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Connexin 43 Enhances the Cisplatin-Induced Cytotoxicity in Mesothelioma Cells

Hiromi Sato and Koichi Ueno

*Graduate School of Pharmaceutical Sciences, Chiba University
Japan*

1. Introduction

The direct cell-to-cell communication is performed by gap junctions (GJ) composed of connexin (Cx), a large protein family with a number of subtypes. GJ channels allow the propagation of electrical impulse and the transfer of small molecules (up to 1,000 to 1,500 Da) between two neighbouring cells (Figure 1). And it has also become clear that different combination of Cxs are expressed in different tissues with temporal specificity during development or tissue differentiation (Paul, 1995). By such a rigid regulation, Cxs contribute to maintain cellular homeostasis in many organs. On the contrary, expression levels of Cx proteins are often decreased in many cancers, and restoring their levels has been shown to have antitumor effects. In this concept, there is a possibility to establish a new cancer therapy based on tumor-suppressive functions of Cxs for refractory cancers, such as mesothelioma.

Malignant mesothelioma is an aggressive and devastating malignancy of the pleura and peritoneum. Although it has been the subject of intense clinical and scientific interest, the case-fatality rate for mesothelioma remains high, and conventional treatments remain inadequate. Systemic chemotherapy is important since it is a whole-body treatment and is useful for patients who cannot be treated with surgery. Cisplatin (CDDP) has been used in clinical mesothelioma therapy, and its chemotherapeutic effect as a single agent (Ryan et al, 1998) as well as in combination with other drugs such as gemcitabine has been examined (Byrne et al, 1999; van Haarst et al, 2002; Vogelzang et al, 2003).

In this chapter, we introduce the tumor-suppressive effect of Cx gene against mesothelioma, especially with regard to chemotherapeutical sensitivity.

2. The possible involvement of Cx43 in mesothelioma

GJ channels are formed by two hemichannels provided by either of neighboring cells. Each hemichannel is a hexameric pore structure made from 6 protein subunits, Cxs. Cx is a protein with four transmembrane spanning domain, two extracellular and one intracellular loop, and N- and C-terminal at the intracellular side (Kumar & Gilula, 1996). The various Cx isoforms differ with regard to their molecular weight due to different length of their C-terminal region. There are 21 Cx morecules which were identified in human so far. With regard to mesothelioma, dysfunction of gap junctional intercellular communication (GJIC)

has been observed in several malignant mesotheliomas cells or tissues (Linnainmaa et al, 1993), and among the many Cx proteins, Cx43 is prominently expressed in nontumorigenic mesothelial tissues (Pelin et al, 1994). Besides that, we found that Cx43 expression was very low in human malignant mesothelioma cells (Figure 2A, left). We also examined GJIC ability using fluorescent dye, Lucifer yellow. The molecular size of Lucifer yellow is small enough to pass through GJ, so the spreading distance of this dye tells the GJIC ability (el-Fouly, 1987). By using this method, it became clear that GJIC function was almost dysfunctional in mesothelioma cells (Figure 2B, above). Taken together, it seems that Cx43 has an important role in the parental mesothelial cells and upregulation of Cx43 could reduce malignancy of mesothelioma.

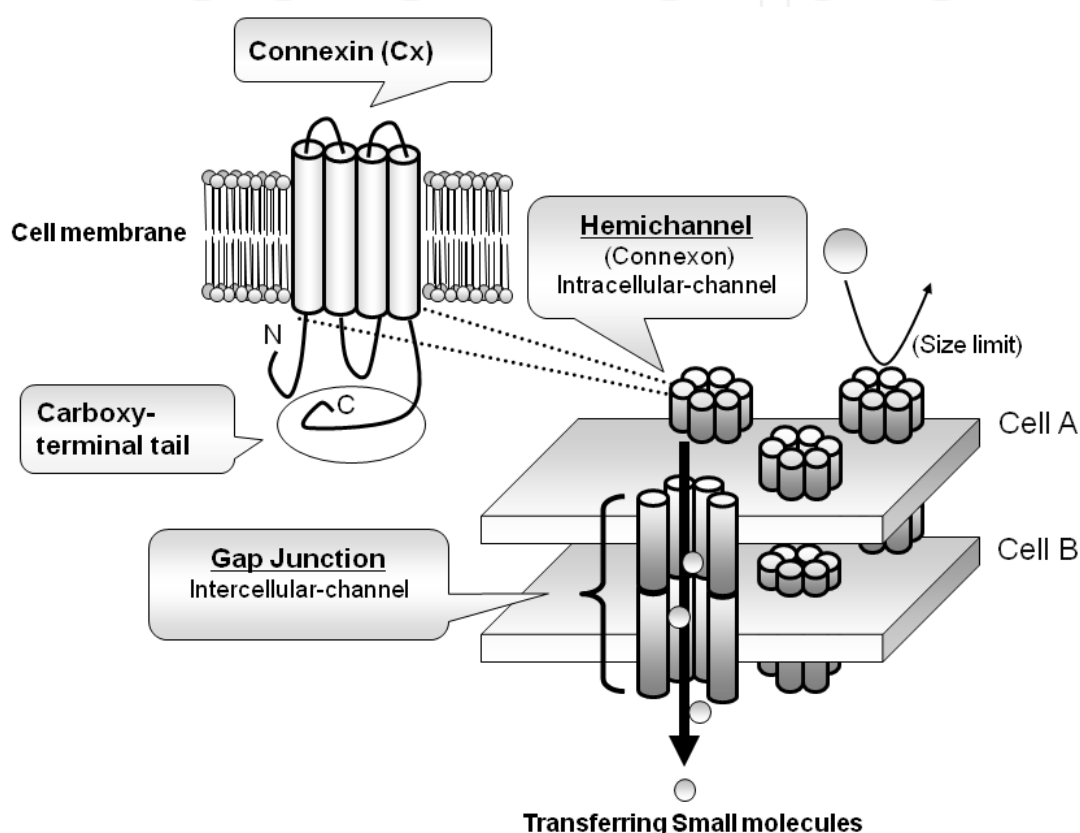


Fig. 1. The structure of connexin (Cx) protein as a component of gap junction (GJ), hemichannel, and as a membrane protein.

