Relief of pain is an integral component of the care of cancer patients. The cancer as well as antineoplastic therapies can cause substantial pain (1, 2). For this reason, cancer patients are often treated concurrently with analgesics and antineoplastic drugs. However, the influence of analgesic agents on the antitumor activity of antineoplastic drugs, and the mechanism of any such effects, are largely unknown.

Cisplatin is one of the most widely used cancer chemotherapy agents. It is particularly effective in the treatment of testicular cancers and is also effective against carcinomas of many other tissues, including those of lung, head and neck, cervix, and ovary. Historically, cisplatin is thought to exert its primary cytotoxic effects via formation of platinum-DNA adducts, leading to G2 cell cycle arrest and apoptosis (3, 4). Recently, other mechanisms have come to light, including rapid formation of DNA-protein cross-links (5) and generation of reactive oxygen species and oxidative stress with a variety of apoptotic, mitochondrial, endoplasmic reticulum, and cell cycle consequences (6).

Gap junctions directly connect the cytoplasms of neighboring cells, thereby mediating direct intercellular movement of cytoplasmic signaling molecules. Almost every cellular and tissue-level process is affected by this communication pathway, including differentiation, migration, and apoptosis (7, 8). Many studies have shown that gap junctions play important roles in cancer biology and drug resistance (9, 10).

It has been reported that cisplatin toxicity is enhanced by the presence of functional gap junctions between the target cells (11). Also, gap junctions were shown to enhance induction of apoptosis induced by etoposide (an agent used with cisplatin in the standard combination bleomycin-etoposide-cisplatin antitumor agents). The role of gap junctions in the modulation of cisplatin toxicity is explored by manipulation of connexin expression, and gap junction presence and function, using clinically relevant concentrations of the analgesics and cisplatin.

Abstract

Purpose: Cancer patients are often concurrently treated with analgesics and antineoplastic drugs, yet the influence of analgesic agents on therapeutic activity of antineoplastic drugs is largely unexplored. This study investigates the effects of three commonly used analgesics, which produce analgesia by different mechanisms, on cytotoxicity induced by cisplatin, a widely used antitumor agent, and the relation between those effects and modulation of gap junction function by the analgesics.

Experimental Design: The role of gap junctions in the modulation of cisplatin toxicity is explored by manipulation of connexin expression, and gap junction presence and function, using clinically relevant concentrations of the analgesics and cisplatin.

Results: Short-term exposure of transformed cells to cisplatin reduced the clonogenic survival in low-density cultures (without gap junction formation) and in high density (with gap junction formation), but the toxic effect was greater at high density. In the absence of connexin expression or with block of connexin channels, cell density had no effect on cisplatin toxicity. Tramadol and flurbiprofen, but not morphine, significantly reduced cisplatin cytotoxicity, but this effect required functional gap junctions between the cells. Tramadol and flurbiprofen inhibited dye-coupling through gap junctions, but morphine did not.

Conclusions: The results suggest that the density dependence of cisplatin toxicity is mediated by gap junctions. They further indicate that tramadol and flurbiprofen depress cisplatin cytotoxicity through inhibition of gap junction activity, and more generally, that agents that depress junctional communication can counteract the effects of antitumor agents. (Clin Cancer Res 2009;15(18):5803-10)
Translational Relevance

Cancer patients are often treated concurrently with analgesics and antineoplastic drugs, yet the influence of the analgesic agents on the therapeutic activity of the antineoplastic drugs is largely unknown. This work investigates the effects of three such analgesics on the cytotoxic efficacy of the antineoplastic agent cisplatin. We find that a major component of the cytotoxicity of therapeutic levels of cisplatin is mediated by gap junctions. We find that two of the tested analgesics (tramadol and flurbiprofen) reduce cisplatin cytotoxicity by decreasing gap junction coupling, whereas another (morphine) had no effect on the toxicity or gap junctions. Thus, analgesics routinely provided concurrently with antineoplastic agents may compromise the effectiveness of those agents, depending on whether they affect junctional communication. This work provides a basis for rational choice of analgesics in chemotherapy. It also suggests that up-regulation of gap junctions may be used to increase the efficacy of anticancer chemotherapies.

