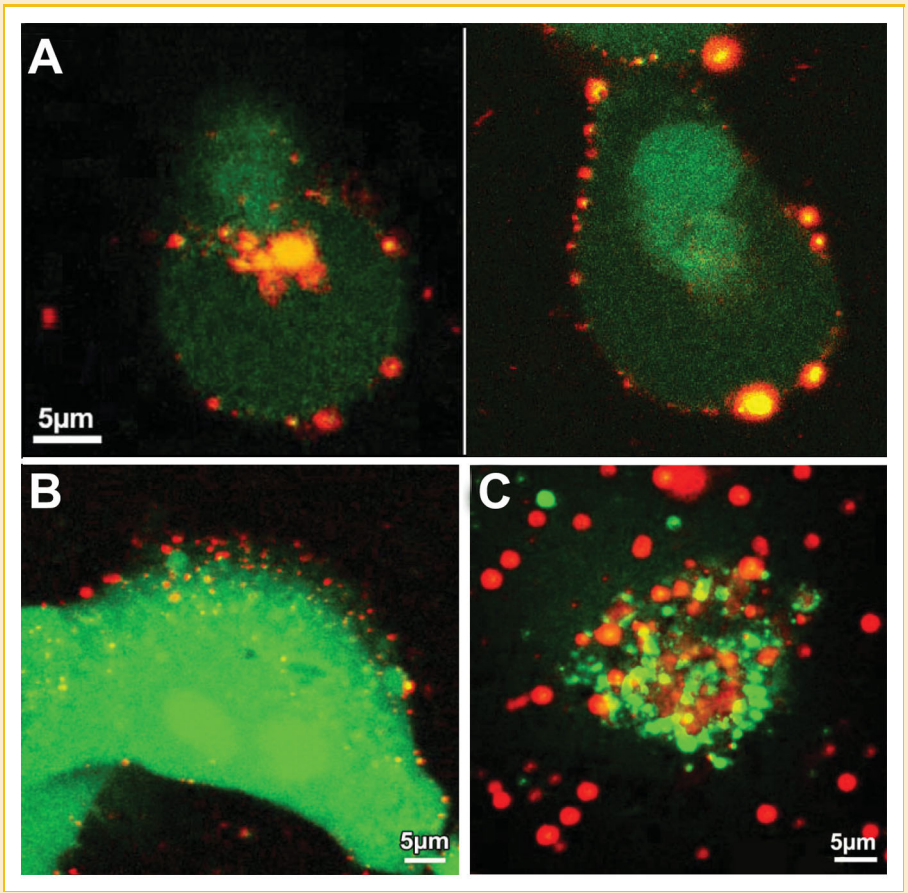

Fig. 3. Confocal analysis of the interaction cells-precipitates (MDA-MB-231). A: Confocal sections (apical and medial) of a cell treated with 100 μM Celecoxib + 0.3 μM DiA in low serum for 20′ and washed; (B) confocal section of CFSE-stained cells (green) treated with 50 μM Celecoxib + 0.15 μM. DiA for 45 min and washed; (C) cell stained with the green lipophilic dye DiO (Invitrogen; 1 μM × 20′ before plating) treated for 20′ with 150 μM Celecoxib + 0.5 μM DiA. For more details see Supplementary Figure S5.

particularly efficient in clearing Celecoxib solutions from toxic precipitates. Moreover, if the solubility of the drug was increased with alkaline pH or organic solvents ([Seedher and Bhatia, 2003] and Supplementary Table S1), this resulted in decreased toxicity, despite the intrinsic toxicity of the procedures used (Fig. 4B–D, Supplementary Fig. S7C,E), while a decrease of the solubility increased the toxicity (Supplementary Fig. S7D). Consistently with the precipitate-dependent model of toxicity, not only the presence of precipitates but also their stickiness appeared essential for the toxic effect. In fact, if precipitates were formed in serum-free medium with Celecoxib 50 and 100 μM and then 10% serum was added, precipitates were not quickly or significantly dissolved, but their adhesiveness was greatly reduced, and cell death significantly delayed (100 μM) or prevented (50 μM; not shown). Moreover, the toxicity was faster on cells in suspension or after repeated agitation of the medium, conditions that favor the encounter precipitates-cells: at 3 h 50 μM Celecoxib killed 70–85% of the cells versus 40 ± 16% in standard conditions.

