The Raf kinase inhibitor BAY 43-9006 reduces cellular uptake of platinum compounds and cytotoxicity in human colorectal carcinoma cell lines

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Raf kinase plays a central role in oncogenic signaling and acts as a downstream effector of Ras in the extracellular signal-regulated (ERK) kinase pathway. BAY 43-9006 (BAY) is a novel signal transduction inhibitor that prevents tumor cell proliferation and angiogenesis through blockade of the Raf/MEK/ERK pathway at the level of Raf kinase and the receptor tyrosine kinases vascular endothelial growth factor receptor-2 and platelet-derived growth factor receptor-β. The present study evaluates the effects of combining BAY and platinum derivatives on human colorectal cancer cells using different incubation protocols. Our data show that the combination of oxaliplatin or cisplatin with BAY results in marked antagonism irrespective of the used application schedule. Furthermore, BAY abrogates the cisplatin-induced G_2 arrest as well as the G_1 arrest induced by oxaliplatin. BAY alone arrests cancer cells in their current cell cycle phase and affects cell cycle regulative genes. Specifically, BAY reduced the protein expression of $p21^{Cip1}$ as well as cyclin D1, and inhibits the expression of cdc2 (cdk1). Utilizing atom absorption spectrometry, BAY significantly reduced cellular uptake of platinum compounds and thereby the generation of DNA adducts. Taken together, co-incubation with BAY results in reduced cellular uptake of platinum compounds and consecutively reduced generation of DNA adducts, and eventually decreased cellular cytotoxicity in human colorectal cancer cells. Our results indicate that the Raf kinase inhibitor BAY 43-9006 might also directly or indirectly interact with platinum transporter proteins *in vitro*. *Anti-Cancer Drugs* 16:129–136 © 2005 Lippincott Williams & Wilkins.

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Introduction

Proliferation, differentiation, survival and apoptosis of all eukaryotic cells are controlled by a highly interactive network of protein kinases and other signal transducers [1,2]. Many receptor tyrosine kinases and cytokine receptors in association with G proteins are known to activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPKs) [1–3]. The Ras/Raf/MEK/extracellular signal-regulated (ERK) signal transduction pathway regulates cell survival and proliferation by transducing signals from growth factor receptors to transcription factors (Fig. 1) [1,3]. Raf activated by Ras phosphorylates and activates MEK1/2 [1–3]. These, in turn, phosphorylate and activate ERK1/2. Activated ERK1/2 directly phosphorylates and modulates the activity of transcription factors including Ets-1, c-Jun and c-Myc [1–3].

Experimental evidence supports a direct role for Raf kinase in the development and maintenance of human malignancies. For instance, the Raf kinases are the direct downstream mediators of the Ras proteins, which are mutated in approximately 30% of human solid tumor types [4,5]. Raf mutations, especially mutations of the B-Raf gene, were detected in a wide range of human tumors, e.g. in 66% of malignant melanomas [6]. Independent of its mutation status, Raf is also activated in tumor cells containing enhanced growth factor signaling pathways, such as those induced by mutant or constitutively expressed EGF receptor family members [5]. In addition, analysis of the transcriptional program induced by Raf in epithelial cells revealed autocrine activation of the EGF receptor to be responsible for the ability of Raf activation to protect transformed cells from apoptosis and is involved in cancerous cell proliferation [1,7].

Hence, the collective evidence suggests that Raf is a valuable anticancer drug target. BAY 43-9006 is a novel and potent small-molecule inhibitor of Raf kinase and the

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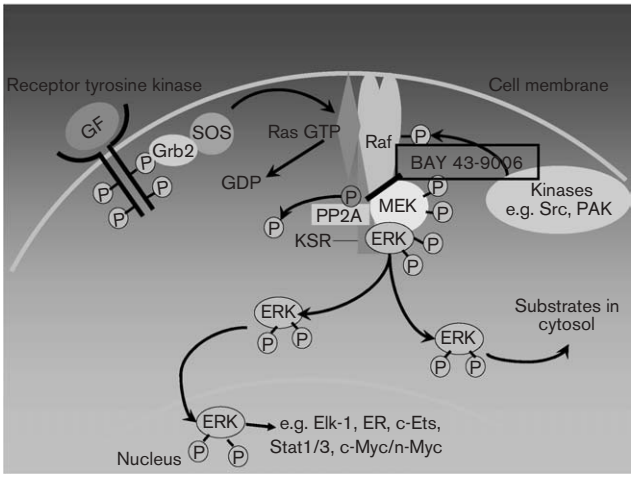
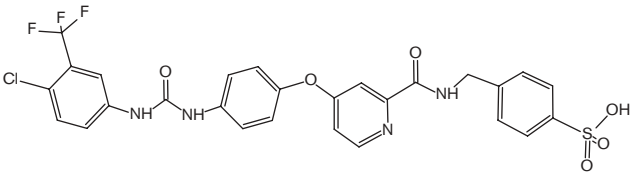
Fig. 1



