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Cisplatin-induced apoptosis involves a Fas-ROCK-ezrin-dependent actin remodelling in human colon cancer cells

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ABSTRACT

In human colon cancer cells, cisplatin-induced apoptosis involves the Fas death receptor pathway independent of Fas ligand. The present study explores the role of ezrin and actin cytoskeleton in relation with Fas receptor in this cell death pathway. In response to cisplatin treatment, a rapid and transient actin reorganisation is observed at the cell membrane by fluorescence microscopy after Phalloidin-FITC staining. This event is dependent on the membrane fluidification studied by electron paramagnetic resonance and necessary for apoptosis induction. Moreover, early after the onset of cisplatin treatment, ezrin co-localised with Fas at the cell membrane was visualised by membrane microscopy and was redistributed with Fas, FADD and procaspase-8 into membrane lipid rafts as shown on Western blots. In fact, cisplatin exposure results in an early small GTPase RhoA activation demonstrated by RhoA–GTP pull down, Rho kinase (ROCK)-dependent ezrin phosphorylation and actin microfilaments remodelling. Pretreatment with latrunculin A, an inhibitor of actin polymerisation, or specific extinction of ezrin or ROCK by RNA interference prevents both cisplatin-induced actin reorganisation and apoptosis. Interestingly, specific extinction of Fas receptor by RNA interference abrogates cisplatin-induced ROCK-dependent ezrin phosphorylation, actin reorganisation and apoptosis suggesting that Fas is a key regulator of cisplatin-induced actin remodelling and is indispensable for apoptosis. Thus, these findings show for the first time that phosphorylation of ezrin by ROCK via Fas receptor is involved in the early steps of cisplatin-induced apoptosis.

1. Introduction

Cisplatin or cis-diamminedichloroplatinum II (CDDP) is one of the most known potent anticancer agents, displaying clinical activity against a variety of solid tumours. Platinum-DNA adducts, which are formed following the uptake of the drug into the cell nucleus, activate several signal transduction pathways and culminate in the activation of apoptosis.[1] However, the contributions of other DNA-independent targets, which have been so far underestimated, could also play an important role in cisplatin cytotoxicity.[2] Elucidating more thoroughly the cytotoxic mechanisms of cisplatin could be a benefit to refine anticancer therapeutic approaches based on this compound.

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Cisplatin-induced cell death implicates both Fas receptor-dependent pathway and mitochondria-dependent pathway. We have previously shown that exposure of HT29 or HCT116 human colon cancer cells to cisplatin induces Fas receptor clustering and activation in a ligand-independent manner.[3] The type I transmembrane protein Fas (CD95/Apo-1) triggers apoptosis in a variety of cell types. Upon engagement, the Fas-associated death domain protein (FADD) and procaspase-8 are rapidly recruited to the intracellular death domain of Fas receptor, forming the Death-Inducing Signalling Complex (DISC), which then leads to activation of a caspase cascade and irreversible apoptosis.[4] Recent studies have shown that apoptosis induced by Fas agonists or chemotherapeutic agents involves the aggregation of the Fas receptor into membrane lipid rafts enriched in cholesterol and sphingolipids.[5–9] Moreover, actin cytoskeleton is involved in Fas-mediated apoptosis, regulating Fas clustering and internalisation[10–12] with a recent demonstration of a key role of RhoA–ROCK-dependent ezrin–radixin–moesin phosphorylation in this cell death pathway.[13]

Actin cytoskeleton is central in the regulation of membrane-associated signalling and membrane trafficking. The ezrin–radixin–moesin (ERM) family proteins constitute the link between plasma membrane proteins and actin cytoskeleton. Among them ezrin has been shown to be involved in Fas-mediated apoptosis.[12]

More recently, we have demonstrated that cisplatin induces a rapid inhibition of Na^+/H^+ exchanger-1 (NHE1), leading to intracellular acidification which promotes acid sphingomyelinase activation, ceramide generation, membrane fluidification and Fas aggregation into lipid rafts.[14] The present study further explores this cascade by evaluating the role of ezrin and actin cytoskeleton in cisplatin-induced apoptosis. We show for the first time that cisplatin induces actin cytoskeleton reorganisation, which depends on membrane fluidification. Concomitantly, cisplatin induces ezrin phosphorylation through ROCK, and co-localisation of Fas receptor with ezrin at the cell membrane of HT29 cells. All these events are dependent on Fas. Altogether, these data suggest for the first time that Fas receptor triggering is indispensable for actin microfilament rearrangement during cell death induced by cisplatin in human colon cancer cells.

2. Materials and methods

2.1. Chemicals

Cisplatin (CDDP) was from Merck, latrunculin A (LTN A) and water-soluble cholesterol (CHOL) were from Sigma–Aldrich. Rho inhibitor (Rho Inh) was from Cytoskeleton. Hoechst 33342 and fluorescein-tagged phalloidin (Phalloidin-FITC) were from Molecular Probes.

2.2. Cell culture and treatments

HT29, HCT116 and SW480 human colon carcinoma cell lines (American Tissue Culture Collection, Biovalley) were cultured in Eagle's minimum essential medium (Eurobio) complemented with 10% foetal calf serum (FCS) (v/v) (GibcoBRL) and 2 mM L-glutamine (GibcoBRL). For all experiments, the cells, growing in exponential phase, were treated with 25 μM CDDP for different times. When indicated, the cells were pre-treated for 2 h or overnight with various chemical compounds.

2.3. Cell death assays

2.3.1. Hoechst staining

Apoptotic index was measured as previously described.[14] At least three independent experiments were performed per inhibitor tested.

2.3.2. Caspase-3 activity

Caspase-3 activity was measured using the substrate DEVD-AMC (N-Acetyl-Asp-Glu-Val-Asp-AMC; Calbiochem) as previously described.[14] Three experiments were performed in triplicate for each experimental condition tested.

2.4. Determination of membrane fluidity by EPR spin-labelling method

The membrane fluidity of cells was determined by a spin-labelling method using electron paramagnetic resonance (EPR) as previously described.[14] A decrease in the membrane order parameter S reflects an increase in the membrane fluidity.

2.5. Analysis of F-actin labelling

After treatment, the cells were fixed in 4% paraformaldehyde (Sigma–Aldrich) for 20 min, washed in PBS 1X and then preincubated with PBS–BSA 2% (w/v)–saponin 0.2% (w/v) for 30 min before staining with fluorescein-tagged phalloidin (Phalloidin-FITC) (1:500, Sigma) for 30 min. The samples were viewed using a fluorescent DMRXA2 Leica microscope with a 40× NA 1.32 lens equipped with standard fluorescent filters. The images of F-actin were acquired with a CoolSNAP ES camera using MetaMorph software.