Taken together, all these evidences strongly supported a causal relationship between precipitation and toxicity, suggesting that cell death is mainly produced by precipitates >0.2 μm, while smaller precipitates can still cause some growth inhibition.

PRECIPITATE-INTERNALIZATION IS ONLY MARGINALLY INVOLVED IN CELL DEATH

Internalization by the endo-lysosomal system can be activated by the cells in order to remove the damaged spots of the plasmamembrane and preserve its integrity [Idone et al., 2008] but can result in damage of the system and intracellular release of lysosomal content. Since internalization is involved in the induction of cell death by calcium phosphate and calcium oxalate crystals [Ewence et al., 2008; Pedraza et al., 2008], this hypothesis was also tested with Celecoxib precipitates, whose internalization was suggested by confocal analysis (Fig. 3B, Supplementary Fig. S6D). However, NH_4Cl (10 mM), which inhibits lysosomal acidification, was totally ineffective, while NaN_3 (0.1–0.2%), used as inhibitor of endocytosis, was only weakly active (Supplementary Fig. S7F and relative legend). These data suggested only a minor role, if at all, of precipitate internalization in Celecoxib toxicity.

MEMBRANE ACCUMULATION OF CELECOXIB IS NOT "PER SE" CAUSE OF TOXICITY

Since membrane accumulation of Celecoxib ([Maier et al., 2009] and unpublished data) could produce toxicity by interfering with the normal membrane structure/function or causing intracellular