Gap junctions (GJ) provide a pathway for communication between neighboring cells. GJ are organized in plaques at the cell membrane surface and arise from 2 connexons (hemichannels) from neighboring cells. Connexons are composed of 6 connexin (Cx) proteins. GJ channels can be permeable to only small molecules (around 1,000 Da), which contribute to keep cellular homeostasis. It has been suggested that the down-regulation of Cx genes is associated with the development of cancers. Besides GJ, hemichannels provide a pathway for the extracellular release of essential homeostasis regulators, like ATP. Moreover, carboxy-terminal (C-tail) of Cx protein has a possibility to modulate gene expression via binding proteins, which can be also modulated by other proteins. Ex) C-tail of Cx43 includes target sites of Src phosphorylation (Giepmans, 2004)

3. Effect of Cx43 mono-therapy

Recent studies have suggested that not all Cx genes could exert a tumor-suppressive effect on a given tumor but rather than that there seems to be Cx-cell type compatibility for this effect (Yano et al, 2006, as cited in Mesnil et al, 1995). That means Cx can exert growth control only in tissue or cell type in which the particular Cx is naturally expressed. Indeed, overexpression of Cx43 in MDA-MB-231 cells results in downregulation of fibroblast growth factor receptor (FGFR)-3, a member of the FGFR family consisting of potent angiogenic factors, which contribute tumor growth and invasion (Qin et al, 2002). According to this concept, we previously investigated the tumor suppressive role of Cx32 in renal cell carcinoma, in which Cx32 expression is downregulated (Yano et al, 2003), using a forced expression clone of Cx32. As expected, we found that Cx32 acts as a tumor suppressor gene in renal carcinoma cells (Fujimoto et al, 2005).

Here, in order to estimate if Cx43 could suppress malignant phenotype of mesothelioma cells, we arranged a Cx43 transfected clone (Cx43 transfectant) and only mock-tranfected clone (mock), as a control. Naïve cell characteristics were compared between Cx43 transfectant and mock. As expected, when attached condition, the growth was significantly inhibited by Cx43 (Figure 2C-i). Next, to examine anchorage-independent growth, the

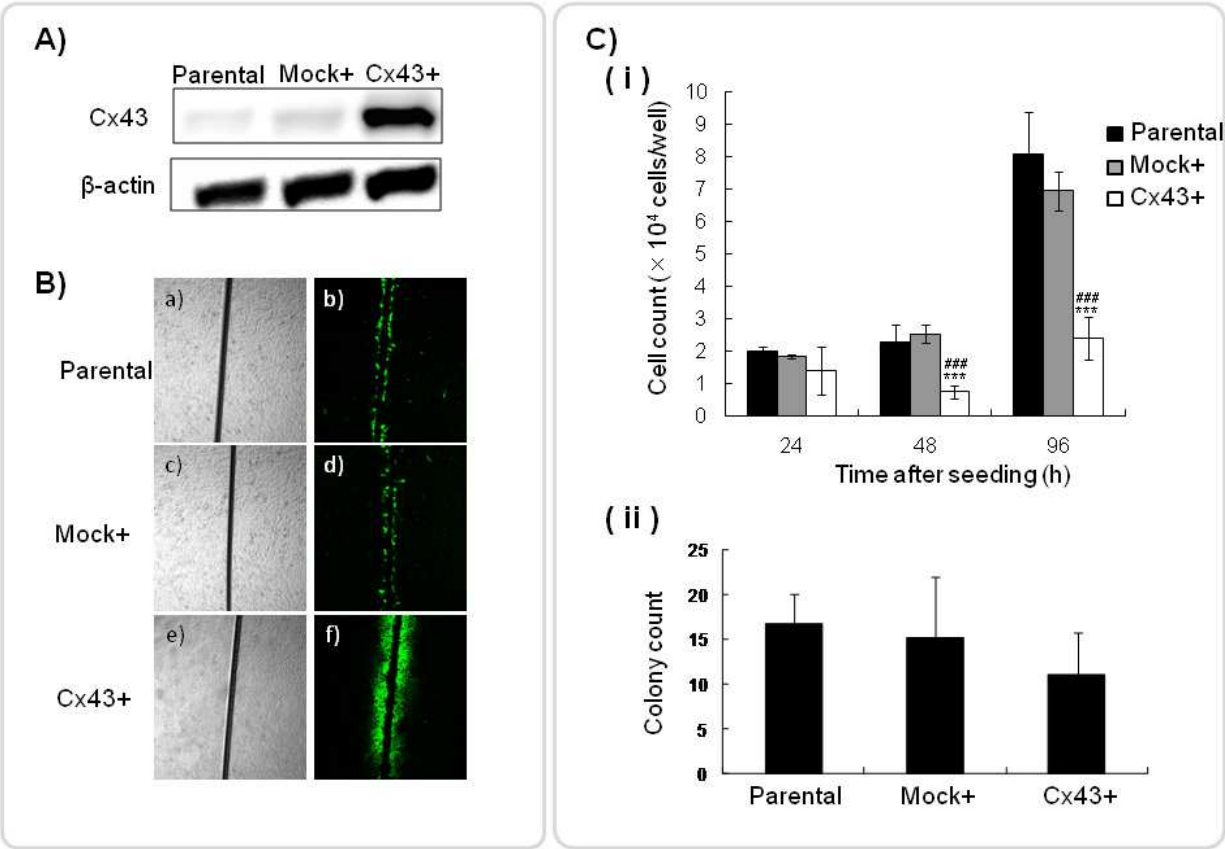


Fig. 2. The naïve role of Cx43 on human mesothelioma cells. **A)** and **B)** show characteristics of mesothelioma cells. Cx43 expression **A)** and GJIC activity **B)** in human mesothelioma cells, parental H28 ('Parental'), mock-transfectant ('Mock+'), and