2.6. Immunofluorescence microscopy analysis

After treatment, the cells were fixed in 4% paraformaldehyde (Sigma–Aldrich) for 15 min, washed in PBS 1X and then preincubated with PBS–BSA 2% (w/v)–saponin 0.2% (w/v) for 30 min before incubation for 2 h with either polyclonal rabbit IgG anti-Fas (1:100, AbCam), mouse monoclonal IgG anti-ezrin (1:100, Biogenesis), or isotype-matched controls. The cells were then washed in PBS and stained for 45 min with TRITC-labelled goat anti-rabbit IgG (Molecular Probes) or FITC-labelled goat anti-mouse IgG (Jackson Immunoresearch Laboratories). The fluorescent images of ezrin and Fas stainings were analysed using a DMRXA Leica microscope, a COHU high performance CCD camera and the metavue software. A Z-series of images has been taken after image acquisition.

2.7. Isolation of membrane microdomains

The membrane microdomains of HT29 cells were isolated as previously described.[8] After ultracentrifugation, 1mL fractions were collected from the top of the gradient. Measurement of cholesterol content was performed with Infinity cholesterol

kit (Konelab S.A.) on each fraction of the gradient. To determine the expression of Fas, FADD, and procaspase-8 and ERM, 40 μL of each fraction were subjected to SDS–PAGE and immunoblot analysis. After blocking for 1 h at room temperature with 5% (w/v) powdered skimmed milk in Tris-buffered saline/Tween [50 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.1% Tween 20 (v/v)], the membranes were incubated with an anti-Fas rabbit polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology), an anti-FADD monoclonal antibody (1:1000 dilution; Transduction Laboratories), an anti-procaspase-8 monoclonal antibody (1:1000 dilution; Immunotech), an anti-ERM polyclonal antibody (1:500, Cell Signalling), or an anti-flotillin-1 monoclonal antibody (1:500; Transduction Laboratories), an anti-TNFR-1 monoclonal antibody (1:1000, Santa Cruz) and an anti-CD71 polyclonal antibody (1:1000, Santa Cruz). The membranes were then washed twice with Tris-buffered saline/Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson Immunoresearch Laboratories) before protein identification using an enhanced chemiluminescence detection kit (Amersham).

2.8. Cell transfection with siRNA

500.000 HT29 cells were transfected with either non-specific siRNA NTS1 (Non-targeting siRNA1, Dharmacon) or siRNA Fas (siGenome Smart Pool siRNA, gene ID 355, Dharmacon) or siRNA ezrin (siGenome Smart Pool siRNA, gene ID 7430, Dharmacon) or siRNA ROCK (siGenome Smart Pool siRNA, gene ID 6093, Dharmacon) by using Dharmafect-4 reagent (Dharmacon) according to the manufacturer's instructions. Briefly, 100 nM siRNA (in 800 μL Dharmafect-4) was applied in a volume of 800 μL opti-MEM (GibcoBRL) on HT29 cells. For Western-blot analysis, the cells were harvested 48 h after transfection. For cell death experiment, following a 48 h-transfection period, the cells were treated with 25 μM cisplatin for 48 h.

2.9. Western-blot analysis

After treatment, the cells were lysed as previously described.[14] Proteins (50 μg) were separated on a polyacrylamide sodium dodecylsulphate-containing gel and transferred to a nitrocellulose membrane (Amersham). After blocking non-specific binding sites for 1 h at room temperature by 5% (w/v) skimmed milk in TPBS (PBS with 0.1% Tween 20 (v/v)), the membranes were incubated for 2 h at room temperature with a rabbit polyclonal anti-Fas antibody (1:500, Santa Cruz), a mouse monoclonal IgG1 anti-Thr567 phospho-ezrin (1:200, Pharmingen), a mouse monoclonal IgG1 anti-ezrin (1:500, Biogenesis), a mouse monoclonal IgG1 anti-ROCK (1:500, Santa Cruz) or a mouse monoclonal IgG2a anti-Hsc70 (1:1000, Santa Cruz). The membranes were then washed twice with TPBS and incubated for 1 h with peroxidase-conjugated goat anti-mouse antibodies or goat anti-rabbit antibodies. Revelation was performed by chemiluminescence.

2.10. RhoA–GTP pull down

RhoA–GTP levels were measured using the RhoA activation assay kit from cytoskeleton. Briefly, the cells were rapidly lysed at 4 °C and incubated with Rhotekin-RBD affinity beads to specifically pull down RhoA–GTP. After washing, RhoA levels were quantified by running bead/protein complexes in Laemmli buffer containing 0.1 M DTT and probing with a mouse monoclonal anti-RhoA (1:500) as recommended by the manufacturer's instructions. Revelation was performed by chemiluminescence.

2.11. RhoA activation assay

To measure RhoA activation, the G-LISA™ activation kit (Kit# BK124, cytoskeleton) was used according the manufacturer's instructions. This assay uses a Rho–GTP-binding protein linked to the wells of a 96-well plate. Active, GTP-bound Rho in cell lysates binds to the wells while inactive, GDP-bound Rho is removed during wash steps. Bound GTP–Rho is detected by incubation with a RhoA-specific antibody followed by a secondary antibody conjugated to HRP and a detection reagent. The signal was read by measuring absorbance at 490 nm using a microplate reader (VersaMax, Molecular Devices).

2.12. Statistical analysis

Statistical analyses were carried out using the unilateral student's *t*-test considering the variances as unequal. The significance is shown as follows: $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$; $^{***}p \leqslant 0.001$.

3. Results

3.1. Cisplatin treatment induces a rapid membrane fluidification-dependent actin cytoskeleton reorganisation which is involved in the related apoptosis

Cisplatin-induced rapid and transient changes in actin organisation with actin polarisation at the edge of the cells or between cell-cell contacts. Actin remodelling was detectable as soon as 30 min and persisted 6 h after cisplatin treatment (Fig. 1A).

Recently, we have shown that cisplatin induced a rapid plasma membrane fluidification, dependent on both NHE1 exchanger and acid sphingomyelinase, and which is required for cisplatin-induced Fas receptor activation in HT29 cells.[8,14] In an attempt to order the early plasma membrane events that occur upon cisplatin exposure, we studied the involvement of plasma membrane fluidification in actin cytoskeleton reorganisation. The results revealed that pretreatment of HT29 cells with LTN A, an agent known to inhibit actin polymerisation, did not modify cisplatin-induced increase in membrane fluidity (as visualised by the decrease of membrane order parameter) (Fig. 1B). However, pretreatment of cells with 30 μg/mL cholesterol (CHOL), a membrane-stabilising agent, inhibited this increase (Fig. 1B). Moreover, pretreatment with CHOL prevented the observed actin reorganisation (Fig. 1C), suggesting that cisplatin-induced actin remodelling depends on the membrane fluidification.