Materials and Methods

Materials. Cisplatin, tramadol, flurbiprofen, and 18-α-GA were from Sigma. Morphine was from First Pharmacy Company of Shenyang, G418, hygromycin, and doxycycline were from Calbiochem. Calcein-acetoxyethyl ester and CM-DiI were from Molecular Probes. Cell culture reagents were obtained from Invitrogen. All other reagents were from Sigma unless stated otherwise.

Cell lines and cell culture. The HeLa cell line expressing Cx32 under the control of a bidirectional tetracycline-inducible promoter was described and characterized previously (18). In this cell line, the Cx32 coding sequence is followed by a sequence coding for a thrombin-cleavable COOH-terminal epitope tag consisting of a hemagglutinin (HA) epitope followed by a 6× (His-Asn) sequence. Connexin expression was induced with 1 μg/ml doxycycline for 48 h.

Cisplatin treatment, 18-α-GA treatment, and survival assays. Stock solutions of cisplatin were prepared freshly at 1 mg/ml in PBS. All exposures to cisplatin were done for 1 h in the dark. 18-α-GA was dissolved in DMSO at 4.7 mg/ml (10 mM/l), diluted to final concentration of 4.7 μg/ml (10 μM/l) in culture medium, and added to the cells either simultaneously with or 1 h before cisplatin treatment, as indicated. Cell survival was assayed by a standard colony-formation assay, adapted for use at high and low cell density, corresponding to conditions in which junctional channel formation was permitted or not, respectively (11). For the high-density condition, cells were seeded at 30,000 cells/cm² so that cultures were 70% to 100% confluent at the time of cisplatin exposure. At this density, there was substantial opportunity for formation of gap junctions; on average, each cell was in contact with three to five others. Cells were treated with cisplatin (0–2.5 μg/ml) for 1 h in the dark. Cells were washed with PBS, harvested by trypanization, counted, diluted, and seeded at 100 cells/cm² into six-well dishes. Colony formation was assessed 5 to 7 days later by fixation and staining with crystal violet. Colonies containing 50 or more cells were scored. For the low-density condition, cells were seeded at low density (100 cells/cm²) directly into six-well plates. At this density, the cells did not have opportunity to form gap junctions, as they did not contact each other, and treated with cisplatin for 1 h after attachment. They were rinsed and assessed for colony formation as above. Colony formation was normalized to the colony forming efficiency of noncisplatin-treated cells. Error bars in the survival analyses indicate SEM based on three independent experiments in all cases. There was no significant difference in plating efficiency between the low-and high-density cultures in the untreated samples and the act of trypanization did not contribute to cell killing by cisplatin (see Supplementary Figure).

“Parachute” dye-coupling assay. This assay for gap junction function was done as described by Goldberg et al. (19) and Kören et al. (18). Donor and receiver cells were grown to confluence. The donor cells were double-labeled with 5 μM/l CM-DiI, a membrane dye that does not spread to coupled cells, and 5 μM/l calcine-acetoxyethyl ester, which is converted intracellularly into the gap junction–permeable dye calcine. The donor cells were then trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The donor cells were allowed to attach to the monolayer of receiver cells and form gap junctions for 4 h at 37°C and then examined with a fluorescence microscope. For each experimental condition, the average number of receiver cells containing dye per donor cell was determined and normalized to that of control cultures. For the studies involving analgesics, the donor and receiver cells were exposed to the analgesics only during the 4- to 5-h period in which the donor cells were plated onto the receiver cell monolayer.