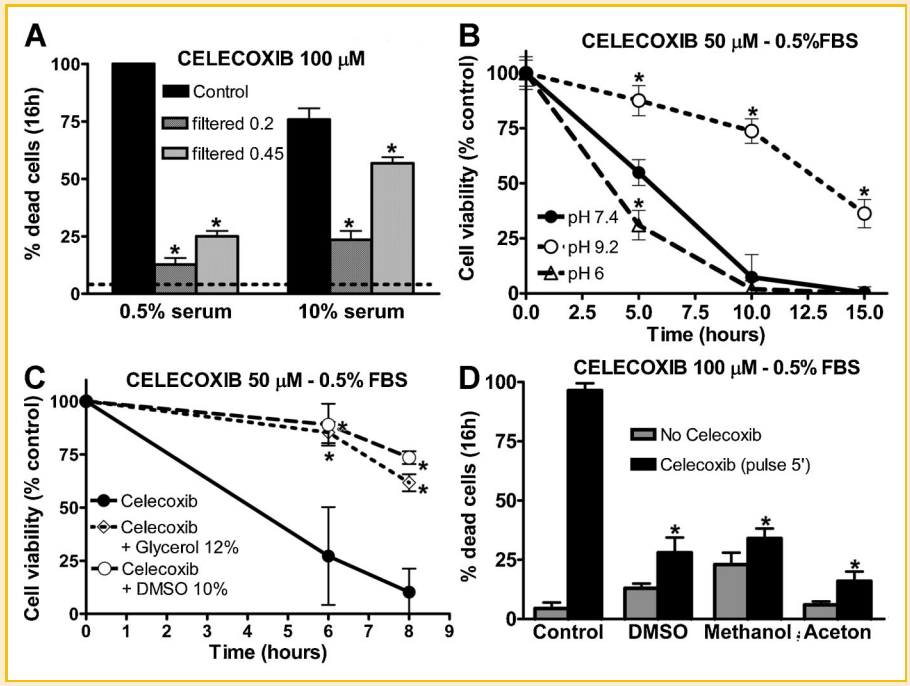

Fig. 4. More evidences in favor of the causal relationship precipitation–toxicity. A: Filtration through 0.2 and 0.45 μm filters significantly reduces cell death in 0.5% and 10% serum. The dashed line indicates basal cell-death in untreated cells. For technical details see Supplementary Figure S7 and relative legend; (C) at alkaline pH higher solubility ([Seedher and Bhatia, 2003] and Supplementary Table S1) significantly reduces Celecoxib toxicity, while at acidic pH the toxicity is slightly increased. Viability is indicated as % of the viability of the respective control cells (see Supplementary Fig. S6C); (D) time course of cell death with 50 μM Celecoxib in the presence of 10% DMSO or 12.5% Glycerol, expressed as % of the viability of the respective control cells. At 8 h viability was 96% (control medium), 35% (10% DMSO), 48% (12.5% Glycerol); (E) effect of organic solvents (DMSO 20%, Methanol 20%, Aceton 10%) on the toxicity of 100 μM Celecoxib applied for 5 min. Notably, all these solvents by increasing Celecoxib solubility ([Seedher and Bhatia, 2003] and Supplementary Table S1) significantly reduced its toxicity, despite their own toxicity.

precipitation, this point was preliminarily analyzed. Cells were repeatedly exposed to high volumes of fresh Celecoxib-containing medium, to push cellular accumulation (unpublished observations). However, no significant increase of cell death was observed if Celecoxib was below the threshold of toxic precipitation, nor membrane-associated precipitates were detected, while higher toxicity was observed after repeated exposition to a low but appreciable density of toxic precipitates (25 μM Celecoxib; Supplementary Fig. S7G).

LONG TIMES OF INCUBATION AT LOW CELL DENSITIES CAN LOWER THE THRESHOLD OF TOXIC PRECIPITATION

Since a few authors reported toxicity at lower concentrations of Celecoxib than those observed here (Supplementary Fig. S8 legend), and this was potentially in favor of precipitate-independent effects, I analyzed the experimental conditions used in those papers. Notably, toxicity with low concentration Celecoxib was observed at longer time points and lower cell densities than those considered here. Thus I hypothesized the involvement of delayed formation of toxic precipitates, by precipitation of super-saturated Celecoxib and aggregation of very small precipitates (Supplementary Fig. S4E). Preliminary analysis at ≥72 h indeed confirmed that some delayed precipitation occurred with 25 and 12.5 μM Celecoxib, together with increased toxicity (Supplementary Fig. S8B,C), to which also contributed precipitate-coating of the plates (not shown). However, this occurred only at very low cell density (≤20,000/ml). At higher cell density, when supersaturated Celecoxib was depleted by membrane accumulation ([Maier et al., 2009] and unpublished observations), late precipitation/toxicity did not occur (Supplementary Fig. S8B,C). Moreover, cell death was never observed with Celecoxib 6 μM (soluble by light scattering), unless extreme or non-accurate experimental conditions were used, which included relevant evaporation of the medium and non-accurate dissolution of the drug from DMSO stocks. These data confirmed the association precipitation-toxicity and further evidenced the risk of artifacts associated with the use of poorly soluble chemicals, in particular with very low cell densities and prolonged incubation times.

ANALYSIS AT PHYSIOLOGICAL LEVELS OF ALBUMIN INDICATES THAT TOXIC PRECIPITATION OF CELECOXIB IS TWO ORDERS OF MAGNITUDE FAR AWAY FROM PLASMA LEVELS REACHED DURING THERAPY

To simulate the conditions present in vivo, precipitation and toxicity of Celecoxib were also tested in pure serum and at physiological levels of albumin, the main plasma protein involved in Celecoxib-binding [Seedher and Bhatia, 2006]. In pure serum (FCS with total protein content estimated 42.5 mg/ml, and bovine serum albumin, BSA, 26 mg/ml, or 390 μM), cell death became visible only at 300 μM, and the threshold of massive cell death was 400 μM (Fig. 5). Insolubility was around 150 μM and visible crystals, indicators of