Pretreatment with LTN A significantly inhibited cisplatin-induced apoptosis as evidenced by the decreased percentage of fragmented and condensed nuclei in HT29 cells treated with CDDP-LNTA (~25%) in comparison with the percentage

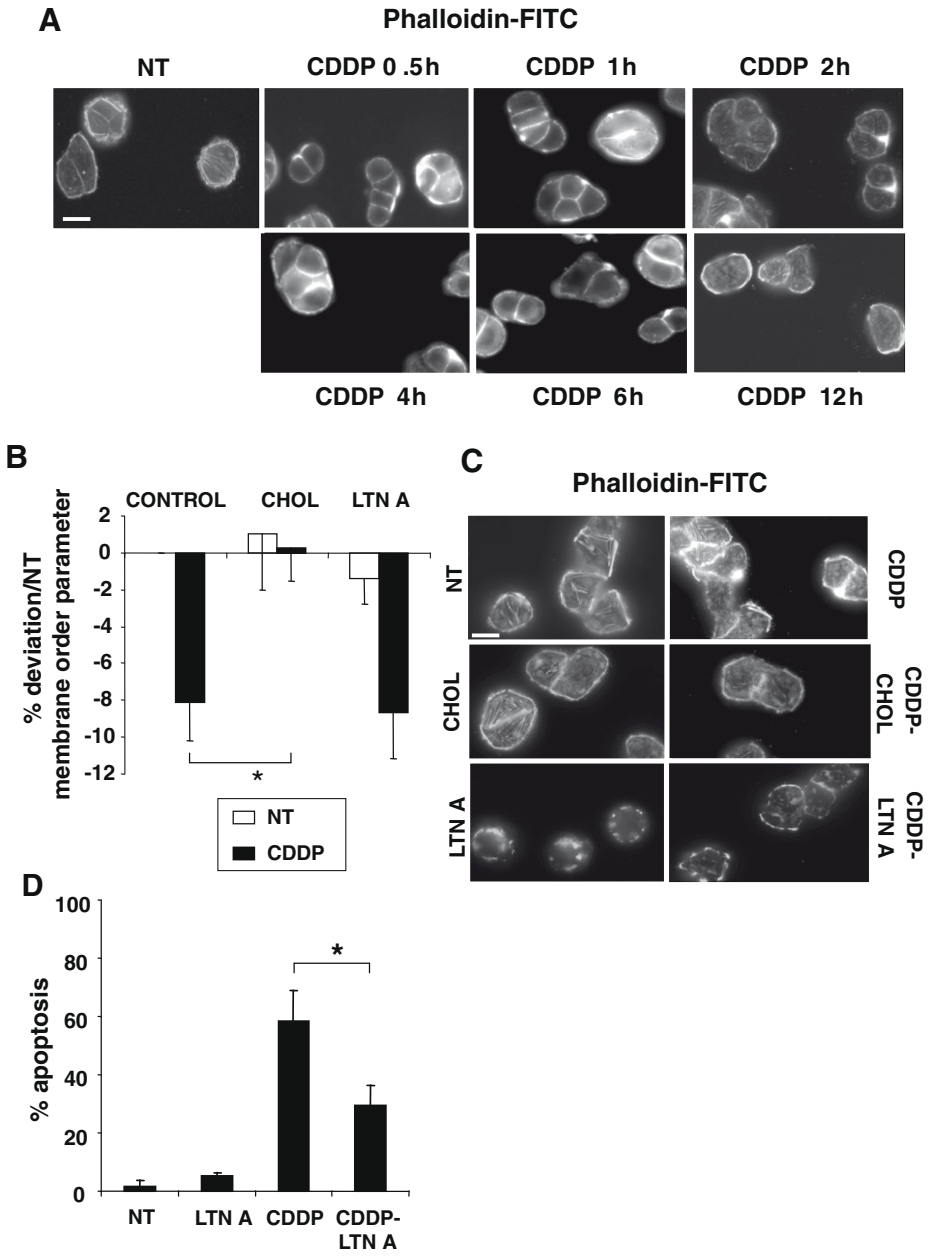

Fig. 1 – Cisplatin-induced apoptosis involves membrane fluidification-dependent actin reorganisation. (A) Cisplatin treatment reorganises actin cytoskeleton. HT29 cells were treated or not (NT) with 25 μM cisplatin (CDDP) for the indicated times. Actin microfilaments were evidenced by fluorescence microscopy using Phalloidin-FITC labelling of F-actin. One representative of three independent experiments is shown. Bars, 10 μM. (B) Pre-treatment with cholesterol but not with latrunculin A inhibits cisplatin-induced increase in membrane fluidity. HT29 cells were pre-treated or not (CONTROL) with 30 μg/mL cholesterol (CHOL) or 100 nM latrunculin A (LTN A) for 2 h, then left untreated or treated with 25 μM CDDP for 1 h. Membrane fluidity was determined by electronic paramagnetic resonance (EPR) using a spin-labelling method. The EPR spectra were used to calculate the membrane order parameter S which is conversely proportional to membrane fluidity. Data are expressed as % deviation relatively to non-treated (NT) cells (mean ± SEM of three independent experiments). $^{*}p \leqslant 0.05$, CHOL-CDDP versus CDDP. (C) Pre-treatment with cholesterol or latrunculin A prevents cisplatin-induced actin reorganisation. HT29 cells were pre-treated or not (NT) with 30 μg/ml CHOL or 100 nM LTN A for 2 h, then left untreated or treated with 25 μM CDDP for 2 h. Actin microfilaments were evidenced by fluorescence microscopy using Phalloidin-FITC staining as previously described. One representative of three independent experiments is shown. Bars, 10 μM. (D) Pre-treatment with latrunculin A reduces cisplatin-induced apoptosis. HT29 cells were pre-treated or not (NT) with 100 nM LTN A for 2 h, then left untreated or treated with 25 μM CDDP for 72 h. Percentages of apoptotic cells were estimated by nuclear chromatin staining with Hoechst 33342. Data are expressed as mean ± SEM of three independent experiments. $^{*}p \leqslant 0.05$, LTN A-CDDP versus CDDP.

of apoptotic cells in HT29 cells treated with CDDP (~60%) (Fig. 1D). These data indicate that cisplatin-induced actin cytoskeleton reorganisation significantly contributes to apoptosis induction.