Schematic representation of the Ras/Raf/MEK/ERK signal transduction pathway regulating cell cycle progression and apoptosis. Extracellular signals (GF) interact with a receptor tyrosine kinase, which then stimulates the activity of Raf via the G-protein Ras. Raf activates MEK1/2, which in turn activate ERK1/2, which eventually phosphorylate a number of proteins that regulate cytoskeletal proteins, metabolism, chromatin remodeling and numerous transcription factors. BAY 43-9006 interacts with Raf and hence inhibits the kinase activity.

Fig. 2



Chemical structure of the Raf kinase inhibitor BAY 43-9006.

first compound in this class which entered clinical studies (Fig. 2) [8]. BAY shows significant antitumoral activity in numerous different human tumor types including colon, pancreatic, lung and ovarian cancer [9,10]. Moreover, anticancer activity has been shown in numerous tumor models with K-Ras mutations and in tumor models that express wild-type Ras, but overexpress growth factor receptors [9,10]. Clinical phase II/III studies with BAY in combination with chemotherapy agents are in progress.

The purpose of this study was to investigate a potential interaction of BAY in combination with platinum derivatives (cisplatin/oxaliplatin) in human colorectal cancer cells. In this way, three consecutive protocols, i.e. (i) cytotoxic drug first, then BAY, (ii) BAY first, then cytotoxic drug, and (iii) BAY and cytotoxic drug together throughout, were used. Furthermore, interaction studies were also performed on the molecular level including cell cycle analyses, cell cycle regulative proteins and evaluation of cellular platinum uptake as well as DNA adducts by flameless atomic absorption spectrometry (AAS) [11].

Methods and materials

Cell lines

The human colon carcinoma cell lines HCT8 and HT 29 were used in this study. Cells were grown in L15 medium (Biochrom, Berlin, Germany) together with 10% fetal calf serum in a humidified atmosphere at 37°C and 5% CO_2.

Antibodies

Monoclonal antibodies used were anti-β-actin, anti-$p21^{Cip1}$, anti-cdk4, anti-cdk6, anti-cyclin D1 and anti-phospho-cdc2 from Sigma-Aldrich (Steinbach, Germany). Anti-mouse and anti-rabbit peroxidase-conjugated antibodies were purchased from Amersham Biosciences (Freiburg, Germany).

SRB assay

Cell cultures were plated in 96-well microtiter plates (500–1500 cells/well) containing 100 µl of growth medium. Cells were pre-incubated with medium for 24 h. Oxaliplatin or cisplatin, respectively, in combination with BAY were added using three different schedules: (i) cytotoxic drug first, then BAY, (ii) BAY first, then cytotoxic drug, and (iii) BAY and cytotoxic drug together throughout. After drug incubation, cells were treated with 10% trichloroacetic acid (TCA) for 2 h at 4°C and thereafter washed with PBS. TCA-treated cells were stained with 0.4% (w/v) sulforhodamine B (SRB), dissolved in 1% acetic acid, for 30 min. SRB was removed and cells washed with 1% acetic acid to remove unbound dye. After incubation with 10 mM unbuffered Tris base (pH 10.5) for 5 min on a plate shaker, OD was measured at 564 nm using a UVmax microtiter plate reader (MRXII; Dynex, Chantilly, VA).

Cell cycle analysis

Cell cycle analyses were performed using flow cytometry. Cells were grown in L15 medium for 24 h. Then medium was replaced by fresh medium containing oxaliplatin (30 µM final concentration) or cisplatin (10 µM final concentration) ± BAY (range 4.0–24 µM final concentration), or with BAY alone (range 4.0–70 µM final concentration). After incubation for 24 h, cells were washed and cell cycle analysis was performed after an additional incubation with either BAY or control medium for 4, 8, 11 and 16 h. A total of 1×10^6 cells were lysed utilizing DNA-Prep ($< 0.1\%$ potassium cyanide, $< 0.1\%$ NaN_3, non-ionic detergents, saline and stabilizers) and stained with DNA-Prep Stain (50 µg/ml propidium iodide, RNase [type II-A, bovine pancreas (4 kU/ml)], $< 0.1\%$ NaN_3, saline and stabilizers). Samples were analyzed by FACS within 30 min after preparation.