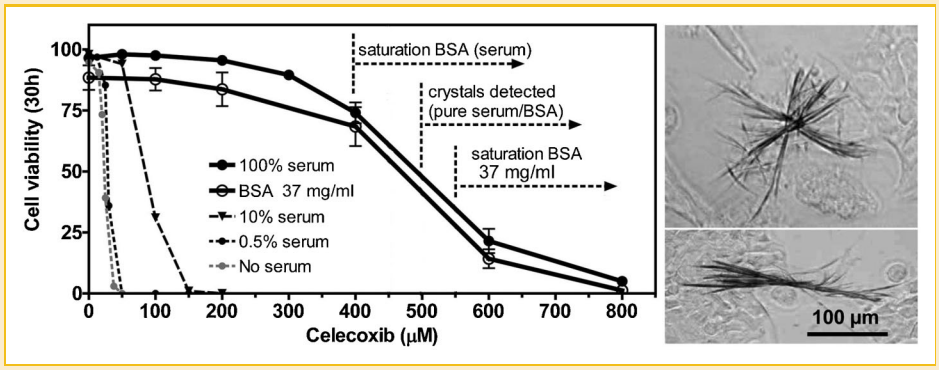

Fig. 5. Toxicity of Celecoxib in pure serum and at physiological levels of albumin. Right: Effect of Celecoxib on the viability of HCT116 in pure serum (FCS) and in medium with 37 mg/ml albumin (BSA, Sigma), compared with lower serum levels. Similar results were obtained with MDA-MB-231 and with human albumin (not shown). Notably, cell death occurs in the proximity of full albumin saturation according to a stoichiometry 1:1 [Seedher and Bhatia, 2006], that is, when free Celecoxib reaches insolubility. Left: Crystals of Celecoxib in pure serum and albumin 37 mg/ml.

gross precipitation, appeared in concomitance with massive cell death, further confirming the association precipitation-toxicity. A similar dose response was observed with albumin 37 mg/ml (Fig. 5), that is, on the low side of the physiological range in humans (range 36–50 mg/ml = 530–750 μM [Seedher and Bhatia, 2006]). This confirmed that albumin is the main responsible of Celecoxib binding and inactivation in serum, albeit 1/3 of the total capacity to inactivate Celecoxib can be produced by other serum components. Interestingly, toxic precipitation occurred in concomitance with saturation of albumin, thus correlating with the levels of free Celecoxib (Fig. 5) [Seedher and Bhatia, 2006]. The eventuality that serum or albumin solutions containing soluble Celecoxib could transfer toxic precipitation to other solutions, tissues or cells was also preliminary tested and excluded. Repeated exposure to Celecoxib (up to 200 μM) in serum or albumin did not cause precipitation on cellular membranes or matrigel, while no precipitation was observed in ultrafiltrates from these solutions (centricon tubes 10 kDa, Millipore).

Overall, these data indicated that the effective threshold of Celecoxib precipitation and toxicity is about two orders of magnitude higher than the concentrations occurring in plasma during therapy [Paulson et al., 2000], and confirmed that in all the tissues that receive Celecoxib from blood, toxic precipitation has no chances to occur.

PRELIMINARY ANALYSIS OF CELECOXIB IN A MODEL OF GASTRIC TOXICITY

Preliminarily tested on the AGS gastric adenocarcinoma cells, commonly used to study the gastrointestinal toxicity of chemicals in vitro, Celecoxib toxicity overlapped with that observed on the other cell types, with slightly faster kinetic: Celecoxib 100 μM killed 100% of the cells in 25 min, 50 μM killed 60–80% of the cells in 3 h, >95% in 5 h. Twenty-five micromolar Celecoxib produced only delayed cell death. In synthesis, in the absence of protective mucus Celecoxib was confirmed to be highly and quickly toxic to gastric epithelial cells in vitro [Tanaka et al., 2005].