3.2. *Ezrin is involved in cisplatin-induced apoptosis and co-localises with Fas receptor*

Recently, it has been proposed that Fas co-localisation with ezrin is an essential requirement for susceptibility to the Fas-mediated apoptosis.[15] By Western-blot analysis, we show that cisplatin induced a redistribution of ERM proteins into HT29 lipid rafts (fractions 1–7, enriched in cholesterol and characterised by flotillin-1 expression and by no expression of CD71), along with Fas, FADD and procaspase-8 (Fig. 2A). Interestingly, TNFR-1 was not redistributed into membrane lipid rafts after cisplatin treatment (Fig. 2A). Moreover, by fluorescence microscopy, a co-localisation of ezrin protein with Fas receptor was detected on the cell membrane of HT29 cells treated with cisplatin for 2 h (Fig. 2B). Finally, we used small interfering RNA (siRNA) targeting ezrin in order to evaluate the role of this protein in the cisplatin-induced apoptotic cascade. We observed that transient transfection with siRNA ezrin (siEzrin) inhibited the expression of ezrin protein (Fig. 2C, see inset) and reduced both cisplatin-induced apoptosis (Fig. 2C, left panel) and caspase-3 activation (Fig. 2C, right panel) by about 50% and 60%, respectively. Moreover, siRNA ezrin transfection inhibited cisplatin-induced actin reorganisation (Fig. 3). These results indicate that ezrin is involved in cisplatin-induced apoptosis and actin cytoskeleton reorganisation.

3.3. *Cisplatin rapidly activates RhoA and induces ROCK-dependent phosphorylation of ezrin*

Inactive ezrin protein resides in the cytoplasm of cells, whereas activated ezrin binds to the integral membrane proteins. Phosphorylation of a threonine residue (T567) within its C-terminal actin-binding domain is considered as a hallmark of ezrin activation. Moreover, the Rho family of GTPases[16] has been shown to contribute to the activation of ERM.[17] In order to study the molecular mechanisms underlying ezrin activation after cisplatin treatment, we evaluated the effect of this anticancer drug on the levels of GTP-bound RhoA. Fig. 4A clearly indicates that cisplatin treatment increased the expression of RhoA–GTP within 15–60 min. We next studied the phosphorylation of ezrin on the T567 residue by Western-blot analysis. These experiments show that cisplatin induced a transient phosphorylation of ezrin from 30 to 60 min after the beginning of cisplatin treatment (Fig. 4B). The Rho kinase ROCK is a major target of the small GTP-binding protein RhoA and a key signalling molecule involved in ezrin phosphorylation and cytoskeleton reorganisation.[18] Pretreatment of HT29 cells with a Rho inhibitor (Rho Inh) or transient transfection with siRNA ROCK (siROCK), which inhibited ROCK protein expression in HT29 cells (Fig. 4D, see inset), prevented cisplatin-induced ezrin phosphorylation (Fig. 4C). Moreover, transient transfection with siROCK inhibited cisplatin-induced apoptosis (Fig. 4D, left panel) and caspase-3 activation (Fig. 4D, right panel). These data strongly indicate that cisplatin-induced apoptosis involves ezrin phosphorylation through a RhoA–ROCK-dependent pathway.

3.4. *Fas is indispensable for ezrin phosphorylation and actin remodelling upon cisplatin treatment*

We have previously shown that the Fas death receptor pathway was involved in cisplatin-induced apoptosis in human colon cancer cells.[3] And it has been recently shown that phosphorylation of ezrin by ROCK was involved in the early steps of apoptotic signalling following Fas triggering.[13] By using transient transfection with siRNA Fas, we confirm the role of Fas in cisplatin-induced apoptosis. The transient transfection with siRNA Fas (siFas) for 48 h strongly decreased the expression of Fas protein in HT29 cells (Fig. 5A, see inset) and significantly reduced cisplatin-induced apoptosis (Fig. 5A, left panel) and caspase-3 activation (Fig. 5A, right panel) by about 50% and 70%, respectively. Moreover, decreased expression of Fas by RNA interference inhibited cisplatin-induced RhoA activation (increased levels of GTP-bound RhoA) (Fig. 5B), cisplatin-induced ezrin phosphorylation (Fig. 5C) and actin reorganisation (Fig. 5D). These results suggest for the first time that cisplatin triggers ezrin phosphorylation and actin reorganisation via Fas receptor, these events being involved in cisplatin-induced apoptosis in HT29 human colon cancer cells.

3.5. *Transient transfection with siRNA targeting Fas or ezrin inhibits cisplatin-induced apoptosis in two other human colon cancer cell lines, HCT116 and SW480*

Transient transfections of HCT116 and SW480 with siRNA targeting Fas (siFas) and ezrin (siEzrin), respectively, significantly reduced cisplatin-induced apoptosis (Fig. 6), which corroborates the results obtained in HT29 cells. Collectively, these data suggest that Fas and ezrin might play a role in cisplatin-induced apoptosis in human colon cancer cells.

4. Discussion

In this study, we show for the first time that cisplatin treatment induces a rapid and transient reorganisation of F-actin microfilaments via Fas death receptor pathway in HT29 cells. Disruption of actin cytoskeleton with LTN A, a toxin isolated from a Red Sea sponge, completely inhibits cisplatin-induced actin microfilament rearrangement. LTN A also significantly reduces apoptosis indicating an important role of actin in cisplatin-induced apoptosis. These data are in agreement with the increasing literature on the involvement of actin cytoskeleton in early and late stages of apoptosis.[19–21] In HT29 cells, we have previously shown that cisplatin-induced apoptosis involves plasma membrane fluidification independent of DNA adduct formation.[14] In order to study the role of membrane fluidity in cisplatin-induced actin polarisation, we used cholesterol, a membrane-stabilising agent, and found that actin reorganisation in HT29 cells was prevented under these conditions, thus suggesting that actin cytoskeleton rearrangement is dependent on membrane fluidification. Recently, Hannun's group has demonstrated that cisplatin