Cell lysis and protein quantification

Cells were incubated with oxaliplatin (30 μM final concentration) or cisplatin (10 μM final concentration) with or without additional BAY (4.0 and 24 μM final concentration). After incubation for 24 h, cells were harvested and incubated with lysis buffer composed of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin (3–7 TIU/mg) and 1.12 mM leupeptin for 30 min on ice. After brief sonification (3 × 5 s) and further incubation on ice for 10 min, extracts were centrifuged at 14 000 *g* for 20 min at 4°C. The amount of protein in the supernatant was determined using the advanced protein assay reagent (ADV01).

Western blotting

A total of 30 μg proteins were separated on 10% NuPage Bis-Tris–HCl buffered polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes. Membranes were saturated in PBS/Tween 20 with 10% non-fat milk and proteins were detected using the appropriate primary antibody at a dilution of 1:1000 and a secondary peroxidase-conjugated anti-mouse or anti-rabbit antibody at a dilution of 1:1000–1:10 000. Blots were developed using the ECL system (ECL-Plus Western blotting detection reagent; Amersham).

AAS

The amount of platinum-induced DNA adducts in human colon carcinoma cells was measured as described previously [11]. Cells were treated either with cisplatin or oxaliplatin (10 and 30 μM final concentration) for 4 h with concomitant or subsequent BAY incubation (24 μM final concentration). After washing the cells, DNA was isolated. Platinum concentration was measured by flameless AAS (SpectrAA-Zeemann 220; Varian, Darmstadt, Germany). The results reflect the total number of Pt atoms per 10^5 nucleotides including standard deviation (SD).

Cellular uptake of platinum in human carcinoma cells was measured according to [11]. Cells were treated either with cisplatin or oxaliplatin for 4 h with concomitant BAY incubation. The results reflect the total number of Pt atoms per 10^6 cells including SD.

Results

Isobologram analyses suggest antagonistic interaction of BAY with platinum compounds

In contrast to clinically used cytotoxic drugs including platinum compounds, BAY is supposed to act primarily cytostatically. Despite a different molecular mechanism of antitumor activity, BAY and platinum compounds showed similar IC_{50} values (8.9 μM for BAY, and 5.2 and 6.7 μM for cisplatin and oxaliplatin, respectively). To screen for potential molecular interactions between these compounds, we performed SRB assays using three different incubation schedules: (i) cytotoxic drug first, then BAY, (ii) BAY first, then cytotoxic drug, and (iii) BAY and cytotoxic drug together throughout.

Simultaneous incubation of BAY with platinum compounds resulted in either mild (oxaliplatin) or moderate (cisplatin) antagonism (Fig. 3A and D). To exclude a possible chemical interaction, we also used various consecutive incubation schedules (Fig. 3B, C, E and F). Independent of the schedule used, BAY markedly reduced oxaliplatin- and cisplatin-induced cytotoxicity. Together, isobologram analyses indicate antagonistic interaction of BAY in combination with oxaliplatin as well as cisplatin.