Cx43-transfectant ('Cx43+') are shown. **a), c), and e)**; phase-contrast image, **b), d), and f)**; dye transfer of Lucifer yellow from the scrape lines. **C)** indicates effect of Cx43 on cell growth **i)** when attached condition or **ii)** when non-attached (anchorage independent) condition. Scale shows cell number (**i)** or colony number (**ii**). Cell growth was significantly inhibited when attached condition in Cx43-transfectant compared to parental (### $P < 0.001$) or mock cells (*** $P < 0.001$).

number of cell colonies formed in three-dimensional agarose gels were counted. This time, however, there was almost no difference between cells (Figure 2C-ii). This finding indicates that the proliferation ability of a single cell, which is needed for survival in a nonmatrix environment, is almost the same between different cell types used in this study. There was also no difference in migration ability (data not shown). One possible reason why restoration of Cx43 could not inhibit cell growth enough is disruption of Cx protein trafficking systems. To put it plainly, Cx protein can not be transported from nucleus to cytoplasmic membrane. Under abnormal localization of Cx protein trafficking, Cxs could not exert tumor suppressive effect neither in GJIC dependent or independent manner, although the reason why it happens is unclear (Trosko & Ruch, 2002). Above all, it is suggested that restoring Cx43 expression alone cannot prevent tumor expansion or metastasis completely.

4. Combination effect of Cx43 and CDDP

As mentioned, mesothelioma is very refractory cancer. Radiotherapy and chemotherapeutic agents are ineffective in many cases despite surgery resection is possible only in case the tumor is very early stage or it is very limited focus. Recently, pemetrexed, a novel multitargeted antifolate, has shown modest activity when used as a single agent and in combination with CDDP in mesothelioma patients. However, although pemetrexed is promising, it has yet to be standardized because of the severity of its side effects and the presence of nonresponders. On the other hand, there are several reports of enhancement efficacy of conventional chemotherapy on cancers by Cxs, which are called bystander effect. Actually, we have reported that cytotoxic effects of conventional chemotherapeutic agents on cancer cells are potentiated by Cx32 in lung cancer (Sato et al, 2007a) and renal cell carcinoma (Sato et al, 2007b). Moreover, Cx43-mediated GJIC has been reported as a major mechanism for the transfer of ganciclovir (nucleoside analogue) to neighboring cells (Elshami et al, 1996). Therefore, we examined the combined effect of Cx43 and a chemotherapeutic agent, CDDP.

4.1 Cx43-dependent induction of the CDDP cytotoxicity

As expected, it was confirmed that mesothelioma cells were resistant to CDDP; approximately 80% of cells survived after exposure to high concentrations of CDDP (Figure 3A). In contrast, Cx43 reduced cell viability and only approximately half of the mesothelioma cells or mock cells survived after being treated with 50 μ M of CDDP (Figure 3A). To confirm the contribution of Cx43 to the enhancement of CDDP-induced damage, we knocked down Cx43 expression by using specific siRNA. As a result, sensitivity to CDDP was attenuated by knockdown of Cx43 (Figure 3B); therefore the involvement of Cx43 in CDDP-induced damage was confirmed.