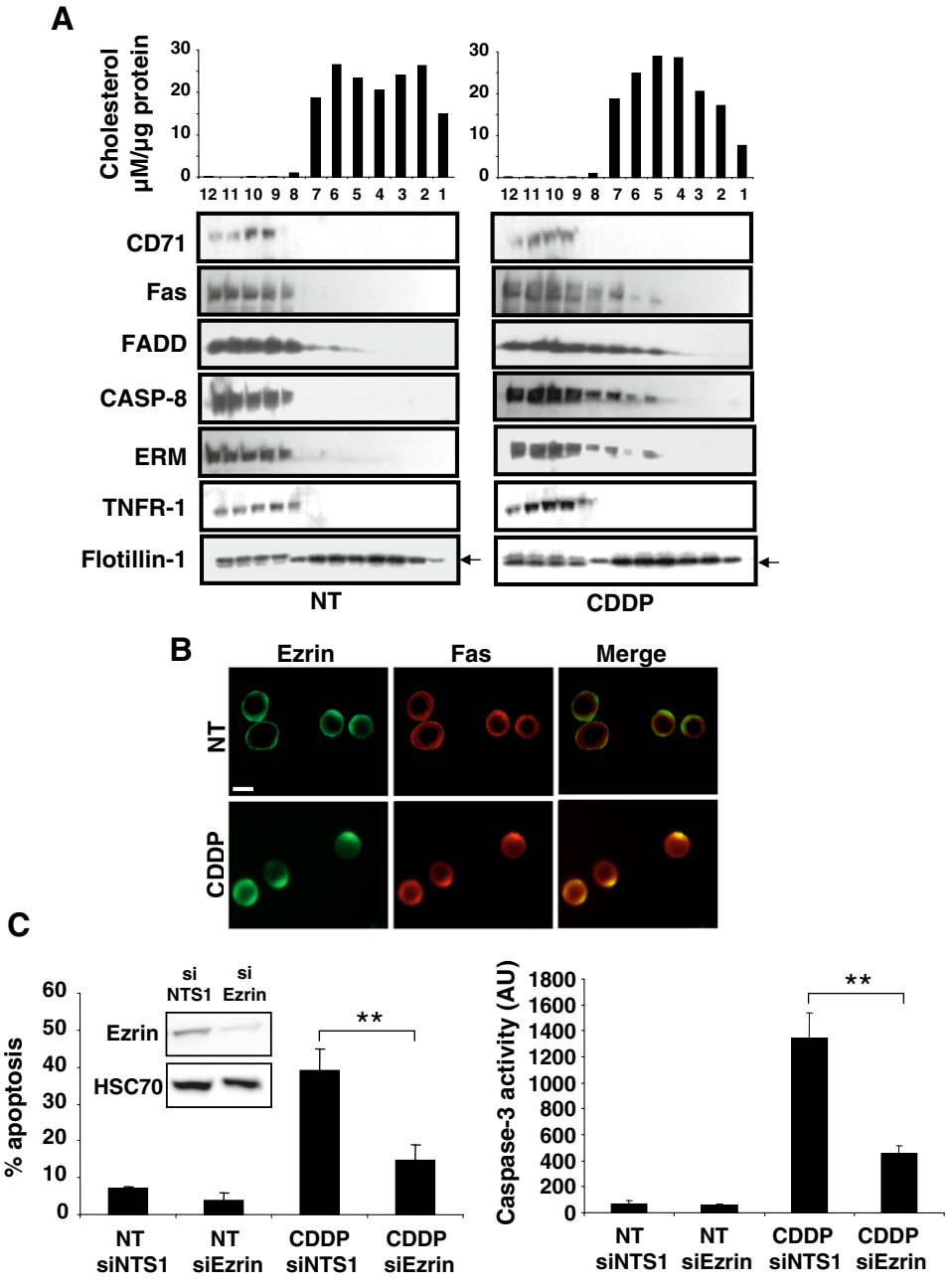

Fig. 2 – Ezrin is involved in cisplatin-induced apoptosis. (A) Cisplatin induces redistribution of ERM (Ezrin Radixin Moesin), Fas, Caspase-8 and FADD into lipid rafts of HT29 cells. HT29 cells were treated or not (NT) with 25 µM cisplatin (CDDP) for 6 h, then lysed in 1% Triton X-100 and fractionated on a linear sucrose density gradient by centrifugation. Cholesterol analysis was performed by a colorimetric assay using Infinity cholesterol kit (top). An equal volume of each collected fraction was submitted to SDS–PAGE before analysis of CD71, Fas, FADD, caspase-8, ERM, TNFR-1 and flotillin-1 expression by Western blot. One representative of three independent experiments is shown (bottom). (B) Cisplatin treatment induces co-localisation of ezrin with Fas receptor at the cell membrane. HT29 cells were treated or not (NT) with 25 µM CDDP for 2 h. Ezrin and Fas receptor expression were evidenced by fluorescence microscopy with an anti-ezrin antibody (Biogenesis) and an anti-Fas antibody (AbCam) staining. One representative of three independent experiments is shown. (C) Interfering with ezrin mRNA expression reduces cisplatin-induced apoptosis. HT29 cells were transiently transfected with siRNA ezrin (siEzrin) or siRNA NTS1 (siNTS1 used as a negative control). For Western-blot analysis (see inset), the cells were harvested 48 h after transfection. For cell death analysis, 48 h after cell transfection, HT29 cells were treated or not with 25 µM CDDP for 48 h. Percentages of apoptotic cells were estimated as in Fig. 1D. Data are expressed as mean ± SEM of three independent experiments. $^{**}p \leqslant 0.01$, siEzrin-CDDP versus siNTS1-CDDP (left panel). Caspase-3 activation was measured in lysates by the cleavage of the DEVD-AMC peptide substrate. Data are expressed in arbitrary units (AU) as mean ± SEM of three independent experiments. $^{**}p \leqslant 0.01$, siEzrin-CDDP versus siNTS1-CDDP (right panel).

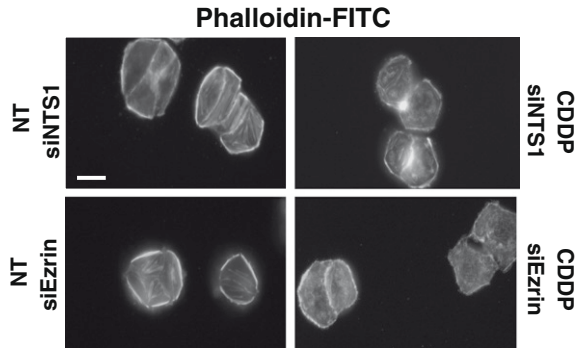

Fig. 3 – Interfering with Ezrin mRNA expression inhibits cisplatin-induced actin reorganisation. HT29 cells were transiently transfected with siRNA Ezrin (siEzrin) or siRNA NTS1 (siNTS1 used as a negative control). For fluorescence microscopy analysis, 48 h after cell transfection, HT29 cells were treated or not (NT) with 25 μM cisplatin (CDDP) for 1 h. Actin microfilaments were evidenced by fluorescence microscopy using Phalloidin-FITC staining. Bars, 10 μM. One representative of three independent experiments is shown.

induces actin remodelling via the acid sphingomyelinase/ceramide pathway in breast cancer cells.[22] These results are in agreement with our present data showing that cisplatin-induced actin reorganisation in HT29 cells is dependent on the plasma membrane fluidification, an event that requires acid sphingomyelinase activation.[14]

Recently, it has been proposed that remodelling of actin cytoskeleton is mainly regulated by ERM proteins.[23] After cisplatin treatment, we demonstrate that ERM proteins as well as DISC components are redistributed into lipid rafts of HT29 cells and that Fas co-localises with ezrin on plasma membrane suggesting a possible interaction between Fas and ezrin. Such a molecular interaction has been previously demonstrated in human T lymphocytes.[12] In fact, a specific CD95-binding domain has been identified in the N-terminal region of ezrin, more precisely in the middle lobe of the ezrin FERM domain.[24] Further co-immunoprecipitation experiments will be necessary to confirm a Fas–ezrin interaction upon cisplatin treatment in HT29 human colon cancer cells. However, transient transfection with specific siRNA ezrin inhibits cisplatin-induced actin reorganisation and significantly reduces the related apoptosis, thus pointing to an important role for ezrin in cisplatin-induced apoptotic process.