BAY abrogates platinum-induced cell cycle arrest

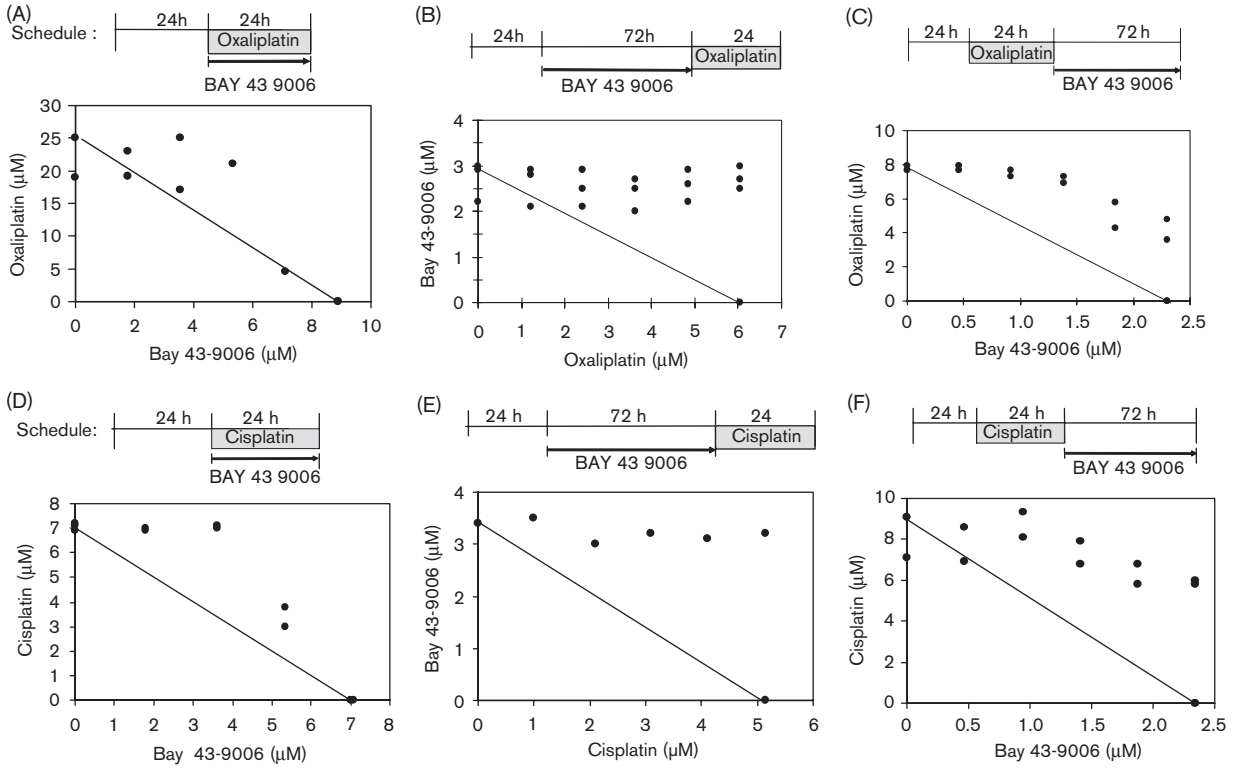
To further investigate potential interactions between BAY and platinum compounds, we performed cell cycle analyses in colon carcinoma cells using flow cytometry (Fig. 4). Incubation of cells with BAY alone arrested cells in their current cell cycle phase (data not shown). In contrast, oxaliplatin induced a G_1 arrest and cisplatin a G_2 arrest, respectively (Fig. 4A and B), which is in accordance with previous reports [12,13].

BAY did not show any effect on platinum-induced cell cycle arrest using consecutive BAY incubation protocols (Fig. 4C and D). In contrast, simultaneous BAY incubation (Fig. 4E and F) resulted in abrogation of both oxaliplatin-induced G_1 arrest and cisplatin-induced G_2 arrest. In addition, the minimum BAY concentration for abrogation of oxaliplatin-induced G_1 arrest was 2-fold higher (8 μM final concentration) compared to cisplatin-induced G_2 arrest (4 μM final concentration).

BAY reduced expression of $p21^{Cip1}$ and cyclin D1

To examine the molecular basis for the BAY-induced abrogation of platinum-induced cell cycle arrest, we performed Western blot analyses on cell cycle regulative proteins. Figure 5 shows that incubation with oxaliplatin (A, 30 μM final concentration) and (to a lesser extend) cisplatin (B, 10 μM final concentration) result in enhanced expression of $p21^{Cip1}$ (Fig. 5A and B, lane 2). Moreover, cisplatin (Fig. 5B) enhances protein expression of cdc2 and (although to a lesser extend) oxaliplatin the expression of cyclin D1 (Fig. 5A and B, lane 2). In contrast, concomitant treatment with BAY reduced (lane 4, 4.0 μM final concentration) or completely inhibited (Fig. 5, lane 5, 24 μM final concentration) the p21 protein expression. It is noteworthy, that a consecutive incubation schema (oxaliplatin/cisplatin first, then BAY incubation) did not show any effect on either p21 protein expression or cyclin D1 and cdc2 (cdk1), respectively

Fig. 3



Isobologram analyses on oxaliplatin and cisplatin in combination with BAY in HCT8 colorectal carcinoma cells. Three different incubation schedules were used: BAY and platinum compounds together throughout (A and D); BAY first, then platinum compounds (B and E); platinum compounds first, then BAY (C and F). Specific incubation schedules are illustrated on each panel.

(Fig. 5, lane 6). The expression of cdk4/cdk6 was not altered (data not shown).