Western blotting. Whole-cell lysates for Western blotting were prepared by washing the cells thrice with cell wash buffer (0.01 mol/l PBS, 0.138 mol/l NaCl, 0.02% Na₂SO₄, pH 7.4) followed by a 2-h incubation in lysis buffer [50 mmol/l NaH₂PO₄, 50 mmol/l NaCl, 5 mmol/l EDTA, 5 mmol/l EGTA, 80 mmol/l N-tocet, β-n-glucopranoside, 1 mmol/l N-mercaptoethanol, 0.5 mmol/l L-di-isopropyl fluorophosphate (pH 7.5)] at 4°C using 0.05 ml/cm². For Western blotting, cell lysates (10 μg) or purified connexin preparations (10 μl) were separated.
Cytotoxicity of cisplatin is cell density dependent. Figure 1 illustrates the effect of short-term cisplatin exposure on survival of Cx32-expressing HeLa cells at low density (100 cells/cm²) and at high density (30,000 cells/cm²), assessed by colony formation. Treatment with cisplatin (from 0.1-2.5 μg/mL) for 1 hour reduced the clonogenic survival of cells at both low density and high density in a concentration-dependent manner. The toxic effect of cisplatin was substantially greater at high density, when the cells are in contact with one another, than at low density. The concentration of cisplatin used is within the therapeutic range reached in tissues during chemotherapy (20).

Density dependence of cisplatin response is mediated by GJIC. The cell density dependence of the cisplatin response in connexin-expressing cells suggests a possible role for intercellular communication mediated by gap junctions. To test the role of GJIC in cisplatin sensitivity, gap junction expression, and function were manipulated by two methods: lack of doxycycline induction of connexin expression (see Materials and Methods) and pharmacologic inhibition of junctional channels. The induction of connexin expression and dye coupling by doxycycline is shown in Fig. 2A and B, respectively. The effect of exposure of connexin-expressing cells to 4.7 μg/mL 18-α-GA, a membrane-permeable blocker of GJIC (21) is shown in Fig. 2B.

Figure 3A shows the effects of doxycycline and cisplatin on the survival of HeLa cells that were not transfected with the Cx32-containing plasmid. At both low and high cell density, doxycycline (1 μg/mL) had no effect on the cell survival. Treatment with cisplatin (0.5 μg/mL) reduced the clonogenic survival of cells at both low density and high density. The inhibition of survival of these cells by cisplatin was not affected by addition of doxycycline. These results show that doxycycline itself had no unexpected effects on HeLa cells or cellular response to cisplatin.

Figure 3B shows the cisplatin survival response in doxycycline-induced and doxycycline-uninduced cells. At high cell density, the cells treated with doxycycline were much more sensitive to cisplatin, with survival half that of doxycycline-untreated cells at 0.5 μg/mL. In contrast, at low cell density, there was no significant difference in cisplatin survival between the doxycycline-induced and doxycycline-uninduced cells (i.e., connexin expressing and nonexpressing, respectively). Pretreatment of Cx32-expressing cells with 18-α-GA at a concentration verified to inhibit dye coupling in these cells (Fig. 2B) reduced the cytotoxicity of cisplatin, yielding substantially increased survival in high-density cultures (Fig. 3C). In contrast, at low cell density dependence of cisplatin response is mediated by GJIC.
cells was assessed 7 days after exposure to cisplatin and analgesics. Figure 4A and B show that tramadol and flurbiprofen have no effect on cisplatin toxicity in low-density cultures, but at high density, they eliminate a substantial portion of the cisplatin toxicity. In contrast, morphine had no effect on either low or high-density cultures (Fig. 4C), even at concentrations far above its analgesic plasma concentration range (16-354 ng/ml). Thus, only tramadol and flurbiprofen decreased the toxicity of cisplatin, and only in the high-density cultures, when there is opportunity for gap junction formation.

Flurbiprofen slightly decreased the cell colony forming ability to similar degrees in high- and low-density cultures relative to controls (~20%; Fig. 4B), suggesting an effect independent of cell contact. Prior studies have reported that some nonsteroidal anti-inflammatory drugs (NSAID) such as flurbiprofen can have antitumor effects (24, 25), consistent with this result.

**Influence of analgesics on gap junction function.** The fact that tramadol and flurbiprofen affected cisplatin toxicity only at high cell density raised the possibility that their protective effects are mediated by gap junction channels. To test this hypothesis, we examined the effects of the three analgesics on dye-coupling between cultured cells.

Junctional coupling was assessed by the parachute assay for dye coupling as described in Materials and Methods. Donor cells labeled with the membrane dye DIL and loaded with the junction-permeable dye calcine were seeded onto unlabeled receiver cells. Experiments were carried out in the presence of analgesics. The seeded donor and receiver cells were incubated for ~4 hours, and the number of receiver cells per donor cell containing calcine determined. Gap junction communication was assessed as the number of receiver cells receiving calcine from a labeled cell, normalized to that for control conditions (without drug).