PRELIMINARY EVIDENCES THAT GASTRIC MUCUS AND THE GASTROINTESTINAL BARRIER CAN EFFICIENTLY BLOCK CELECOXIB PRECIPITATES

The capacity of gastric mucus to block toxic precipitates was also preliminarily analyzed. Interestingly, gastric mucin in solution showed the ability to inactivate Celecoxib with an efficiency 25% of that of BSA, and even a tiny and loose mucin film, residual from incubation of cells with a 2% mucin solution, was sufficient to protect cells from 50 μM Celecoxib for several hours (at 30 h cell death was 48 ± 12% vs. 100% in control cells). Moreover, preliminary evidences obtained with ex vivo mouse stomachs, indicated that Celecoxib precipitates (red stained with DiA) were completely blocked by the tight mucus layers (not shown), as predicted by the low mesh size [Phillipson et al., 2008]. This confirmed that gastric mucus (and in general gastrointestinal mucus) can prevent toxicity to the epithelium and confine precipitates to the luminal content. The action of the gastrointestinal epithelium as further barrier in preventing systemic delivery of precipitates was tested in vitro using monolayers of cells (HCT116, AGS) grown on transwell membranes of 3 μm pore size (Corning) [Nigsch et al., 2007]. The latter efficiently blocked toxic precipitates, thus confirming and extending the evidences shown in Supplementary Figure S7B.

DISCUSSION

THE TOXICITY OF CELECOXIB IS PRECIPITATE-DEPENDENT

In an attempt to define the mechanisms involved in in vitro cancer cell killing by Celecoxib, I performed a preliminary analysis of cell growth and death using different cancer cell lines. The latter well fitted with previous studies [Williams et al., 2000; Jendrossek et al., 2003; Kulp et al., 2004; Tomisato et al., 2004a; Tanaka et al., 2005; Fukada et al., 2007; Schonthal, 2007; Chuang et al., 2008; Maier et al., 2009; Rudner et al., 2010; Schiffmann et al., 2010; Jendrossek, 2011; Reed et al., 2011] and confirmed the COX-independency of Celecoxib toxicity. However, remarkably, the formation of crystals in cultures of cells killed by Celecoxib, and the dramatic sharpness of

the dose-response, suggested a correlation between precipitation and toxicity of the drug, leading to further investigation of this correlation and thus to a change of the focus of the entire study.

Notably, precipitation is a potential confounding factor in the analysis of the biological effects of many chemicals in vitro [Pan et al., 2001; Alsenz and Kansy, 2007]. First, it is cause of nonlinear dose-responses. Second, precipitates can damage the cells and/or induce cellular reactions and signaling unrelated with the molecular actions of the substances under study [Morgan et al., 2001; Pan et al., 2001; Morgan and McCarthy, 2002; Alsenz and Kansy, 2007; Nigsch et al., 2007; Ewence et al., 2008; Pedraza et al., 2008]. From this perspective, studies specifically dedicated to precipitate-dependent signaling and toxicity include calcium phosphate and calcium oxalate crystals [Ewence et al., 2008; Pedraza et al., 2008], β-amiloid aggregates [Finder and Glockshuber, 2007], transfection reagents, and more in general lipid aggregates as liposomes and micellae [Mayhew et al., 1987]. Third, most of the precipitate-dependent effects observed with a drug in vitro are unlikely to occur after gastrointestinal filtration and systemic absorption, and thus are mostly confined to in vitro observations or artifacts [Nigsch et al., 2007]. For all these reasons, solubility analysis is a standard preliminary step in drug development [Pan et al., 2001; Alsenz and Kansy, 2007; Nigsch et al., 2007].

With a limit of solubility reported between 2.6 and 18 μM ([Seedher and Bhatia, 2003] and Supplementary Table S1), Celecoxib is well known to be scarcely water-soluble. However, most of the in vitro effects on growth and viability have been observed between 10 and 100/200 μM, that is, in a potentially insoluble range, without solubility verification. Thus, precipitation of Celecoxib was here accurately analyzed and correlated with its toxicity. Taking into account the variability of solubility measurements, the present data well fitted with previous studies (Supplementary Table S1), and for the first time defined the solubility of Celecoxib in cell culture media with serum. Remarkably, the analysis highlighted a striking correlation, on a molar basis, between precipitation and toxicity. The causal relationship between these phenomena was successively demonstrated using independent strategies to observe the interaction precipitates-cells, to keep out precipitates from the medium, to reduce their stickiness, or to enhance Celecoxib solubility. Overall, these data strongly pointed to cell damage by precipitates as the effective reason of the toxicity of the drug, thus suggesting that the latter is devoid of intrinsic molecular toxicity, at least in the cell types and experimental conditions tested in the present study.