BAY reduces cellular uptake of platinum compounds and the amount of DNA adducts

To investigate the interaction of BAY and platinum compounds on the molecular level, we determined the amount of cisplatin- and oxaliplatin-induced DNA adducts with or without concomitant or likewise consecutive BAY incubation (Fig. 6). DNA–platinum adducts were measured by AAS. Cells were treated with cisplatin (Fig. 6A, 10 μM final concentration) or oxaliplatin (Fig. 6B, 30 μM final concentration) for 4 h.

As expected, the amount of cisplatin-induced DNA adducts was significantly higher, compared to oxaliplatin (Fig. 6, column 1) [14]. For both platinum derivatives, a significant reduction of DNA adducts was detected when cancer cells were exposed to BAY simultaneously (Fig. 6, column 2). In contrast, no significant reduction of DNA adducts was measured using consecutive incubation schedules, i.e. incubation with a platinum compound first and thereafter incubation with BAY (Fig. 6, column 3). Furthermore, consecutive BAY incubation did not affect reversal/repair of DNA adducts for both cisplatin (Fig. 6A, column 3) and oxaliplatin (Fig. 6B, column 3).

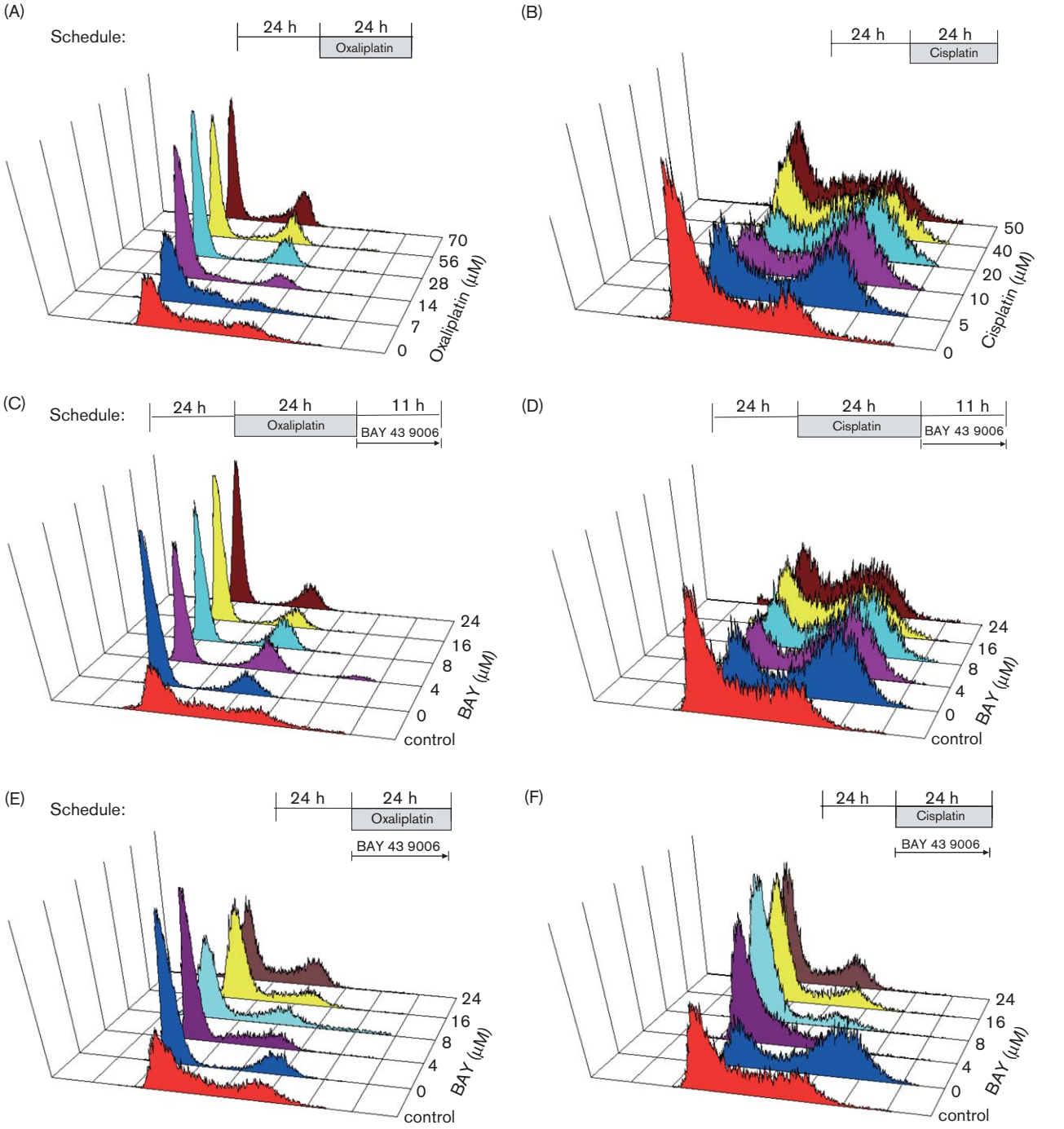
We next questioned whether the reduction of DNA adducts upon BAY co-incubation is the result of reduced cellular uptake of platinum compounds. Figure 7 shows that cellular uptake of cisplatin was significantly higher, compared to oxaliplatin. Co-incubation with BAY resulted in markedly reduced cellular uptake for both oxaliplatin (Fig. 7A) and cisplatin (Fig. 7B).