In the cytoplasm, ezrin is maintained in an inactive conformation through an intramolecular interaction between their N-terminal and C-terminal domains. The activation of ezrin occurs through conformational changes triggered by events including the activation by Rho GTPases and the phosphorylation of a conserved threonine (T567) in the C-terminal domain (for review see)[15,25] allowing its interaction with membrane proteins and F-actin.[12] Indeed, the Rho family may directly affect cell susceptibility to apoptosis by modulating the actin cytoskeleton.[26] In order to understand the origin of ezrin activation, we study the involvement of the small G protein RhoA that has been shown to be activated by Fas in Jurkat cells.[13] Our present study demonstrates that cisplatin induces an early and transient increase in the levels of GTP-bound RhoA, thus suggesting an activation of RhoA in our model. Different arguments support the idea that RhoA is a potent inducer of the serine/threonine kinase Rho kinase ROCK known to phosphorylate ezrin on the threonine 567 residue.[23] Accordingly, we show that cisplatin-induced ezrin phosphorylation is inhibited by pretreatment with a Rho inhibitor and is dependent on ROCK since transient transfection with specific siRNA ROCK totally prevents this event. Moreover, siRNA ROCK reduces by about 70% cisplatin-induced apoptosis, thus suggesting a major role for ROCK in this cell death pathway. Taken together, our data therefore suggest that cisplatin activates the RhoA–ROCK pathway, leading to ezrin phosphorylation, actin reorganisation and apoptosis. In another way, Hannun's group demonstrates that cisplatin induces actin remodelling through acid sphingomyelinase-dependent ezrin dephosphorylation, leading to a cytoplasmic localisation of this protein in MCF-7 breast cancer cells.[22] The discrepancy between these data and our results could be explained by the difference between the cell types and by the involvement of the Fas death pathway in cisplatin-induced apoptosis in human HT29 colon cancer cells.[3,8]

Cisplatin has been shown to bind to actin, leading to polymerisation alterations in a dose-dependent manner.[27] However, such a direct action is unlikely to occur under our experimental conditions since membrane fluidification, due to NHE1-dependent acid sphingomyelinase activation,[14] was found to be necessary for cisplatin-induced actin reorganisation. Recent data have shown that Fas engagement leads to Rho GTPases family activation which in turn activates ROCK and triggers actin reorganisation.[13,26,28] These results led us to investigate the involvement of this receptor in cisplatin-induced actin remodelling. We observe that transient transfection with specific siRNA Fas prevents cisplatin-induced increase in RhoA–GTP levels, actin reorganisation and ezrin phosphorylation and reduces both apoptosis and caspase-3 activation in HT29 cells by about 60%. This suggests for the first time that Fas receptor triggering is indispensable for actin microfilament rearrangement during cell death induced by cisplatin.

The exact role of actin in cisplatin-induced apoptosis requires definition. Recently, it has been shown that ezrin and actin were not required for Fas-rafts association but ezrin-mediated cytoskeleton association initiated receptor internalisation, a prerequisite step for the intracellular formation of DISC and apoptosis.[11] Thus, actin cytoskeleton might be required for the intracellular amplification of Fas receptor signalling, through DISC formation, leading to cell death.

In summary, these results provide new insights into the molecular ordering of the early events that occur independent of DNA adduct formation in cisplatin-induced apoptosis. Cisplatin treatment increases membrane fluidity, then relocalises Fas receptor into lipid rafts and ceramide-enriched membrane domains and concomitantly phosphorylates ezrin via ROCK leading to F-actin reorganisation and apoptosis (Fig. 7).

In conclusion, several studies have shown that cisplatin-induced apoptosis depends not only on cisplatin interactions with DNA,[1] but also on interactions with mitochondrial DNA

