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ORIGINAL PAPER

# A chemical inhibitor of Apaf-1 exerts mitochondrioprotective functions and interferes with the intra-S-phase DNA damage checkpoint

**Laura Mondragón · Lorenzo Galluzzi · Shahul Mouhamad · Mar Orzáez · José-Miguel Vicencio · Ilio Vitale · Alejandra Moure · Angel Messeguer · Enrique Perez-Paya · Guido Kroemer**

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**Abstract** QM31 represents a new class of cytoprotective agents that inhibit the formation of the apoptosome, the caspase activation complex composed by Apaf-1, cytochrome *c*, dATP and caspase-9. Here, we analyzed the cellular effects of QM31, as compared to the prototypic caspase inhibitor Z-VAD-fmk. QM31 was as efficient as Z-VAD-fmk in suppressing caspase-3 activation, and conferred a similar cytoprotective effect. In contrast to Z-VAD-fmk, QM31 inhibited the release of cytochrome *c* from mitochondria, an unforeseen property that may contribute to its pronounced cytoprotective activity. Moreover, QM31 suppressed the Apaf-1-dependent intra-S-phase DNA damage checkpoint. These results suggest that QM31 can interfere with the two known functions of Apaf-1, namely apoptosome assembly/activation and intra-S-phase cell cycle arrest. Moreover, QM31 can inhibit mitochondrial outer membrane permeabilization, an effect that is independent from its action on Apaf-1.

**Keywords** Apoptosis · Caspases · Cell cycle · Cisplatin · DNA damage · Mitochondria · Non-small cell lung cancer (NSCLC)

**Abbreviations**

| | |
|---|---|
| Apaf-1 | Apoptotic protease activating factor 1 |
| AnnV | Annexin V |
| ATM | Ataxia telangiectasia mutated kinase |
| ATR | Ataxia telangiectasia and Rad3 related kinase |
| CARD | Caspase activation recruitment domain |
| CDDP | Cisplatin |
| Chk1 | Checkpoint kinase 1 |
| Cyt *c* | Cytochrome *c* |
| $\Delta\Psi_m$ | Mitochondrial transmembrane potential |
| $DiOC_6(3)$ | 3,3′ dihexiloxalocarbocyanine iodide |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| IMS | Mitochondrial intermembrane space |

L. Mondragón · L. Galluzzi · S. Mouhamad · J.-M. Vicencio · I. Vitale · G. Kroemer
U848, INSERM, 94805 Villejuif, France

L. Mondragón · L. Galluzzi · S. Mouhamad · J.-M. Vicencio · I. Vitale · G. Kroemer (✉)
Institut Gustave Roussy, PR1, 39 rue Camille Desmoulins, 94805 Villejuif, France
e-mail: kroemer@igr.fr

L. Mondragón · L. Galluzzi · S. Mouhamad · J.-M. Vicencio · I. Vitale · G. Kroemer
Université Paris-Sud/Paris XI, 94270 Le Kremlin Bicêtre, France

L. Mondragón · M. Orzáez · E. Perez-Paya
Department of Medicinal Chemistry, Centro de Investigacion Principe Felipe, 46013 Valencia, Spain

S. Mouhamad
U841, INSERM, 94010 Créteil, France

S. Mouhamad
Hôpital Henri Mondor, 94010 Créteil, France

A. Moure · A. Messeguer
Department of Chemical and Molecular Nanotechnology, IQAC-CSIC, 08034 Barcelona, Spain

E. Perez-Paya
Instituto de Biomedicina de Valencia, CSIC, 46010 Valencia, Spain

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|---|---|
| MOMP | Mitochondrial outer membrane permeabilization |
| NSCLC | Non-small cell lung cancer |
| OM | Mitochondrial outer membrane |
| PARCS | Pro-apoptotic protein required for cell survival |
| PGA | Poly-L-glutamic acid |
| PI | Propidium iodide |
| siRNA | Small interfering RNA |
| Z-VAD-fmk | Z-Val-Ala-Asp(OMe)-fluoromethylketone |

## Introduction

The development of cytoprotective agents that avoid pathogenic cell loss constitutes a major challenge for drug development. Apoptosis constitutes the cell death modality that is best characterized in the context of embryogenesis, organogenesis and adult tissue homeostasis. Moreover, apoptosis is induced by numerous pathological insults including infectious agents, iatrogenic damage (such as anti-cancer chemotherapy and radiotherapy), toxic agents (such as heavy metals and environmental toxins), ischemia and degenerative processes [1–3]. Therefore, both acute and chronic processes that involve the loss of proliferating or post-mitotic cells might be attenuated or even avoided by efficient anti-apoptotic drugs [4].