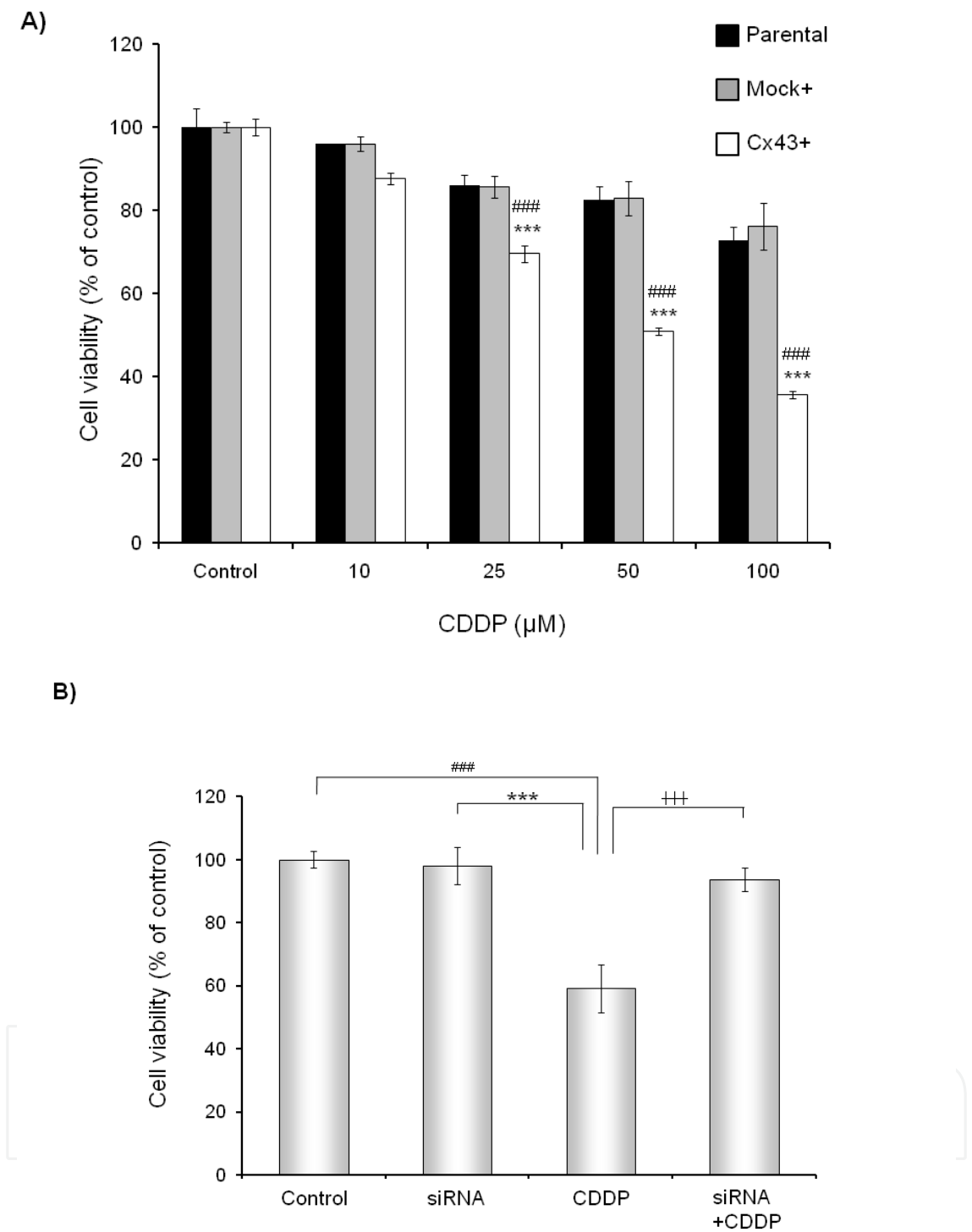


Fig. 3. Effect of Cx43 on CDDP-induced cytotoxicity. **A)** shows CDDP cytotoxicic effect on human mesothelioma cells, parental H28 ('Parental'), mock-transfectant ('Mock+'), and Cx43-transfectant ('Cx43+'). The cell viability after CDDP treatment was significantly decreased in Cx43-transfectant compared with parental cells (###*P* <0.001) or mock cells (****P* <0.001). **B)** shows effect of Cx43-knockdown on Cx43-transfectant cell growth after CDDP treatment. The cell viability in cells co-treated with siRNA and CDDP ('siRNA+CDDP') recovered to control level. ###, ***, and +++ *P* <0.001; Significant differences compared with only treated with CDDP group ('CDDP').

4.2 Molecular mechanism of Cx43 which contributes to the CDDP cytotoxicity

Here, it is confirmed that Cx43 significantly enhances CDDP growth inhibiting effect and apoptosis might be induced, that is, Cx43 upregulates the sensitivity to CDDP in mesothelioma cells. Then considering the involvement of GJIC, molecular mechanism of this phenomenon was investigated via cell cycle distribution, apoptotic factors, and Src which could interact with Cx43 protein.

4.2.1 Gap junction dependent manner

To determine the influence of GJIC on CDDP-induced cytotoxicity, we used a GJ blocker, 18 β -glycyrrhetic acid (GA). After 4 h of culturing with GA, CDDP treatment followed, then cell viability was determined using WST-1 Reagent. As a result of Lucifer yellow dye spreading assay, GJIC inhibiting effect by GA was observed in Cx43-transfectant (Figure 4A). However, such inhibition of GJIC had almost no effect on cell viability detected by WST-1 assay (Figure 4B). Thus, inhibition of GJ-dependent function with a specific inhibitor did not abrogate CDDP-induced cytotoxicity. These results suggest that Cx43-mediated enhancement of cytotoxicity might be independent of GJ function.

On the other hand, there are several reports concerning the relation of GJIC and CDDP cytotoxicity. One of the previous reports suggests a possibility that CDDP could affect GJIC, it might be regulated by MAP kinase-dependent phosphorylation of specific sites of Cx43 (Procházka et al, 2007). Because it is known that CDDP toxicity is modulated by a MAP kinase pathway besides the established mechanisms (Park et al, 2002; Woessmann et al, 2002) and that Cxs are regulated by multiple mechanisms, including MAP kinase dependent phosphorylation (Jo et al, 2005; Lampe & Lau, 2004; Wang et al, 2000). Procházka et al showed a new platinum complex, LA-12, had strong GJIC inhibiting effect (Procházka et al, 2007). They also found the inhibitory effect of GJIC was induced by hyperphosphorylation of Cx43 protein which decrease the localization of cell membrane, which correlated with activation of MAP kinase pathway proteins. On the other hand CDDP exerted only a low effect on GJIC though the drug caused activation of MAP kinase protein (Procházka et al, 2007). Another report which supports Procházka et al showed that Cx43 level was elevated in CDDP-resistant cell line (Li et al, 2007). Li et al indicated the conflicting reports in which the role of Cxs in cell survival were different; in case GJ component protein, they have been implicated the bystander effect, but Cx43 protein itself has also been shown to be important in cell survival, although this function is poorly characterized. They also suggested that although the selection of CDDP resistance might have favored cells that expressed high level of Cx43, continued expression of this gene might decrease drug resistance by promoting a bystander effect, and finally concluded that Cx43 played a role in drug sensitivity in any case (Li et al, 2007). Meanwhile other reports showed that compounds like CDDP suppressed intercellular communication (Jo et al, 2005; Wang et al, 2000).

Taken together, it is still unclear if GJIC involves to the enhanced effect of CDDP by Cx43. Further investigation of this concern which magnify more elaborate apoptotic pathway field will be needed.

4.2.2 Gap junction independent manner

In this section, several factors which probably concern the mechanism of Cx43 induced cytotoxicity of CDDP are suggested irrespective of GJIC.