FROM PRECIPITATE BINDING TO CELL DEATH

Previous findings demonstrated that major membrane-permeability changes are the basic event in Celecoxib-induced cell death [Tomisato et al., 2004ab; Tanaka et al., 2005; Lichtenberger et al., 2006; Katsu et al., 2007]. Interestingly, besides an action on ion channels, Celecoxib was demonstrated to directly permeabilize liposomes, through the formation of pores >0.67 nm [Tomisato et al., 2004a; Tanaka et al., 2005; Katsu et al., 2007]. Since such pores are incompatible with viability, these data clearly evidenced that, independently of intracellular signaling or membrane channels/transporters, Celecoxib can directly induce loss of membrane integrity and thus cell death. Remarkably, the present analysis well fits with those data, and points to precipitate-dependent membrane damage as the cause of membrane permeabilization. In fact, liposome permeabilization is reported with EC20 of 20 μM, EC50 = 50 μM, that is, in coincidence with the onset of gross/visible precipitation (15–20 μM) in serum-free buffers. This is extremely unlikely to be a simple coincidence. In fact, I clearly show that Celecoxib precipitates possess membrane-damaging properties, while efficient and irreversible binding of precipitates to liposomes was confirmed by microscopy (unpublished observations).

Compared with liposomes living cells have more complex and resistant membranes, and also possess reaction and repair mechanisms that counteract membrane damage [Idone et al., 2008; Keyel et al., 2011]. Confocal analysis showed that Celecoxib ≥100 μM can directly and brutally disrupt the cells, overcoming their resistance, and this was confirmed by the very quick permeabilization to viability dyes. However, when the insult is milder cells can activate repair mechanisms that counteract full permeabilization. Thus, depending on the size and number of damaged spots, this results in different timing and pattern of cell death or in membrane repair if the damage is not excessive. In this context, limited permeability changes and other reactions to the insult of precipitates have enough time to activate signaling elements that can lead to or contribute to cell death, for example, Ca++ ions [Tanaka et al., 2005].

In agreement with this model, and in line with previous findings [Tomisato et al., 2004a; Tanaka et al., 2005; Lichtenberger et al., 2006; Katsu et al., 2007], Celecoxib produced numerous membrane-permeability changes, which better fitted with direct membrane permeabilization than pharmacological actions on channels/transporters (unpublished results). However, at variance with previous studies [Tanaka et al., 2005] neither Ca++ signaling, implicated in both apoptosis and necrosis, nor other ions appeared essential to cell death (unpublished results). Moreover, in contrast with a current opinion, I have found that Celecoxib does not induce specific apopotic signaling. Morphological features of apoptosis and limited caspase activation can only be observed in selected experimental conditions (unpublished results). All these evidences well fit with necrotic-like cell death directly resulting from extensive/irreparable membrane damage [Babiychuk et al., 2011]. In other words, if unable to repair the damage, cells lose their integrity and die, while apoptotic and other death signaling pathways are not essential for cell death to occur.

Albeit interesting, the analysis of permeability changes ad death pathways was too complex to be included in the present article, which was designed to address very basic points: the involvement of precipitates in Celecoxib toxicity and the risk that the latter is confined to an in vitro artifact.