In contrast to necrosis, apoptosis involves a highly organized ("programmed") sequence of biochemical events that accompany the cellular demise. The "point-of-no-return", which defines the boundary between life and death, is determined by two closely interwoven processes. First, a panoply of distinct damage (pro-apoptotic) signals converge on mitochondria and promote mitochondrial outer membrane permeabilization (MOMP). MOMP allows for the release into the cytosol of proteins that are usually retained within the mitochondrial intermembrane space (IMS) by the intact mitochondrial outer membrane (OM). Among these proteins, cytochrome *c* (Cyt *c*) is the major trigger for the second decisive biochemical event in the intrinsic (mitochondrial) apoptotic pathway, i.e., caspase activation. In the presence of dATP (or ATP), cytosolic Cyt *c* (which in the IMS functions as an electron shuttle of the respiratory chain) [5, 6] interacts with apoptosis protease activating factor 1 (Apaf-1) [7], thereby favoring its heptamerization into a supramolecular complex known as the "apoptosome" [8, 9]. Within the apoptosome, oligomerized Apaf-1 recruits and allosterically activates pro-caspase-9 [10], which in turn proteolytically processes the principal executioner caspase of apoptosis, i.e., caspase-3 [11]. The mitochondrial release of Cyt *c* [12] (and of other proteins that have nonapoptotic roles within the IMS) [5, 6], as well as caspase activation [13], then results in the progressive impairment of mitochondrial function. This includes the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) that is required for maintaining the vital redox and bioenergetic activities of mitochondria [14]. Both MOMP and caspase activation suffice to seal the cell's fate, and both stimulate each other in multiple feed-forward signaling loops that amplify pro-apoptotic stimuli [12, 13, 15].

Caspases and mitochondria are pharmacological and genetic targets for cytoprotection. Thus, several major pharmaceutical companies have generated inhibitors of the proteolytic activity of caspases. Moreover, drugs that are able to inhibit MOMP have been shown to exert relevant cardioprotective or neuroprotective effects in animal models of infarction, stroke and trauma [3, 4]. Recently, we have started a novel approach to identify apoptosome inhibitors based on diversity-oriented chemical libraries. These molecular collections were screened for compounds that would inhibit the activation of caspase-9 in vitro, as driven by a chemically defined complex composed of highly purified Cyt *c*, recombinant Apaf-1 and pro-caspase-9 proteins, and dATP [16]. The first lead compound identified by these screening efforts was a linear pseudopeptoid ("peptoid 1"), which exhibited potent effects in cell-free assays but low cellular uptake and modest efficacy in cell-based tests [16]. The conjugation of peptoid 1 to cell penetrating peptides (e.g., penetratin, HIV-1 Tat) [17] or to a water soluble polymeric carrier (poly-L-glutamic acid, PGA) [18] resulted in improved internalization and anti-apoptotic activity. Similar goals were achieved by reducing the conformational mobility of peptoid 1 via backbone cyclization, which lead to the synthesis of QM31 (previously named "compound 2") [16, 19]. As previously shown by our group, QM31 is able to inhibit anthracyclin-induced apoptosis in a variety of transformed human cell lines of epithelial and mesenchymal origin, and to preserve the viability and function of primary myocardiocytes in conditions of hypoxia. Moreover, QM31 efficiently inhibits cell death induced by doxycycline-inducible Bax overexpression in human osteosarcoma cells [19]. These functional assays performed on intact cells suggest that QM31 may constitute the first representative of a new class of cytoprotective agents that inhibit the apoptosome yet have no direct effect on the proteolytic activity of caspases.

Apaf-1, the central component of the apoptosome, is subjected to major conformational changes during mitochondrial apoptosis. These modifications are influenced by transient interactions between Apaf-1 and ATP (which upon hydrolysis to ADP inhibits the apoptosome) [20], Cyt *c*, as well as several other cofactors [21]. Apaf-1 exerts also a completely different function, which is clearly separated from its role within the apoptosome. In response to

genotoxic stress, Apaf-1 translocates to the nuclear envelope, depending on the activation of DNA damage sensors such as the ataxia telangiectasia mutated (ATM) and/or the ataxia telangiectasia and Rad3 related (ATR) kinases [22]. Knockout or knockdown of Apaf-1 attenuates the DNA damage-induced activating phosphorylation of the checkpoint kinase Chk1, and weakens the intra-S-phase DNA damage checkpoint, thereby facilitating chromosomal instability [22, 23]. The depletion of one particular interactor of Apaf-1, i.e., pro-apoptotic protein required for cell survival (PARCS), has been reported to reduce caspase activation in a cell-free assay, and to block proliferation of non-transformed cells (but not of multiple tumor cell lines) [24]. Similarly to PARCS, Aven (another Apaf-1 binding partner) is able to inhibit caspase activation in cell-free extracts [25]. Moreover, it has been recently shown that Aven cooperates with ATM in a positive feedback loop for the transduction of the DNA damage signal [26]. Nevertheless, the exact contribution of PARCS and Aven to the Apaf-1-dependent intra-S-phase DNA damage checkpoint has not been determined thus far.

Based on these premises, we decided to investigate the cellular effects of QM31, in particular by focusing on potential mitochondrial and cell cycle effects that could be related to the complex biology of the Apaf-1 system. Here, we provide evidence that QM31 exerts unexpected MOMP-inhibitory ("mitochondrioprotective") functions and that QM31 weakens the Apaf-1-dependent intra-S-phase DNA damage checkpoint.