The data in Fig. 5A and B show that morphine, up to 20 μg/ml, had essentially no effect on the dye coupling between cells. However, both tramadol and flurbiprofen markedly inhibited the dye spread from donor cells to receiver cells in a dose-dependent manner. Thus, only the two agents that inhibited gap junctions decreased the toxicity of cisplatin, and only in the high-density cultures, whereas morphine, which had no effect on gap junctions at the concentration used, did not affect cisplatin toxicity under any conditions. Cisplatin alone or in combination with the analgesics had no effect on GJC (Fig. 5B).

**Effects of tramadol and flurbiprofen on connexin expression.** To determine whether tramadol and flurbiprofen affected connexin expression, expression of Cx32 in cells following induction with doxycycline and exposure to the agents was assessed by Western blotting. As shown in Fig. 6A, treatment with 10 μg/ml tramadol for 1 h increased the level of Cx32 but prolonging the treatment time to 4 and 48 hours did not alter the expression level compared with controls. Therefore, at 1 hour, the time-frame of exposure to cisplatin, tramadol increased connexin expression while decreasing coupling, demonstrating that alteration of connexin expression by tramadol was not responsible for the inhibition of dye coupling. The level of Cx32 expression was unchanged with exposure to 10 μg/ml flurbiprofen (Fig. 6B) or to morphine (data not shown) at 1, 4, and 48 hours. This suggests tramadol and flurbiprofen reduce junctional function by direct or indirect effects on channel gating and/or stability of junctional structures, rather than by altering connexin expression levels.

density, there was no significant effect of 18-α-GA on the cisplatin response. Overall, the effects of GJC reduction, induced by either absence of connexin expression or by 18-α-GA inhibition of gap junctions, on cisplatin toxicity only at high cell density support the idea that the enhancement of cisplatin toxicity at high density is mediated by GJC.

**Effect of analgesics on cytocotoxicity of cisplatin.** To assess the role of analgesics in modulating the effects of chemotherapeutic reagents, we examined the effects of morphine, tramadol, and flurbiprofen on cisplatin-induced cytotoxicity in HeLa cells expressing Cx32. Cells seeded at high or low cell density were treated with each reagent for 1 hour, followed by exposure to 0.5 μg/ml cisplatin plus analgesic for 1 hour. The clonogenic survival of HeLa
Discussion

Consistent with previous reports (11–13), we show that there is a significant gap junction dependent component of cisplatin toxicity. This component is absent in low-density cultures, which lack junctional contacts. Importantly, we find that in high-density cultures, where there is substantial intercellular contact, inhibition of GJIC either by preventing connexin expression or use of the connexin channel inhibitor 18-α-GA, reduces cisplatin-induced cytotoxicity, exclusively in high-density cultures.

Specifically, the present study investigates the influence of three analgesics, morphine, tramadol, and flurbiprofen, which produce analgesia by different mechanisms, on the cytotoxic action of cisplatin in HeLa cells expressing Cx32. At analgesic concentrations, tramadol and flurbiprofen counteract the cytotoxicity of cisplatin but morphine does not. The analgesic-induced reduction of cisplatin toxicity is seen only in cells with functional gap junctions and not in high-density cultures either not expressing connexin or where the gap junctions are pharmacologically inhibited, or in low-density cultures. This establishes a GJIC-dependent mechanism for the effect of tramadol and flurbiprofen on cisplatin toxicity. The mechanism was revealed by studies showing that tramadol and flurbiprofen, but not morphine, inhibited gap junction function in these cells. The present data lead to the idea that some analgesics, or other drugs commonly used concurrently with cisplatin or other antineoplastic agents in clinical settings, reduce GJIC and thereby reduce the antineoplastic efficiency of cisplatin for tumors with GJIC.