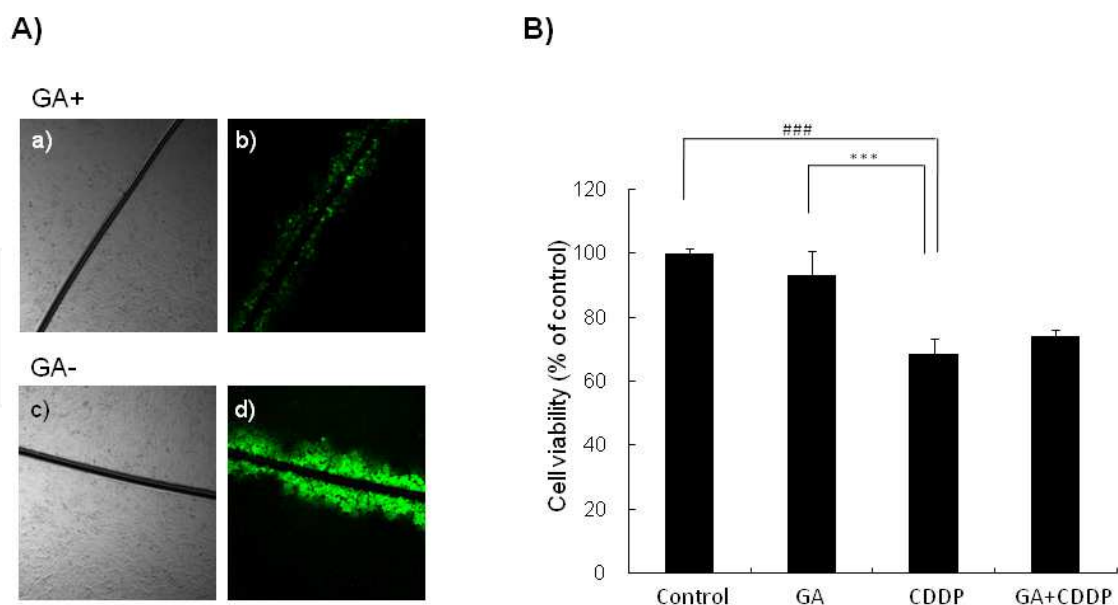


Fig. 4. Effect of GJ blockade on CDDP-induced cytotoxicity. **A)** GJ inhibitor (GA) downregulated GJIC in Cx43-transfectant. 'GA+'; cells treated with GA for 4 h, 'GA-'; cells treated with only 0.1% dimethylsulfoxide (DMSO, vehicle for GA). **a)** and **c)**; phase-contrast image, **b)** and **d)**; dye transfer of Lucifer yellow from the scraped lines. **B)**; Cell viability after CDDP treatment in Cx43-transfectant was detected by WST-1 assay. Downregulating effect of CDDP did not recover by pre-treatment of GA 4 h. 'Control'; cells treated with only 0.1% DMSO (vehicle for CDDP), 'GA'; cells pre-treated with GA for 4 h followed 0.1% DMSO treatment for 48 h, 'CDDP'; cells treated with only CDDP for 48 h, and 'GA+CDDP'; cells pre-treated with GA for 4 h followed CDDP treatment for 48 h. ### $P < 0.001$; Significant difference between 'Control' and 'CDDP', *** $P < 0.001$; significant difference between 'GA' and 'CDDP'.

4.2.2.1 Regulation of cell cycle distribution by Cx43

We also checked cell cycle distribution after exposure to CDDP in mesothelioma cells. In both parental mesothelioma cell or Cx43-transfectant, an increase in the G_1 -phase population was observed after CDDP treatment for more than 24 h (Figure 5A). On the other hand, the S-phase population increased by Cx43. And we also compared the sub- G_1 peak between cells. A higher sub- G_1 population was induced by Cx43, indicating upregulation of apoptosis. It has been reported that DNA modification by CDDP activates the G_1 checkpoint (Un, 2007). This checkpoint exists to halt cell cycle progression in the event of DNA damage to allow time for repair before initiating DNA replication (Wang et al, 2001). However, even those cells which retain G_1 checkpoint eventually die when they are exposed to persistent treatment. Therefore it is suggested that G_1 arrest represents a critical determinant of CDDP cytotoxicity (Un, 2007). In our study, G_1 arrest was observed in both parental mesothelioma cells and Cx43-transfectant (Sato et al, 2009). Therefore, the G_1 checkpoint does not appear to have broken down in these cells. As expected, an obvious induction of apoptosis was observed by Cx43. This observation might be explained by the additive effect of Cx43 on cell cycle distribution, inducing S-phase arrest. Thus, two surveillance mechanisms possibly work independently in the G_1 and S checkpoints, making it easy to suppress progression of the cell cycle and induce apoptosis in mesothelioma.