## Materials and methods

### Cell culture, siRNA transfection and treatments

Non-small cell lung cancer (NSCLC) A549 cells were routinely maintained in F-12 medium supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes buffer, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Cells were transfected with an Apaf-1-specific siRNA (siApaf-1, sense 5′-GAGCAGCUAUGCUGAUUAAdT dT-3′) or with an irrelevant control siRNA (siUNR, sense 5′-GCCGUAUGCCGGUUAAGUdTdT-3′), by means of Oligofectamine™ transfection reagent (Invitrogen), according to the manufacturer's recommendations. Media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA). Unless otherwise indicated, chemicals were purchased from Sigma–Aldrich (St. Louis, USA).

For apoptosis induction experiments, cells were either left untreated or incubated with 50 μM Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-fmk) for 1 h, followed or not by the administration of 50 μM *cis*-diammineplatinum(II) dichloride (cisplatin, CDDP). When required, 5 μM QM31 was administered 1 h after CDDP addition, and cells were maintained in culture for 48 h. For cell cycle arrest assays, cells that had previously been transfected with the indicated siRNAs (or left untreated) for 48 h were incubated with 2.5, 5 or 7.5 μM QM31 for 1 h, followed or not by the administration of 20 μM CDDP for a total time of 24 h. For chromosomal instability determinations, cells were pre-treated or not with 5 μM QM31 for 1 h and then subjected to 5 J/m$^2$ UVC irradiation (upon removal of the culture medium), followed by culture in normal conditions for 24 h. During the irradiation period, control cultures were left untreated in the absence of the culture medium.

### Cytofluorometry, immunofluorescence microscopy and immunoblotting

Caspase-3 activation was monitored by staining the cells with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody specific for active caspase-3 (Becton Dickinson, Franklin Lankes, USA). To quantify apoptosis-associated parameters, live cells were co-stained with 40 nM 3,3′ dihexiloxalocarbocyanine iodide ($DiOC_6(3)$, from Molecular Probes-Invitrogen), which measures mitochondrial transmembrane potential ($\Delta\Psi_m$), and 1 μg/ml propidium iodide (PI), which identifies cells with ruptured plasma membrane [27]. To assess phosphatidylserine exposure and to quantify the release of cytochrome *c* from mitochondria, the Annexin V-FITC kit (Miltenyi Biotec GmbH, Bergisch Galdbach, Germany) and the Innocyte™ Flow Cytometric Cytochrome *c* Release kit (Calbiochem, San Diego, USA) were employed, respectively, according to the manufacturer's recommendations. For cell cycle analysis, cells were fixed in 80% ice-cold ethanol and labeled with PI (50 μg/ml) in the presence of 500 μg/ml RNAse. Cytofluorometric determinations were performed with a FACSCalibur or a FACScan equipped with Cell Quest Pro software (Becton Dickinson).

Immunofluorescence assessments were carried out as previously reported [22, 28]. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% sodium dodecylsulfate and labeled with primary antibodies specific for cytochrome *c* (mouse monoclonal, #556432, BD Pharmingen, San Diego, USA), or phospho-Chk1 (Ser317, rabbit polyclonal, #2344, Cell Signaling Technology, Beverly, USA), according to the manufacturers' instructions. Then, goat anti-rabbit or anti-mouse antibodies coupled to Alexa Fluor® 568 (emitting in red) or Alexa Fluor® 488 (emitting in green) fluorochromes (Molecular Probes-Invitrogen) were used for revelation. Nuclei were counterstained with 10 μM Hoechst 33342 (Molecular Probes-Invitrogen) and slides were observed on a LSM 510

confocal fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). Quantitative immunofluorescence assessments were carried out on a DMIR2 inverted fluorescence microscope equipped with a DC300F camera (Leica Microsystems GmbH, Wetzlar, Germany).

For immunoblotting determinations, cells were lysed on ice in a buffer containing 1% NP40, 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM dithiothreitol, and 10 μg/ml aprotinin, leupeptin, pepstatin followed by centrifugation (20 min, 14,000 rpm) and collection of supernatants. Total protein extracts were then separated on pre-cast 4–12% polyacrylamide gradient gels (Invitrogen) and analyzed following standard immunoblotting procedures [29, 30], by using primary antibodies specific for Apaf-1 (mouse monoclonal, #Mab868, R&D Systems, Minneapolis, USA), Chk1 (rabbit polyclonal, #2345, Cell Signaling Technology), phospho-Chk1 (Ser317, rabbit polyclonal, #2344, Cell Signaling Technology) or *β*-actin (mouse monoclonal, #MAB1501, Chemicon International, Temecula, USA), which was employed to ensure equal loading of lanes.