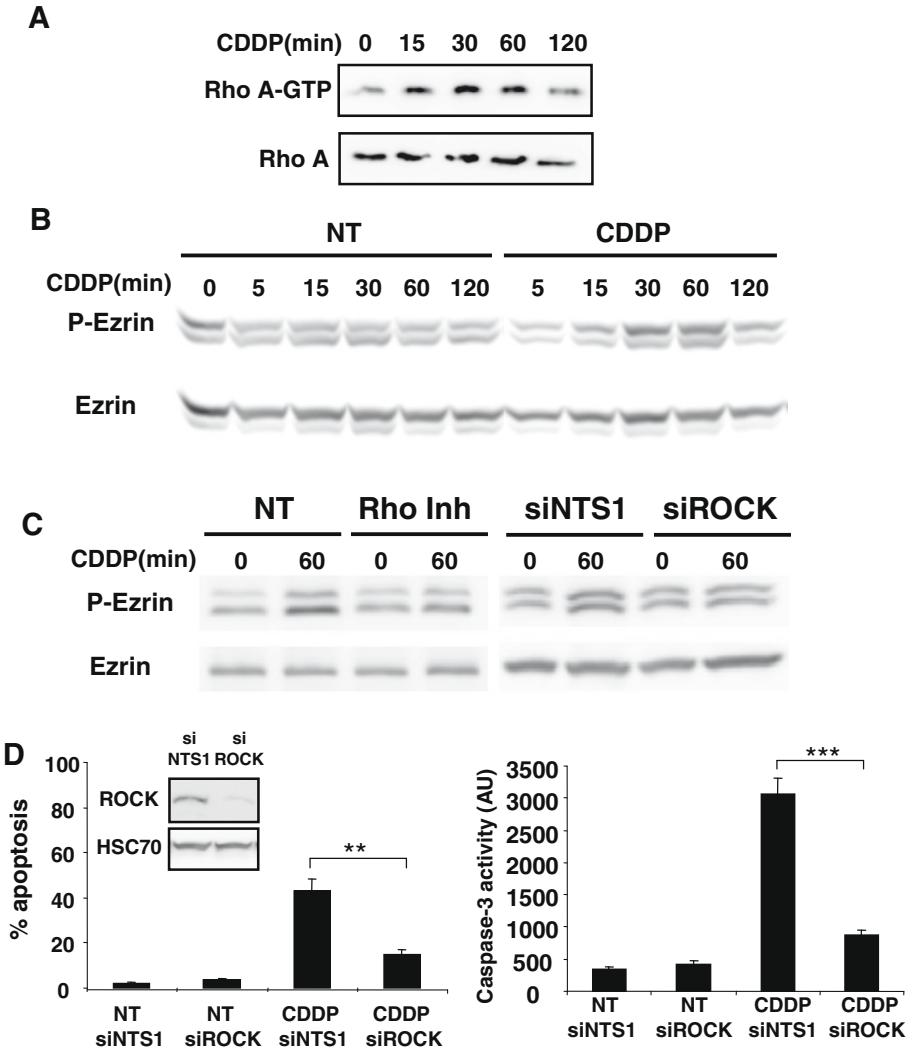

Fig. 4 – Cisplatin activates RhoA and induces ezrin phosphorylation on threonine 567 residue. (A) Cisplatin increases RhoA–GTP level in HT29 cells. HT29 cells were treated or not (0) with 25 μM cisplatin (CDDP) for the indicated times. The GTP-binding fraction of RhoA was pulled down as described in Materials and Methods. The bead/protein complexes and whole-cell lysates were immunoblotted to detect RhoA level. Equal gel loading and transfer efficiency were checked by protein hybridisation with anti-RhoA antibody. One representative of three independent experiments is shown. (B) Cisplatin induces a transient phosphorylation of ezrin. HT29 cells were treated or not with 25 μM CDDP for the indicated times. Phosphorylation of ezrin (P-Ezrin) was studied by Western-blot analysis using a mouse monoclonal anti-phospho-ezrin (Thr567). Ezrin expression was used as a loading control. One representative of three independent experiments is shown. (C) RhoA–ROCK-dependent ezrin phosphorylation is involved in cisplatin-induced apoptosis. HT29 cells were pre-treated or not (NT) with 0.1 μg/ml Rho Inh overnight, then left untreated or treated with 25 μM CDDP for 60 min. HT29 cells were transiently transfected with siRNA ROCK (siROCK) or siRNA NTS1 (siNTS1 used as a negative control). 48 h after cell transfection, HT29 cells were treated or not (NT) with 25 μM CDDP for 60 min. Phospho-ezrin expression was evidenced by Western-blot analysis as previously described in (B). One representative of three independent experiments is shown. (D) Interfering with ROCK mRNA expression significantly inhibits cisplatin-induced apoptosis. HT29 cells were transiently transfected with siRNA ROCK (siROCK) or siRNA NTS1 (siNTS1 used as a negative control). For Western-blot analysis (see inset), the cells were harvested 48 h after transfection. For cell death analysis, 48 h after cell transfection, HT29 cells were treated or not with 25 μM CDDP for 48 h. Percentages of apoptotic cells were estimated as in Fig. 1D. Data are expressed as mean ± SEM of three independent experiments. $^{**}p \leqslant 0.01$, siROCK-CDDP versus siNTS1-CDDP (left panel). Caspase-3 activation was measured as in Fig. 2C. Data are expressed in arbitrary units (AU) as mean ± SEM of three independent experiments. $^{***}p \leqslant 0.001$, siROCK-CDDP versus siNTS1-CDDP (right panel).

and voltage-dependent anion channels in the mitochondrial membrane.[29] More recently, our group has found that the exchanger Na^+/H^+ (NHE1) is another potential target for cisplatin cytotoxicity at the plasma membrane.[14] We now demonstrate that F-actin cytoskeleton reorganisation via Fas death pathway is also critical for cisplatin-induced apoptosis.