IMPLICATIONS OF THE PRESENT FINDINGS FOR WHAT CONCERNS THE GASTROINTESTINAL TOXICITY OF CELECOXIB

As generally accepted, the gastrointestinal side effects of NSADs are mainly caused by COX-1 inhibition, which blocks the prostaglandin-dependent production of protective mucus [Silverstein et al., 2000; Scarpignato and Hunt, 2010]. Despite this, COX-2 selective inhibitor, albeit safer than non-selective

NSAIDs, are not devoid of gastrointestinal toxicity ([Silverstein et al., 2000; Hippisley-Cox et al., 2005] and http://www.celebrex.com). Interestingly, besides COX inhibition, direct damage of the mucus layer and cytotoxicity to the gastrointestinal epithelia has been observed with different NSAIDs, Celecoxib included [Tomisato et al., 2004b; Lichtenberger et al., 2006]. At the light of the precipitate-dependent model, direct gastric toxicity by Celecoxib might be produced by precipitates. In fact, following oral assumption, the upper gastrointestinal epithelia can be transiently exposed to very high luminal concentrations of partially dissolved drug. In the stomach, taking into account a luminal volume between 100 ml (including some water to swallow the capsule) and 1 L [Sherwood, 1977] and Celecoxib capsules of 200–400 mg [Brautigam et al., 2001; Maier et al., 2009], the average concentration of the drug can peak 0.5–10 mM, which is compatible with the formation/presence of toxic precipitates. In favor of this is also the evidence that direct gastric cytotoxicity by NSAIDs has a relevant exception in Rofecoxib [Tomisato et al., 2004b], which is non-toxic in vitro ([Kazanov et al., 2004; Schiffmann et al., 2008], and Supplementary Fig. S1D) and whose precipitates have low affinity for the cellular membranes (unpublished observations). However, since precipitate-dependent toxicity requires direct contact of the cells with insoluble Celecoxib, a major limiting factors for luminal toxicity is the presence of a protective layer of mucus ([Phillipson et al., 2008] and see Results 3.9 Section). Toxicity might only occur in spots of higher vulnerability where the protective mucus is weakened by COX-1 inhibition, preexisting lesions, or incipient damage ([Tomisato et al., 2004b; Lichtenberger et al., 2006; Scarpignato and Hunt, 2010] and www.celebrex.com). Moreover, toxic precipitates are probably inactivated (at least partially) by a dense luminal content after a meal. On the other hand, low pH and other factors, among them bile acids, can potentiate the toxicity of Celecoxib precipitates ([Lichtenberger et al., 2006; Scarpignato and Hunt, 2010] and unpublished observations). Overall, the identification of the factors that can cooperate with or protect from precipitate-dependent toxicity, might allow reducing the gastrointestinal side effects. This makes the precipitate-dependent hypothesis an important base for future studies on the gastrointestinal toxicity of Celecoxib, which might be extended to other NSAIDs.

IMPLICATIONS OF THE PRESENT FINDINGS FOR WHAT CONCERNS THE ANTITUMOR EFFECT OF CELECOXIB

Taken together, the present data suggest that, in contrast with one of the current opinions [Schonthal, 2007; Jendrossek, 2011], direct cytotoxicity by Celecoxib might not have a significant role in its antitumor effect in vivo, thus confirming previous criticism [Williams et al., 2000] and indicating that COX-dependent mechanisms might be the only effective antitumor players. In synthesis, if toxic precipitates can be formed in the upper gastrointestinal tract, they are unlikely to persist or be de novo generated after systemic absorption of the drug, or to be present/active in distal intestine.

In fact, in a standard therapeutic regime plasma levels of Celecoxib can transiently peak up to 4 μM, but are frequently comprised between 0.5 and 2 μM [Brautigam et al., 2001; Maier et al., 2009]. These concentrations, which mainly reflect accumulation by albumin binding [Seedher and Bhatia, 2006], are at least two orders of magnitude lower than the threshold of toxic precipitation in the presence of physiological levels of albumin and other plasma components (Fig. 5). Thus, in all the tissues where Celecoxib is delivered by blood and in equilibrium with it, toxic extracellular precipitation is extremely unlikely to occur (see Fig. 5 and relative Results Section). Even though limited accumulation occurs in some tissues, this is mainly due to cell-membrane binding, which does not produce "per se" precipitation or toxicity (see Results). In fact, the available biodistribution studies indicate that the concentration of Celecoxib in whole blood is about four times the plasma levels, reflecting about six times cellular accumulation in erythrocytes ([Paulson et al., 2000] and unpublished data). The peak concentration in other tissues is generally $\leq$5 times the plasma levels [Paulson et al., 2000], that is, lower or equal to total blood, thus suggesting that a simple equilibrium is reached with blood [Maier et al., 2009].