## Results and discussion

### Comparison of the cytoprotective effects of QM31, Z-VAD-fmk and Apaf-1 knockdown

Apaf-1 is required for two distinct responses to the DNA damaging agent cisplatin (CDDP), namely induction of apoptosis and cell cycle arrest in the S phase [22]. We therefore studied the response to CDDP of non-small cell lung cancer (NSCLC) A549 cells in the continuous presence of the chemical Apaf-1 inhibitor QM31 or of the pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-fmk). After 48 h of treatment, the proteolytic maturation of caspase-3 was monitored by staining fixed and permeabilized cells with a fluorescein isothiocyanate (FITC)-conjugated antibody that recognizes the large p17 and p20 subunits of active caspase-3, followed by cytofluorometric analysis (Fig. 1a, b). As expected, both Z-VAD-fmk and QM31 relevantly reduced the percentage of cells displaying caspase-3 activation upon CDDP treatment (Fig. 1b). When the fate of cells was monitored by the combined staining with the vital dye propidium iodide (PI, which is excluded from cells with intact plasma membranes) and FITC-labeled Annexin V (AnnV, which binds to phosphatidylserine moieties exposed on the surface of dying cells) (Fig. 1c, d), we found that Z-VAD-fmk and QM31 were equally efficient in reducing the frequency of dead ($PI^+$) and dying cells ($PI^-/AnnV^+$) (Fig. 1d) The fact that QM31 is slightly less potent than Z-VAD-fmk in inhibiting caspase activation (Fig. 1b), yet displays an analogous efficacy in preventing cell death (Fig. 1d), suggests that the cytoprotective effects of QM31 may not be exclusively mediated by caspase inhibition.

### Mitochondrioprotective effect of QM31

Intrigued by the cellular events underlying the aforementioned discrepancy, we compared the effect of QM31 and Z-VAD-fmk on mitochondrial alterations that precede cell death, and in particular on MOMP and $\Delta\Psi_m$ dissipation. MOMP was monitored by assessing the mitochondrial release of Cyt *c*, either by in situ immunofluorescence microscopy (by which Cyt *c* release is documentable as a change from a cytoplasmic, speckled pattern of fluorescence to a diffuse signal that apparently involves both the cytoplasm and the nucleus, Fig. 2a, b), or by cytofluorometry (according to a protocol that allows to detect Cyt *c* liberation as a reduced staining of whole cells, thanks to the washout of cytoplasmic Cyt *c* prior to labeling, Fig. 2c, d). Using either of these two methods, we found that CDDP-induced MOMP was only partially inhibited by Z-VAD-fmk, yet strongly reduced by QM31 (Fig. 2b, d). The loss of $\Delta\Psi_m$ and subsequent cell death were also determined by simultaneous staining with the $\Delta\Psi_m$-sensitive cationic dye 3,3′ dihexiloxalocarbocyanine iodide ($DiOC_6(3)$) and the vital dye PI (Fig. 2e, f). In accord with published observations [31, 32], Z-VAD-fmk was able to stabilize the $\Delta\Psi_m$, even in cells that had undergone MOMP. Z-VAD-fmk and QM31 were equally potent in maintaining high $\Delta\Psi_m$ levels (Fig. 2f). Altogether, these data indicate that QM31 has the unexpected capacity to prevent MOMP, which may be related to the ability of Apaf-1 to bind to antiapoptotic Bcl-2 family proteins [33, 34].

### Inhibition of the intra-S-phase DNA damage checkpoint by QM31

The experiments described above have been performed in conditions in which the pro-apoptotic effect of CDDP is well discernible (50 μM CDDP, 48 h of treatment). In sub-apoptotic conditions (20 μM CDDP, 24 h of treatment), CDDP causes the arrest of NSCLC A549 cells in the S phase of the cell cycle, which is characterized by a gradual increase from a 2N DNA content (at the $G_1$-S transition) to a 4N one (in cells that are entering mitosis). The absence of Apaf-1 function prevents this cell cycle blockade in multiple model organisms [22, 23]. As assessed by cytofluorometric analysis of cell cycle distribution, this manifestation of the intra-S-phase DNA damage checkpoint was gradually subverted by increasing concentrations of QM31 (Fig. 3a, b). An Apaf-1-specific siRNA (Fig. 3c)