Taken together, BAY reduced cellular uptake of platinum compounds and generation of DNA adducts in human colorectal cancer cells, which eventually decreased cellular cytotoxicity.

Discussion

BAY is a novel dual-action Raf kinase and vascular endothelial growth factor receptor (VEGFR) inhibitor that prevents tumor growth by combining two anticancer activities: inhibition of tumor cell proliferation and tumor

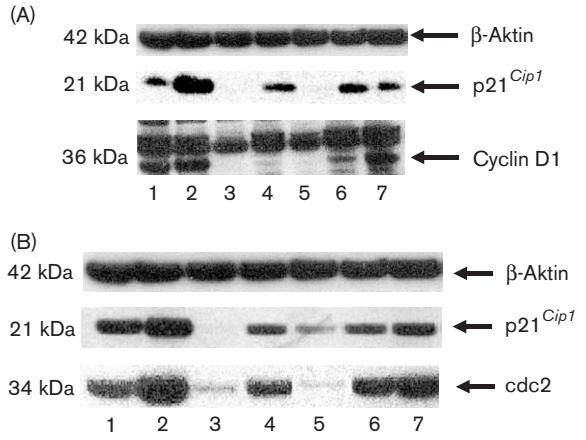
Fig. 4



Cell cycle analyses in colon carcinoma cells by flow cytometry. Cells were treated either with oxaliplatin or cisplatin for 24 h (A and B) or with subsequent BAY incubation for 11 h (C and D) or with simultaneous BAY incubation (E and F). Final concentrations were 30 μM for oxaliplatin and 10 μM for cisplatin, respectively. Control: no drug added. The incubation scheme is shown on top of each panel.

angiogenesis [9,10]. *In vitro*, reduced MEK and ERK phosphorylation was demonstrated without direct inhibition of MEK or ERK kinase activity [9]. BAY also inhibited phosphorylation and, therefore, activation of several receptor tyrosine kinases involved in angiogenesis and tumor progression, including VEGFR-2, VEGFR-3, platelet-derived growth factor receptor-β, Flt3 and c-Kit, as well as p38α, a member of the MAPK family [10]. BAY has demonstrated significant and broad activity against human tumor xenograft models of colon, pancreatic and