Notably, significantly higher concentrations are reported in the stomach, directly exposed to luminal Celecoxib, which peaks 22 times the plasma levels [Paulson et al., 2000], with a possible gradient associated with even higher accumulation in the epithelium. Since gastric epithelial cells are highly sensitive to Celecoxib ([Tanaka et al., 2005] and present results) this should result in general and dramatic gastric toxicity, which quite surprisingly does not occur. In fact the overall incidence of gastrointestinal events with Celecoxib is surprisingly low both in men and experimental animals ([Silverstein et al., 2000] and http://www.celebrex.com). This indicates that toxicity is extremely unlikely to occur in other tissues, where Celecoxib levels are significantly lower. Notably, the evidence relative to the gastric epithelium further supports the precipitate-dependent model of toxicity and the non-molecular nature of the cytotoxic effect. Moreover, it confirms that simple cellular accumulation does not produce toxicity.

On the other hand, toxic precipitates in the luminal gastrointestinal compartment are unlikely to cross the gastrointestinal barrier, which acts as a very efficient system of filtration [Nigsch et al., 2007]. Moreover, blood vessels, plasma, and blood cells would act as further filters to inactivate and prevent systemic delivery of eventually escaped precipitates. Precipitates are also unlikely to be present or active in distal intestine. In colon, where tumor prevention is well documented, accumulation of Celecoxib is in fact equivalent to total blood, thus indicating delivery through blood and not by luminal content [Paulson et al., 2000; Lee et al., 2012]. Even in case residual insoluble Celecoxib is delivered to the distal intestine, precipitates would be inactivated by the dense luminal content and the mucosa would be protected by the mucus layer. Notably, the evidence that Celecoxib has antitumor properties in districts unequivocally reached after systemic absorption [Jendrossek, 2011] and that Rofecoxib is also tumor-preventive [Ashok et al., 2011; D'Arca et al., 2010], despite its negligible cytotoxicity in vitro ([Kazanov et al., 2004; Schiffmann et al., 2008] and Supplementary Fig. S1D) and in vivo [Tomisato et al., 2004b], confirms that direct tumor cell killing by NSAIDs is not necessary for tumor prevention to occur.

Nonetheless, indirect pro-apoptotic/necrotic actions of Celecoxib, caused by inhibition of pro-survival or pro-angiogenic

prostaglandins in sensitive tumor cells and micro-environments, or by other prostaglandin-dependent mechanisms [Cha and DuBois, 2007; Greenhough et al., 2009], can still occur in vivo but should not be confused with direct cytotoxicity.

CONCLUSIONS

In conclusion, by demonstrating the involvement of a precipitate-dependent mechanism of membrane damage the present article sheds new light on the cytotoxicity of Celecoxib on tumor cells in vitro. This finding challenges the hypothesis of the involvement of true pharmacological actions, and raises a serious concern about the occurrence of such a toxicity on tumor cells in vivo. Overall, the original analysis and criticism contained in this article open an important discussion, and represent an important basis for future studies on Celecoxib. True molecular actions of the drug should be, in fact, accurately discriminated from the precipitate-dependent ones, and the correspondence between in vitro and in vivo effects should be defined at the light of the precipitate-dependent model. Also studies that evaluate the interaction between Celecoxib and other drugs should take into account this model, since other chemicals might interfere with the precipitation of Celecoxib (e.g., co-precipitation) or with the binding precipitates-cells. Moreover, beyond Celecoxib this article defines a type of critical analysis that can be extended to other poorly soluble chemicals, to draw more reliable conclusions from in vitro studies with the latter.

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