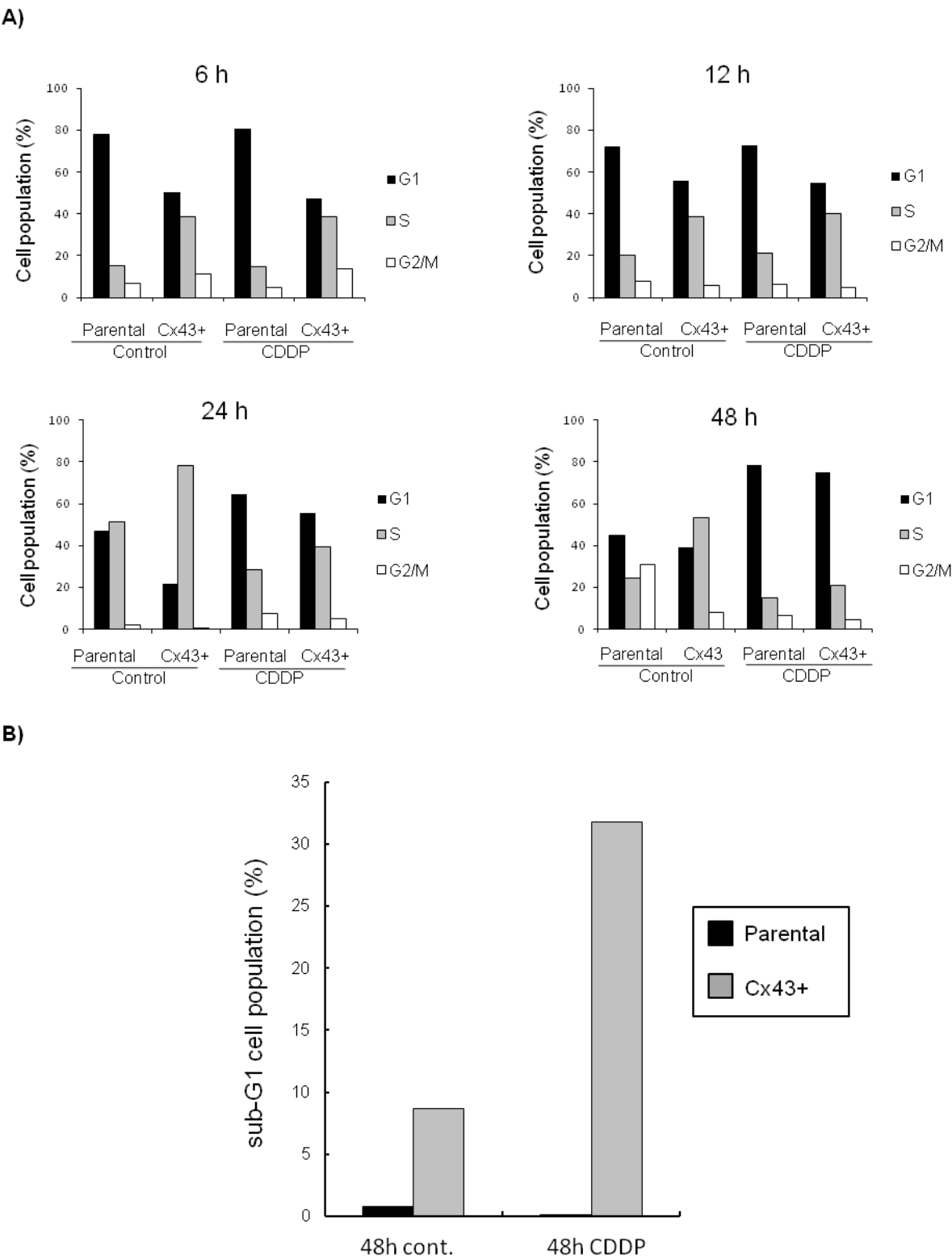


Fig. 5. Effect on cell cycle distribution by Cx43. **A)** shows changes in cell cycle distribution in parental mesothelioma cells ('Parental') and in Cx43-transfectant cells ('Cx43+') after 25 μ M of CDDP stimulation. Cells were treated with CDDP for the indicated periods, and 20,000 cells of each group were analyzed by flow cytometry. Control cells were treated with only 0.1% dimethylsulfoxide (vehicle for CDDP).

S-phase arrest was observed in Cx43-transfctant. **B)** shows effect on the induction of sub-G₁ population (estimated apoptosis) of mesothelioma cells 48 h CDDP treatment. Strong induction of sub-G₁ population was observed in Cx43 transfectant.

4.2.2.2 Effect of Cx43 on apoptotic factors

Proteins of the Bcl-2 family play an important role in the regulation of programmed cell death. Overexpression of Bcl-2, an anti-apoptotic factor, is associated with resistance to various cytotoxic agents, while the Bax protein is a pro-apoptotic member of the Bcl-2 family. In our study, no differences were noted in the levels of Bcl-2 among parental mesothelioma cells, mock, and Cx43-transfectant (Sato et al, 2009). On the other hand, Bax expression was strongly upregulated in Cx43-transfectant compared to the other two. Thus, the Bcl-2 family balance appeared to lean toward enhancing apoptosis by Cx43.

The Bcl-2 family proteins are involved in most of apoptosis pathway, so they are attractive target for cancer therapy (Kim et al, 2004). A previous study reported little or no expression of Bcl-2 but high expression of Bcl-xL and a pro-apoptotic protein, Bax, in mesothelioma histological sections and cells (Soini et al, 1999). Our results showed a different trend: Bcl-2 but not Bcl-xL was detected (data not shown). However, the important finding was the balance between pro- and anti-apoptotic factors. Any approach that changes the balance in favor of apoptosis may have a therapeutic benefit. Our results suggest that Cx43 influences the balance between pro- and anti-apoptotic factors in the direction of apoptosis, possibly contributing to the improved sensitivity of cancer cells to CDDP. However, some studies have reported low Bcl-2 and high Bax expression in mesothelioma samples. A previous study helped the current understanding of apoptosis regulation by raising the following possibilities: one is Bax mutation, which makes it nonfunctional and blocks its pro-apoptotic effect (Narasimhan et al, 1998); and the other is breaking out of antagonists to Bax as previously suggested (Yu et al, 2008). To clarify this issue, a functional assay of Bax in mesothelioma cells that also compares that of Cx43-transfectant is required.

4.2.2.3 Interactive effect of Cx43 with Src

Cx43 is known to interact with the proto-oncogene product, Src. Src can directly phosphorylate Cx43 (Loo et al, 1995). Including mesothelioma, the Src family of nonreceptor tyrosine kinases are overexpressed in various human tumors, which often involves tumor progression and metastatic potential. In fact, a previous report revealed that total c-Src is highly expressed in some mesothelioma cells (Tsao et al, 2007). On the other hand, it has also shown that Cx43 regulates Src kinase through interaction of the Cx43 C-terminal region, irrespective of GJIC function (Giepmans et al, 2001).

Src activity is regulated by tyrosine phosphorylation. Phosphorylation of Tyr416 in the activation loop of the kinase domain upregulates enzyme activity. We assayed for Src levels and activation in mesothelioma cells using antibodies which recognized total-Src and phospho-Src protein (an activated form, phosphorylated at Tyr416). Tyr416 antibody detects endogenous levels of the Src family proteins, when phosphorylated at Tyr416. As a result, both phospho-Src and total-Src were decreased in Cx43-transfectant, although there were no