Loss of GJIC is widely regarded to correlate with tumorigenic phenotypes, but there are exceptions. More important, it is now clear that connexins can play distinct roles in specific stages of cancer progression. Specifically, increased level of connexin expression and of GJIC are correlated with invasiveness, extravasation, and metastasis in a variety of cancer cells, including HeLa cell, melanoma cells, lung carcinoma, breast carcinoma, and glioma. It has also been noted that primary tumors that are initially GJIC impaired become GJIC competent at the metastatic stage (8). Thus, in addition to cancer cells that retain some level of GJIC, the results reported here are quite relevant to therapies directed at inhibition of several stages of tumor progression.

Inhibition of gap junctions by analgesics would be expected to have little effect on tumors at stages that have limited GJIC. For tumor cells with reduced GJIC, development of drugs and methods that can recover or increase GJIC provide a new and potent way to enhance treatment of these tumors. Several compounds, notably 4-phenylbutyrate, an inhibitor of histone deacetylases, have been shown to increase GJIC of otherwise GJIC-impaired tumor cells, which enhances toxic bystander effects as well as tending to restore growth control (26). If tramadol and flurbiprofen are used concurrently with 4-phenylbutyrate or other such agents, the antineoplastic efficiency of these agents may be reduced by the GJIC inhibition produced by the analgesics.

For the experiments with the analgesics, cells were exposed for 1 hour to analgesics, followed by 1-hour exposure to both analgesic and cisplatin, with toxicity assessed by clonogenic survival 7 days later. Therefore, any modulation of cisplatin toxicity by changes in GJIC must occur during the 1-hour exposure to cisplatin or during GJIC recovery. With exposure to 18-α-GA, tramadol, or flurbiprofen, there was substantial, but not complete, inhibition of the toxic effect of cisplatin. Therefore most of the intercellular transmission of cisplatin-induced toxic signaling takes place within or shortly after the 1 hour of cisplatin exposure.

Cisplatin has been shown to rapidly up-regulate expression of Bax, a member of the pro-apoptotic Bcl-2 family, as well as cell cycle regulators P53 and P21, within this timeframe (27). Thus, there is ample opportunity for transmission via gap junctions during this time period of chemical signals that promote toxicity in response to cisplatin exposure.

The concentration of cisplatin used in this study is low relative to that used in most in vitro studies (0.5 μg/mL or 1.6 μmol/L), which are typically more than an order of magnitude higher, but is approximately the peak plasma concentration achieved during chemotherapy (20). This accounts for the fact...
that killing is not complete in the present study, and by design allows us to reveal clinically relevant modulation of cisplatin’s effects that would be obscured by the use of higher, supratherapeutic concentrations. The use of this paradigm argues that the GJIC-dependent effects we see are clinically relevant. That is, at higher, clinically irrelevant concentrations, all cells would be killed directly by cisplatin and one would not expect GJIC to play a significant role in the end point. At the clinically appropriate concentrations used, however, toxic products generated in one cell can enter another via gap junctions and thus enhance the likelihood of cell death in a cell that might not otherwise be so affected, and in turn, it may generate its own toxic products in a positive feedback mechanism.

It is not surprising that pharmacologic agents can have effects on cell physiology distinct from what is thought to be their primary action. The present article reveals that a previously unknown downstream effect of at least two analgesics has important consequences for any process that involves intercellular communication—in this case the efficacy of antineoplastic agents.

The present study makes clear that there is a Cx32-composed gap junction–dependent component of cisplatin toxicity, presumably mediated by gap junction–mediated intercellular diffusion of toxic factors. In addition to Cx32, it has been shown that increasing cell-cell communication in Cx43 overexpressing tumor cells enhances drug sensitivity (11). Such transfer through gap junction channels has been widely reported for several connexins (28). To date, there is no reported study on the effect of analgesics on GJIC mediated by Cx32 or other connexin channels. It should be noted, however, that the pores of Cx43 channels are as wide or wider than Cx32 pores, so they should mediate similar intercellular molecular communication. To obtain information about precisely which tumors may be affected by these analgesics, it is necessary to investigate the effects of tramadol and flurbiprofen on GJIC mediated by other connexin channels and in different tumor cell types.