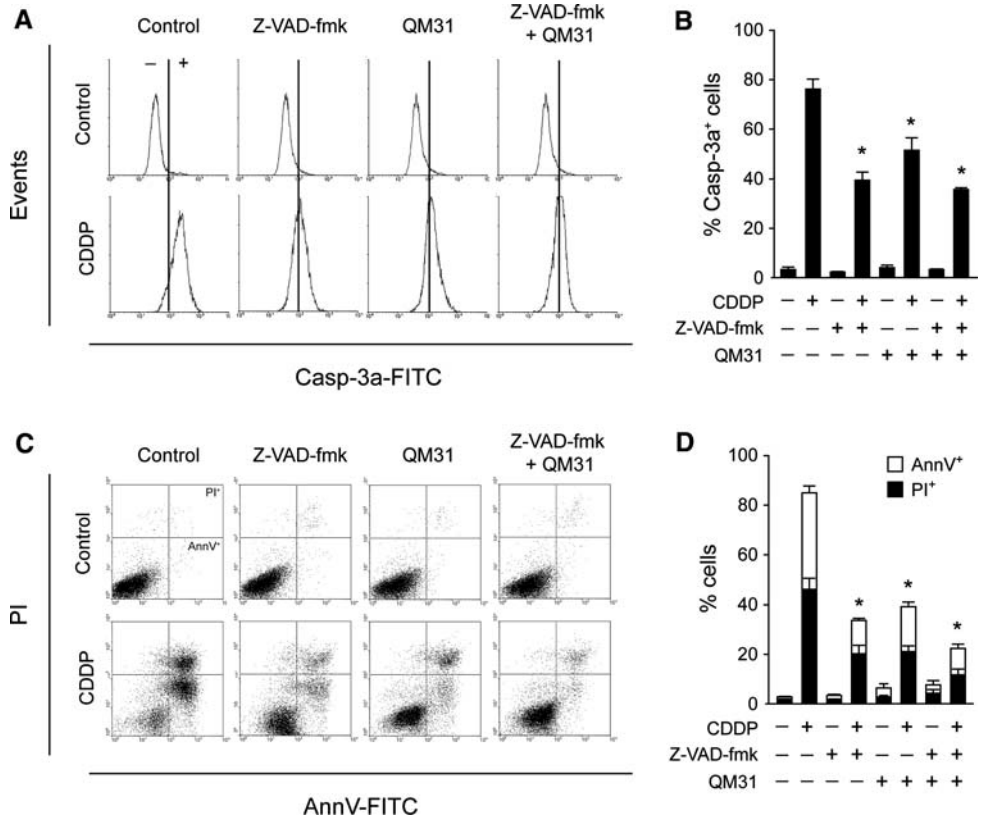


**Fig. 1** Non-small cell lung cancer (NSCLC) A549 cells were left untreated or incubated with 50 μM Z-VAD-fmk for 1 h, followed or not by the administration of 50 μM cisplatin (CDDP). When required, 1 h later, QM31 was added at the final concentration of 5 μM and cells were maintained in culture for additional 48 h prior to the cytofluorometric assessment of apoptosis-related parameters. Panels **a** and **c** depict representative caspase-3 activation profiles and Annexin V/propidium iodide (AnnV/PI) dot plots, respectively. Quantitative data are reported in panels **b** and **d** (mean ± SEM, $n = 4$). In panel **b**, columns indicate the percentage of cells which stained positive for active caspase-3 (Casp-3a$^+$ cells). In panel **d**, black and white columns illustrate the percentage of dead (PI$^+$) and dying (PI$^-$/AnnV$^+$) cells, respectively. Asterisks indicate statistically significant data (Student's $t$ test, $P < 0.05$), as compared to CDDP-treated cells. FITC, fluorescein isothiocianate

also reduced the number of cells retained in the S phase of the cell cycle following CDDP sub-apoptotic stimulation (Fig. 3b). The degree of this reduction (~10–15%) could have been negatively influenced by the incomplete siRNA-mediated downregulation of Apaf-1 (Fig. 3c), yet was in line with previously published results [22, 23]. In this setting, siRNA-mediated partial depletion of Apaf-1 and QM31 administration failed to exhibit additive or synergistic effects (Fig. 3c), suggesting that the Apaf-1-specific siRNA and QM31 affect the same molecular pathway of CDDP-induced cell cycle blockade. One of the principal mediators of the Apaf-1-dependent intra-S-phase DNA damage checkpoint is the checkpoint kinase Chk1 [22]. Chk1 activation in S phase cells delays DNA replication, stabilizes stalled replication forks and blocks mitotic entry [35]. As detectable by both immunoblotting (Fig. 4a) and immunofluorescence staining (Fig. 4b), QM31 partially reduced the activating phosphorylation of Chk1 following CDDP-triggered DNA damage (Fig. 4c).

## Concluding remarks

Apaf-1 is a rather large protein (130 kDa) that carries multiple functional domains, namely an *N*-terminal caspase activation recruitment domain (CARD), the CED4 homology domain (which include the dATP/ATP-binding motif), as well as 13 C-terminal WD-40 repeats, which allow for its interaction with Cyt *c* and for its oligomerization [36, 37]. Deletion mapping revealed that all these domains are necessary for caspase-9 activation in mammalian cytosolic extracts and intact cells [38–40]. In contrast, both the CARD domain and the WD-40 repeats are dispensable for the contribution of Apaf-1 to the intra-S-phase DNA damage checkpoint [22]. QM31 has been originally identified while searching for agents that could prevent pro-caspase-9 processing in vitro, by the apoptosome reconstituted with purified components [17]. We suggest—based on the functional experiments that are presented in this article—that QM31 alters the global