Fig. 5



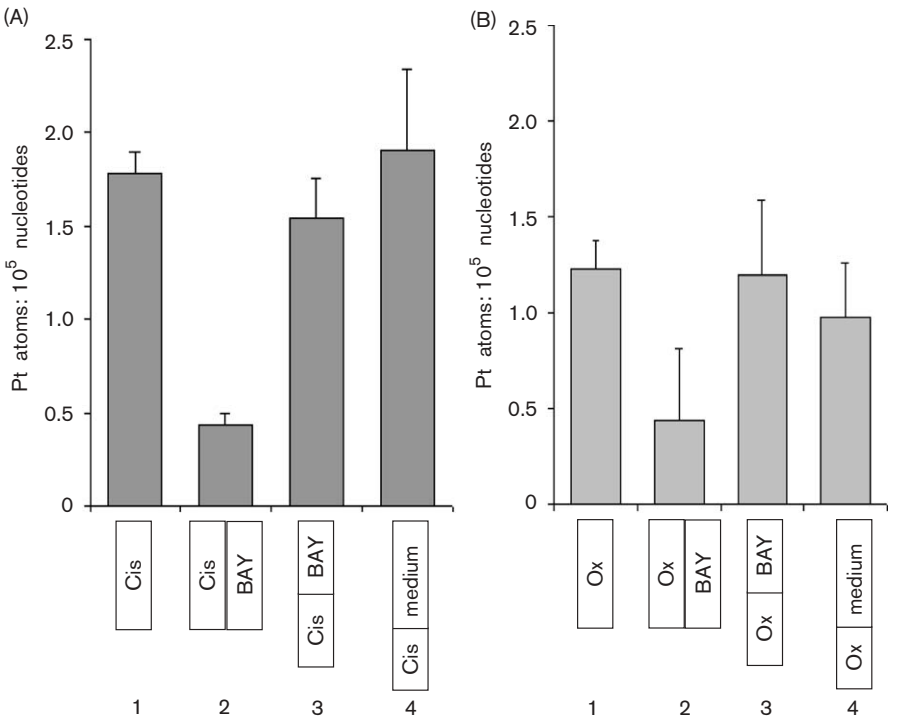
Western blot analyses of cell cycle regulative proteins. (A) Lane1, control; lane 2, oxaliplatin (30 μM final concentration); lane 3, BAY (24 μM final concentration); lane 4, oxaliplatin combined with BAY (4 μM final concentration); lane 5, oxaliplatin combined with BAY (24 μM final concentration); lane 6, oxaliplatin followed by BAY (24 μM final concentration); lane 7, DMSO. (B) Lane1, control; lane 2, cisplatin (10 μM final concentration); lane 3, BAY (24 μM final concentration); lane 4, cisplatin combined with BAY (4 μM final concentration); lane 5, cisplatin combined with BAY (24 μM final concentration); lane 6, cisplatin followed by BAY (24 μM final concentration); lane 7, DMSO.

non-small cell lung origin with mutations in B-Raf or K-Ras [9]. A number of clinical studies with BAY have already been finished. As well as showing preliminary antitumor activity, cumulative clinical experience is consistent in that the cytotoxicity profile of BAY is generally mild and different from clinically established cytotoxic drugs [15].

Platinum compounds like cisplatin and oxaliplatin are highly active anticancer agents. Cisplatin is curative against most testicular cancers and is highly active against a wide range of other tumor types, notably ovarian, bladder carcinoma and non-small cell lung carcinoma [16]. Oxaliplatin is part of first-line chemotherapy regimens in the treatment of colorectal cancer [17]. The distinct toxicity profile of BAY provides a rationale for combination therapy with platinum compounds.

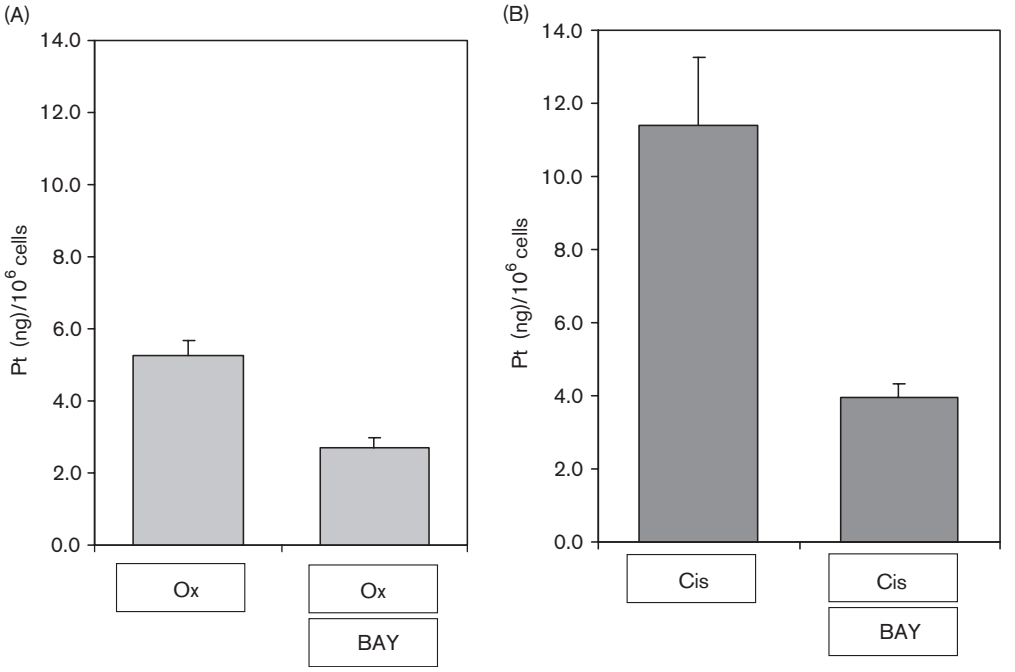
Our data suggest antagonistic interaction of cisplatin and oxaliplatin in combination with BAY on cellular level. The results of our studies indicate that antagonism is based on BAY-mediated reduction of cellular uptake of platinum compounds, causing a marked decrease of DNA–platinum adducts which eventually reduces platinum-mediated cytotoxicity.