Tramadol is a nonopioid analgesic, for which the mechanism of analgesia is still unclear. It has very low affinity for μ-opioid receptors but does inhibit noradrenaline and 5-HT transporters (29–31). These transporters are absent in HeLa cells (32, 33), so...
its effects on gap junctions must be mediated by other mechanisms. Flurbiprofen is an NSAID, exerting its analgesic, anti-inflammatory, and antipyretic effects through inhibition of both cyclooxygenase (COX)-1 and COX-2 activity. HeLa cells are well known to express both COX-1 and COX-2 (34), so in theory, its effects on junctional coupling could be mediated via this mechanism. There is no direct evidence that alteration of COX activity affects gap junction function, however, a recent study indicates that COX-2 and Cx43 are coregulated and colocalized (35). Whether COX activity is involved in the effect of flurbiprofen on GJIC function requires further exploration.

Morphine exerts its therapeutic effects by mimicking the action of endogenous opioid peptides at opioid receptors (36); however, HeLa cells are devoid of opioid receptors (37).

Recent evidence suggests a correlation between the increase in connexin expression and function that accompanies neuropathic pain and nerve injury with pain perception. Specifically, reduction of gap junction function under these conditions is correlated with decreased pain perception (38–40). Carbenoxolone, a nonselective gap junction inhibitor, produced analgesia in several experimental pain models (41, 42). These results suggest the possibility that reduction of gap junction function may be involved in the analgesic mechanism of some drugs. To date, there are no reports on the effects of analgesics on GJIC except for a study showing that morphine had no effect on the junctional dye permeability of astrocyte primary cultures (43), consistent with our finding that morphine has no effect on GJIC mediated by Cx32 gap junctions.

The present results, showing that tramadol and flurbiprofen at their analgesic concentrations reduce junctional permeability, and the previously mentioned work, showing that a gap junction blocker produces analgesia, provide support for the hypothesis that inhibition of GJIC is a component of the analgesia produced by at least some nonopioid analgesics.

Flurbiprofen treatment induced mild cytotoxicity at both high and low cell density, an effect independent of gap junctions. As noted, flurbiprofen is an NSAID, and exerts its analgesic effects by inhibiting prostaglandin synthesis by inhibition of COX-1 and COX-2 (44, 45). A large body of literature shows that COX-2 is highly expressed in cell lines of cancers of the prostate, bladder, colon, esophagus, skin, pancreas, lung, and breast cancer as well as head and neck cancer (46). On the basis of these and other studies, it has been proposed that inhibition of COX-2 suppresses malignancy. In fact, several NSAIDs are known to exert antitumor effects (47). Therefore, we suggest that the mild inhibition of cell growth by flurbiprofen we observed may be through suppression of COX-2.

Some NSAIDs, including flurbiprofen, can have effects unrelated to COX inhibition (48), such as on signaling pathways involving β-catenin, which may be responsible for downstream effects on gap junctions as well as COX-independent toxic effects (49). Thus, the effects of NSAIDs regarding cisplatin toxicity are likely to involve a variety of factors, specific to each setting, some of which may enhance toxicity and some of which may exert a protective effect via gap junction inhibition. In this context, it is worth noting that in a test of a wide variety of NSAIDs in combination with various chemotherapeutic drugs on several human cancer cell lines, cisplatin was found not to have a synergistic effect with NSAIDs (including flurbiprofen), and conversely, flurbiprofen did not have a synergistic effect with any of the tested chemotherapeutic agents (including cisplatin; ref. 50).

By showing the mechanism of interplay between analgesics, gap junction function, and cisplatin toxicity, this work provides a cautionary note and a basis for rational choice of analgesics in chemotherapy, and more generally highlights the importance of basic cell biology in developing an integrated approach to pharmacologic intervention. The fact that some analgesics (tramadol and flurbiprofen), but not others (morphine), counteract the cytotoxicity of cisplatin suggests for example that, where possible, morphine should be used rather than tramadol or flurbiprofen in this context. Alternatively, if tramadol or flurbiprofen are used, it may be advisable to increase the dose of antitumor drugs to preserve their effectiveness. The present results also suggest the possibility that up-regulation or maintenance of gap junction functionality may be used to increase the efficacy of anticancer chemotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Tramadol and Flurbiprofen Depress the Cytotoxicity of Cisplatin via Their Effects on Gap Junctions

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