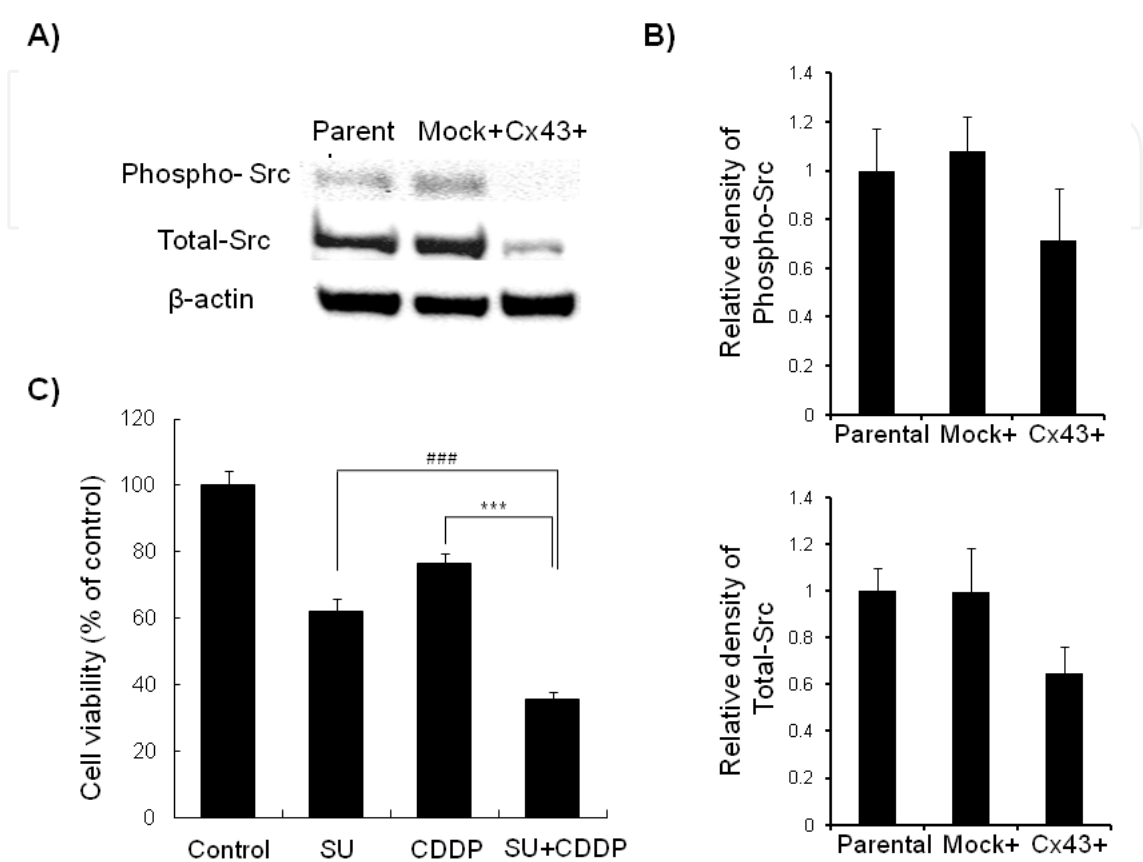


Fig. 6. Downregulation of Src protein level and activity by Cx43. **A)** shows expression of total src ('Total Src') and phosphorelated Src (Phospho-Src, active form), in human mesothelioma cells, parental H28 ('Parental') , only mock-transfected cells ('Mock+'), and Cx43-transfectant ('Cx43+'). β-Actin was used as the internal standard. **B)** shows densitometric data for phosphorelated Src (upper) and total Src (lower). Both Total Src and Phosphorelated Src were decreased by Cx43. **C)** shows cell viability after CDDP treatment in parental mesothelioma cells detected by WST-1 assay. CDDP cytotoxic effect was enhanced by Src inhibitor, SU6656. 'Control'; cells treated with only 0.1% DMSO (vehicle for CDDP), 'SU'; cells treated with only SU6656, 'CDDP'; cells treated with only CDDP, and 'SU + CDDP'; cells co-treated with SU6656 and CDDP. ###*P* < 0.001; Significant difference between 'SU' and 'SU+CDDP', ****P* < 0.001; significant difference between 'CDDP' and 'SU+CDDP'.

significant differences among cells (Figure 6A, 6B). Then, we further investigated whether the inhibition of Src activity affects the cytotoxicity of CDDP in mesothelioma cells by using a specific Src kinase inhibitor, SU6656. It was revealed that when the cells were treated with both CDDP and SU6656, cell viability significantly decreased as compared to that when the cells were treated with CDDP or SU6656 alone (Figure 6C). Our results showed that not only phospho-Src level but also total-Src protein level was decreased in Cx43-transfectant, suggesting that Cx43 somehow suppressed the Src protein expression, which in turn suppressed the total amount of its activation form. One report which supports our results showed that the effect of activated Src on survival after CDDP treatment was reversed by forced overexpression of Cx43 using human Cx43 cDNA transfection model (Peterson-Roth et al). They finally suggested that a novel application for Src kinase inhibitors in combination with CDDP, i.e., one of Src inhibitor, dasatinib has the potential to sensitize tumor cells overexpressing activated Src as well as to sensitize tumor cells without activated Src that are in direct contact with Src-activated cells via GJIC, and also another combination such as proteasome inhibitors with CDDP treatment in order to up-regulate Cx43 expression which sensitize to CDDP.

Interestingly, a previous report showed that some selective inhibitors of Src kinases are specific inhibitors of cell cycle progression into the mid-S phase (Mizenina & Moasser, 2004), which correlates with our result of cell cycle distribution. Therefore, Cx43 may inhibit Src activation, which is reflected as S-phase arrest, resulting in the suppression of cell growth which was observed in Cx43-transfectant. Moreover, under the *in vivo* condition, Src upregulates vascular endothelial growth factor (VEGF), the most important factor in angiogenesis. Tumors must undergo angiogenesis for survival and for metastatic spread in a limited physiological environment (Folkman, 1971). Indeed, a previous report showed that a novel Src inhibitor, M475271, significantly inhibited VEGF-induced HUVEC proliferation, migration and angiogenesis (Ali et al, 2005). It is, therefore, possible that Cx43 has a more suppressive role in growth and metastasis *in vivo*, where angiogenesis always contributes to tumor survival.

It has also been indicated that simultaneous inhibition of Src and its downstream factor, signal transducer and activator (Stat) 3, results in synergistic death of mesothelioma cells (Johnson et al, 2007; Tsao et al, 2007). Moreover, CDDP inactivates Stat 3 by modulating Janus kinase (JAK) 2 through dephosphorylation of JAK/Stat in cancer cells (Song et al, 2004). Taken together, inhibition of Src by Cx43 and inactivation of Stat 3 by CDDP could induce synergistic death of mesothelioma cells.