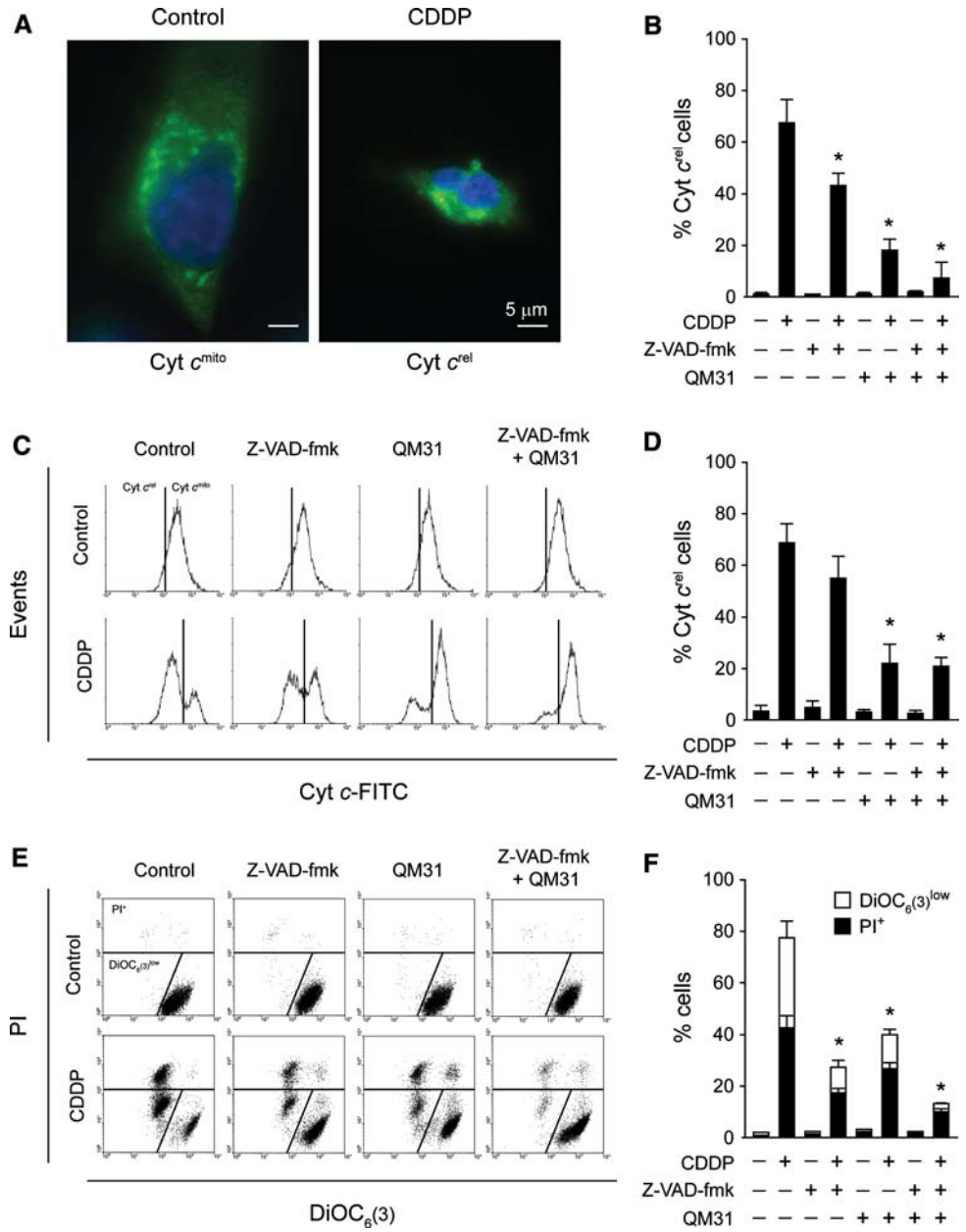


**Fig. 2** Non-small cell lung cancer (NSCLC) A549 cells treated as in Fig. 1 were fixed, permeabilized and stained for the immunofluorescence microscopy-assisted detection of cytochrome *c* (Cyt *c*) release (**a**, **b**), or subjected to the cytofluorometric determination of parameters that reflect the mitochondrial status during cell death (**c**–**f**). In panel **a**, representative immunofluorescence microscopy pictures are shown to exemplify mitochondrial (Cyt $c^{mito}$) versus released (Cyt $c^{rel}$) Cyt *c* (green signal). Nuclei appear in blue due to Hoechst 33,342 chromatin counterstaining. White bars indicate picture scale. Columns in panel **b** report the percentage of cells (mean ± SEM, $n = 4$) which exhibited diffuse Cyt *c* staining (Cyt $c^{rel}$ cells). Panels **c** and **e** illustrate representative Cyt *c* release profiles and $DiOC_6(3)$/propidium iodide ($DiOC_6(3)$/PI) dot plots, respectively. Quantitative data are reported in panels **d** and **f** (mean ± SEM, $n = 4$). In panel **d**, columns indicate the percentage of cells (mean ± SEM, $n = 4$) exhibiting Cyt *c* release (Cyt $c^{rel}$ cells). In panel **f**, white and black columns report the percentage of cells characterized by mitochondrial transmembrane dissipation alone ($DiOC_6(3)^{low}/PI^-$) or in combination with plasma membrane breakdown ($PI^+$). Asterisks mark statistically significant results (Student's *t* test, $P < 0.05$), as compared to cells treated with cisplatin (CDDP) alone. FITC, fluorescein isothiocianate