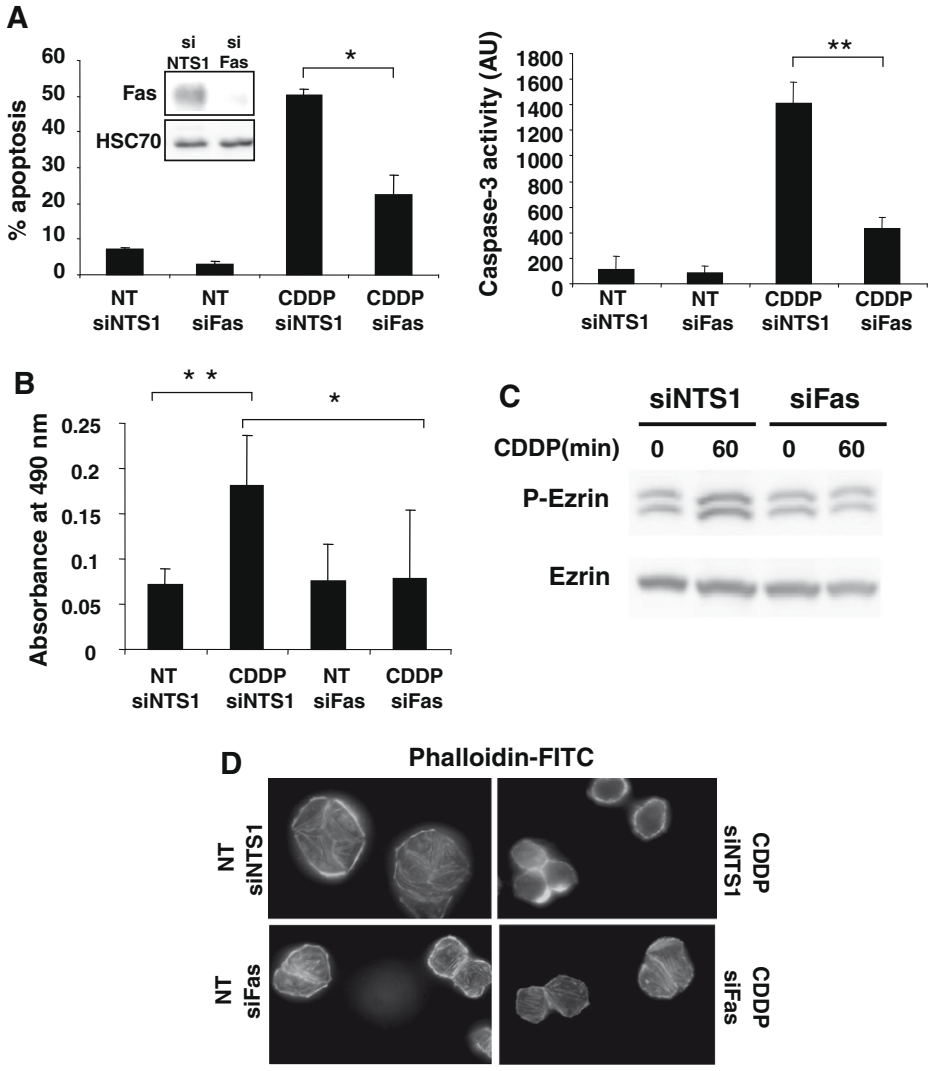

Fig. 5 – Fas is involved in cisplatin-induced actin reorganisation (A) Interfering with Fas mRNA expression reduces cisplatin-induced apoptosis. HT29 cells were transiently transfected with siRNA Fas (siFas) or siRNA NTS1 (siNTS1 used as a negative control). For Western-blot analysis (see inset), the cells were harvested 48 h after transfection. For cell death analysis, 48 h after cell transfection, HT29 cells were treated or not with 25 μM cisplatin (CDDP) for 48 h. Percentages of apoptotic cells were estimated as in Fig. 1D. Data are expressed as mean ± SEM of three independent experiments. $^{*}p \leqslant 0.05$, siFas-CDDP versus siNTS1-CDDP (left panel). Caspase-3 activation was measured as in Fig. 2C. Data are expressed in arbitrary units (AU) as mean ± SEM of three independent experiments. $^{**}p \leqslant 0.01$, siFas-CDDP versus siNTS1-CDDP (right panel). (B) Interfering with Fas mRNA expression inhibits cisplatin-induced RhoA activation. HT29 cells were transiently transfected with siRNA Fas (siFas) or siRNA NTS1 (siNTS1 used as a negative control). Forty-eight hours after cell transfection, HT29 cells were treated or not (NT) with 25 μM CDDP for 30 min and RhoA activation was measured with a G-LISA™ kit. Data shown are absorbance over background signal expressed as mean ± SEM of three independent experiments. $^{**}p \leqslant 0.01$, siNT1-CDDP versus siNTS1-NT; $^{*}p \leqslant 0.05$, siFas-CDDP versus siNTS1-CDDP. (C) Interfering with Fas mRNA expression inhibits cisplatin-induced ezrin phosphorylation. HT29 cells were transiently transfected with siRNA Fas (siFas) or siRNA NTS1 (siNTS1 used as a negative control). Forty-eight hours after cell transfection, HT29 cells were treated or not (NT) with 25 μM CDDP for 1 h. Phospho-ezrin (P-Ezrin) expression was evidenced by Western-blot analysis as previously described in Fig. 4B. One representative of three independent experiments is shown. (D) Interfering with Fas mRNA expression inhibits cisplatin-induced actin reorganisation. HT29 cells were transiently transfected with siRNA Fas (siFas) or siRNA NTS1 (siNTS1 used as a negative control). For fluorescence microscopy analysis, 48 h after cell transfection, HT29 cells were treated or not (NT) with 25 μM CDDP for 2 h. Actin microfilaments were evidenced by fluorescence microscopy using phalloidin-FITC. Bars, 10 μM. One representative of three independent experiments is shown.

Malignant cells often have altered expression of Fas or low F/G-actin ratio (increased non-polymerised G-actin with concomitantly decreased polymerised double-helical F-actin)[30] which could contribute to cisplatin resistance in cancer cells.

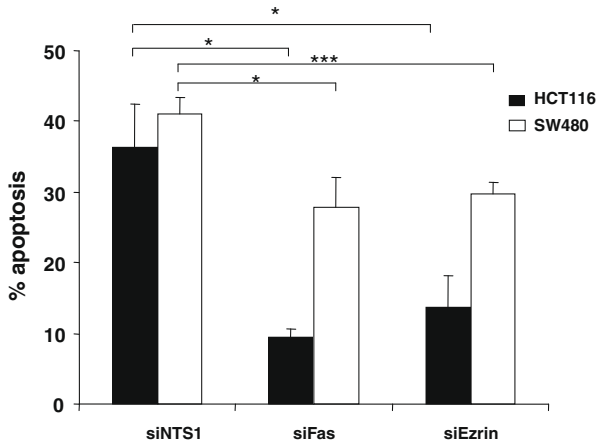

Fig. 6 – Transient transfection with siFas or siEzrin significantly inhibits cisplatin-induced apoptosis in HCT116 and SW480 human colon cancer cells. HCT116 and SW480 cells were transiently transfected with siNTS1, siFas or siEzrin for 48 h, then treated with 25 μM CDDP for 48 h. Percentage of apoptosis was determined as in Fig. 1D. Data are expressed as mean ± SEM of three independent experiments. $^{*}p \leqslant 0.05$, $^{***}p \leqslant 0.001$, siFas-CDDP versus siNTS1-CDDP or siEzrin-CDDP versus siNTS1-CDDP.

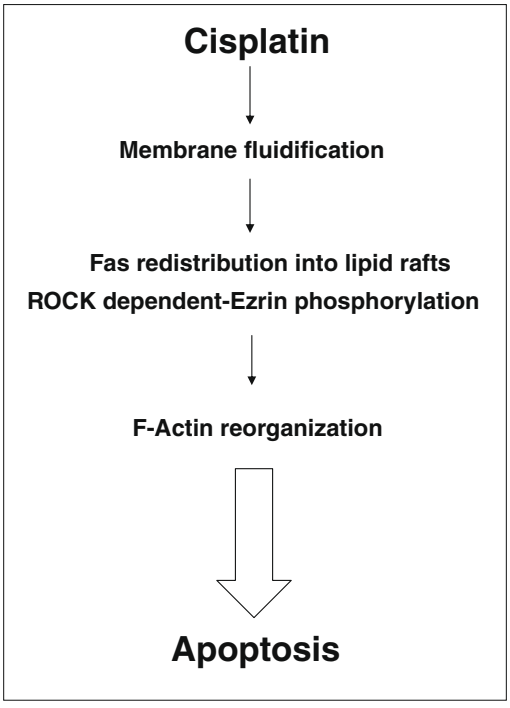

Fig. 7 – Hypothetical scheme showing the molecular ordering of the early Signalling events in cisplatin-induced apoptosis.

In fact, the Fas death pathway has been considered as a possible target for cancer treatment[31] and a loss of drug-induced activation of the CD95-signalling pathway has been observed in a cisplatin-resistant testicular germ tumour cell line,[32] suggesting that a functional CD95-signalling pathway may be an important factor determining cisplatin sensitivity. Altogether, these findings may serve to define new therapeutical strategies based on cisplatin therapy taking into account the functionality of the Fas death receptor pathway that could be disrupted at several steps in human cancers.

Conflict of interest statement

None declared.

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