5. Conclusion

From the view of previous studies including our data, we concluded that Cx43 could improve the chemoresistance of mesothelioma cells to CDDP mainly in a GJIC-independent manner (Figure 7). Also there might be another possible factors, such as hemichannel activity. In next step for clinical application, it should be considered that what kind of methodologies can be used which make possible to regulate Cx43 expression and its activities. There are still many concerns, however, this finding will help in overcoming resistance to current chemotherapy for malignant mesothelioma.

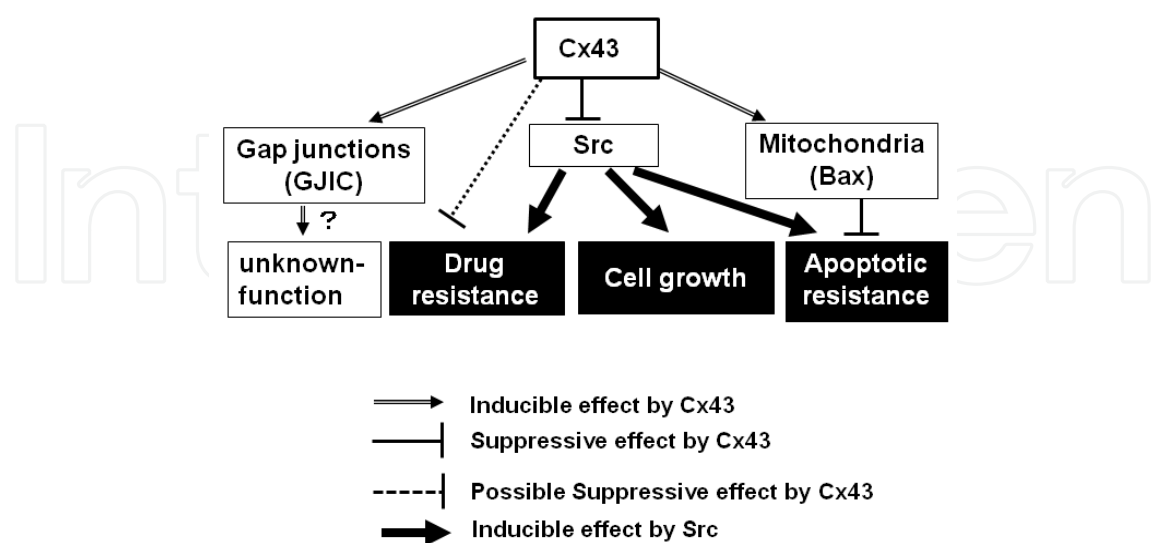


Fig. 7. A scheme of a new potential therapy against mesothelioma using Cx43 functions

6. Acknowledgment

We express our hearty thanks to Dr. Tomohiro Yano, a professor of Toyo University in Japan, and Dr. Christian C. Naus, a professor of the University of British Columbia in Canada, for their valuable advices and technical assistance. This study was supported by Grant-in-Aid for Young Scientists (Start-up) from the Japanese Society for the Promotion of Sciences, and by Special Funds for Education and Research (Development of SPECT Probes for Pharmaceutical Innovation) from the Ministry of Education, Culture, Sports, Sciences, and Technology, Japan.

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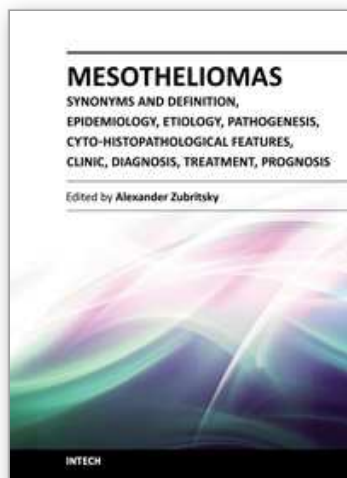
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Mesotheliomas - Synonyms and Definition, Epidemiology, Etiology, Pathogenesis, Cyto-Histopathological Features, Clinic, Diagnosis, Treatment, Prognosis

Edited by Dr Alexander Zubritsky

ISBN 978-953-307-845-8

Hard cover, 244 pages

Publisher InTech

Published online 03, February, 2012

Published in print edition February, 2012

Mesotheliomas are mysterious mesothelial tumors in that they are relatively rare, difficult to diagnose, with a large number of synonyms, and the etiology and pathogenesis of the disease are still not fully disclosed. This problem attracts the attention of various specialists in the field of medicine and biology every year. In recent years there has been a significant increase of mesothelioma morbidity in most of the countries, due to the further industrialization of society. In this regard, this book has been published with the participation of an international group of experts with rich experience from around the world . The book consists of 14 chapters containing the most advanced achievements of all aspects of the various types of mesotheliomas, both in humans and domestic animals, at a high methodological level. This book is intended for biologists and all health care workers, mostly oncologists of different profiles, as well as students of medical educational institutions engaged or even just interested in the problems of mesotheliomas.

How to reference

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Hiromi Sato and Koichi Ueno (2012). Connexin 43 Enhances the Cisplatin-Induced Cytotoxicity in Mesothelioma Cells, Mesotheliomas - Synonyms and Definition, Epidemiology, Etiology, Pathogenesis, Cyto-Histopathological Features, Clinic, Diagnosis, Treatment, Prognosis, Dr Alexander Zubritsky (Ed.), ISBN: 978-953-307-845-8, InTech, Available from: <http://www.intechopen.com/books/mesotheliomas-synonyms-and-definition-epidemiology-etiology-pathogenesis-cyto-histopathological-features-clinic-diagnosis-treatment-prognosis/connxin-43-enhances-the-cisplatin-induced-cytotoxicity-in-mesothelioma-cells>

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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
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Phone: +86-21-62489820
Fax: +86-21-62489821

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