conformation of Apaf-1 in a way that it impinges on both functions of Apaf-1, as a DNA damage checkpoint protein and as a constituent of the apoptosome. As an alternative explanation, QM31 might directly affect the nuclear function of Apaf-1 or disturb the interactions between Apaf-1 and those co-factors (perhaps including PARCS and/or Aven) that might mediate its dual implication in caspase activation and cell cycle blockade [24, 25, 41].

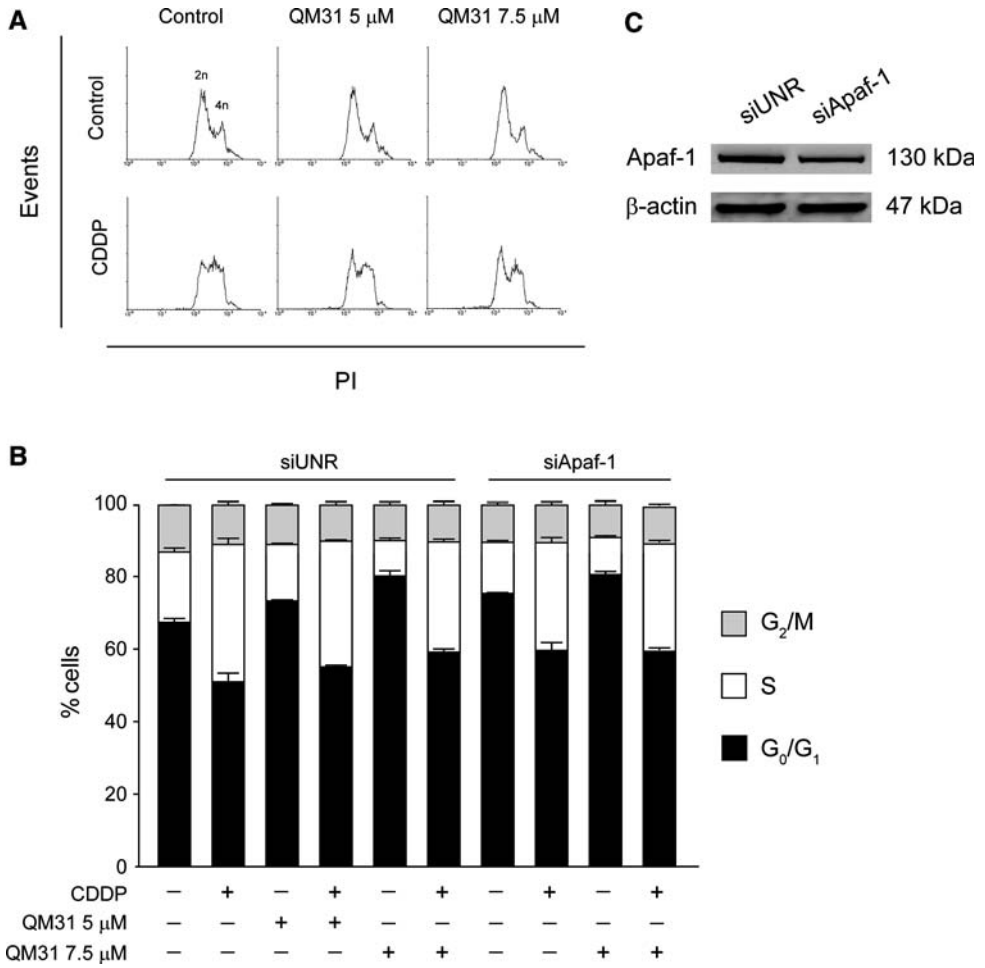


**Fig. 3** Non-small cell lung cancer (NSCLC) A549 cells were left untreated or transfected with a control (siUNR) or an Apaf-1-specific (siApaf-1) siRNA for 48 h, and then incubated with the indicated concentration of QM31 for 1 h. Thereafter, cells were cultured in the absence or in the presence of 20 μM cisplatin (CDDP) for additional 24 h, followed by cytofluorometric analysis of the cell cycle (**a**, **b**) or immunoblotting to check for Apaf-1 downregulation (**c**). Panel **a** reports representative cell cycle profiles, as obtained in a single experiment upon propidium iodide (PI) staining. 2*n* and 4*n* indicate the peaks corresponding to the $G_0/G_1$ and $G_2/M$ phases, respectively. In panel **b**, black, white, and grey columns illustrate the percentage of cells in the $G_0/G_1$, S and $G_2/M$ phases of the cell cycle, respectively (mean ± SEM, $n = 3$). Please note how the percentage of S-phase cells recorded upon CDDP administration is gradually reduced by increasing concentrations of QM31, down to a minimum that matches the value of siApaf-1-transfected cells. Panel **c** illustrates the partial downregulation of Apaf-1 observed after the transfection of A549 cells with siApaf-1 as compared to siUNR. An antibody recognizing *β*-actin was employed to control equal loading