Fig. 6



Amount of platinum-induced DNA adducts in human colon carcinoma cells measured by AAS. Cells were treated either with cisplatin (10 μM final concentration, A) or oxaliplatin (30 μM final concentration, B) for 4 h with concomitant or subsequent BAY incubation (24 μM final concentration). Results reflect the total number of Pt atoms per 10^5 nucleotides including SD.

Fig. 7



Cellular uptake of platinum in human carcinoma cells measured by AAS. Cells were treated either with oxaliplatin (30 μM final concentration, A) or cisplatin (10 μM final concentration, B) for 4 h with or without concomitant BAY incubation (24 μM final concentration). Results reflect the total number of Pt atoms per 10^6 cells including SD.

The action of cisplatin in cell killing is now well established [18]. Although cisplatin can react with a variety of cellular macromolecules, there is strong evidence that the most important target is DNA [18,19]. Cisplatin can form both intrastrand and interstrand DNA cross-links, with intrastrand purine:purine representing the majority of the adducts [19].

Our results indicate that BAY does not affect repair/reversal of DNA–platinum adducts. After formation of DNA adducts upon incubation with platinum compounds, BAY post-incubation did not enhance the 'off-rate' of platinum adducts.

As is the case with other anticancer agents, reduced accumulation of cisplatin is frequently observed in cisplatin-resistant cell lines. Several authors have suggested that decreased uptake of cisplatin is an important factor that can result in drug resistance [20]. Recently, Ishida *et al.* reported that CTR1, a high-affinity copper transporter, is the major protein for cisplatin uptake in yeast [21]. The final key point in the demonstration that CTR1 plays an important role in cisplatin influx was that *ctr1* mutations result in a reduction of cisplatin levels in yeast cells, but do not alter efflux of cisplatin [21].

The next obvious question is whether mammalian homologs of yCTR1 are also able to import cisplatin into cells and whether the mammalian homologs play an important role in intracellular cisplatin levels. Mammalian homologs of yCTR1 have been identified [22,23], in part, through their ability to complement yeast *ctr1* mutants. Interestingly, murine CTR1 plays an essential role in embryonic development; mouse embryos lacking mCTR1 die at mid-gestation, presumably from the lack of functioning of copper-dependent enzymes [24,25]. Ishida *et al.* used mouse cells carrying two, one or no functional copies of mCTR1, and assessed cisplatin sensitivity and drug accumulation [21]. Their results showed cisplatin accumulation and sensitivity was proportional to the number of functional CTR1 alleles [21]. Furthermore, homozygous *ctr1* mutant cells were 8-fold resistant to cisplatin and exhibited a 70% reduction in cisplatin accumulation [21]. These results clearly established CTR1p as a major factor in the uptake of cisplatin into mammalian cells and a potentially critical protein in cellular sensitivity to cisplatin.

The human CTR1 protein is localized to the plasma membrane and exists as a homomultimer [26]. The energy for translocation is unlikely to be directly derived from ATP hydrolysis, since the CTR1p lacks consensus motifs for ATP binding [27]. Hence, hCTR1-mediated copper transport is an energy-independent process, and is stimulated by extracellular acidic pH and high K^+

concentrations [26]. These data may reflect copper and platinum transport by a proton co-transport mechanism.

BAY is a small molecule inhibitor of Raf and other protein kinases [9,10]. It is not yet clear whether reduced cellular uptake of platinum on BAY co-incubation is based on direct interaction of BAY with CTR1p. Moreover, BAY-mediated inhibition of plasma membrane H^+-ATPase activity might dissipate the proton gradient necessary for cellular uptake of copper and platinum compounds.

The clinical significance of the reduced cellular uptake of platinum compounds on BAY co-incubation remains blurred. Since clinically achievable concentrations for both cisplatin and BAY are in general lower compared to the concentrations used in our experiments, antagonistic drug interaction might be clinically less significant. However, although BAY was selected for Raf kinase specific inhibition, our data indicate a possible collateral inhibition of other non-kinase proteins.

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