QM31 is a lead compound for the development of a new class of cytoprotective agents. Although QM31 weakens the Apaf-1-dependent DNA damage checkpoint, it is unlikely that QM31 would favor deleterious genomic instability, for two reasons. First, the deficiency of Apaf-1 alone has not been reported to increase the frequency of tumors in *apaf-1*$^{-/-}$ mice [42, 43], suggesting that even the life-long absence of Apaf-1 is not carcinogenic. Second, QM31 did not act as a clastogen on its own (data not shown), in line with the observation that Apaf-1-deficient cells do not tend to acquire chromosomal aberrations, at least in the absence of exogenously-triggered DNA damage.

In comparison to the prototype pan-caspase inhibitor Z-VAD-fmk, QM31 was relatively less efficient in preventing the activation of caspase-3, yet afforded a similar degree of cytoprotection. This correlates with the unexpected property of QM31 to stabilize mitochondria and to prevent MOMP. Since MOMP is (one of) the principal decision point(s) that mark the frontier between a cell's death and life [3], it appears plausible that MOMP-inhibitory agents would mediate a high degree of cytoprotection. As it stands, however, the mechanisms through which QM31 confers mitochondrioprotection remain elusive. Novel molecular partners of Apaf-1 are being continuously discovered, among which members of the Bcl-2 protein family [33, 34]. Bcl-2 proteins are renowned for their MOMP-regulatory activities, and hence may provide a molecular link between QM31-mediated apoptosome and MOMP inhibition.

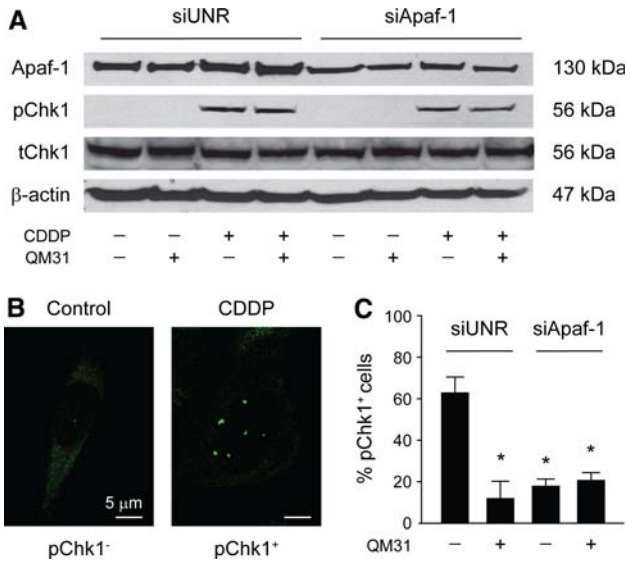


**Fig. 4** Non-small cell lung cancer (NSCLC) A549 cells were transfected for 48 h with a control (siUNR) or an Apaf-1-specific (siApaf-1) siRNA, and then left untreated or incubated with 5 μM QM31 for 1 h. Finally, cells were treated or not with 20 μM cisplatin (CDDP) for 24 h, prior to immunoblotting (**a**) or immunofluorescence microscopy (**b**, **c**) for the assessment of the phosphorylation status of Chk1. Panel **a** illustrates the protein levels of phosphorylated (pChk1) and total (tChk1) Chk1, as well as those of Apaf-1 and $\beta$-actin (which was monitored to check equal loading). Panel **b** reports representative immunofluorescence microphotographs of untreated and CDDP-treated cells, which exhibit negative ($pChk^-$) and positive ($pChk^+$, nuclear bright dots) staining for phosphorylated Chk1, respectively. White bars indicate scale. In panel **c**, columns depict the percentage (mean ± SEM, $n = 3$) of $pChk^+$ cells recorded upon the administration of CDDP to siUNR- and siApaf-1-transfected cells in the absence or presence of QM31. Asterisks indicate values that are significantly different (Student's $t$ test, $P < 0.05$) from those observed in siUNR-transfected, CDDP-treated cells

In multiple models of intrinsic apoptosis the absence of Apaf-1 does not prevent Cyt *c* release [44–46]. This reinforces the notion that the mitochondrioprotective effects of QM31 are not due to a generalized inhibition of all Apaf-1 functions, but rather to a selective interference in the interaction between Apaf-1 and some (but not all) of its molecular partners. As an alternative, QM31 may afford mitochondrioprotection by inhibiting caspase-mediated feed-forward signaling circuitries that are able to amplify MOMP [12, 13]. Irrespective of this unresolved question, QM31 may be the first agent that inhibits caspase activation through a direct effect on apoptosome components as well as through the inhibition of Cyt *c* release from mitochondria.

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