Published in final edited form as:
Int J Cancer. 2012 June 1; 130(11): 2703–2714. doi:10.1002/ijc.26308.

Luteolin induces apoptosis in multidrug resistant cancer cells without affecting the drug transporter function: involvement of cell line-specific apoptotic mechanisms

Prema S. Rao[1], **Arun Satelli**[1], **Majid Moridani**[2], **Marjorie Jenkins**[3], and **U. Subrahmanyeswara Rao**[1,§]

[1]Department of Biomedical Sciences Texas Tech University Health Sciences Center, Amarillo, TX 79106

[2]Department of Pharmaceutical Sciences Texas Tech University Health Sciences Center, Amarillo, TX 79106

[3]Department of Internal Medicine Texas Tech University Health Sciences Center, Amarillo, TX 79106

Abstract

Bioflavonoids are of considerable interest to human health as these serve as antioxidant and anticancer agents. Although epidemiological and experimental studies suggest that luteolin, a natural bioflavonoid, exhibits chemopreventive properties, its effectiveness as an antiproliferative agent against multidrug resistant (MDR) cancers is unclear. We thus assessed the antiproliferative effects of luteolin and associated molecular mechanisms using two MDR cancer cell lines that express high levels of P-glycoprotein and ABCG2. In this paper, we demonstrate that luteolin induces apoptosis in P-glycoprotein- and ABCG2-expressing MDR cancer cells without affecting the transport functions of these drug transporters. Analysis of various proliferative signaling pathways indicated that luteolin-induced apoptosis involves reactive oxygen species generation, DNA damage, activation of ATR→Chk2→p53 signaling pathway, inhibition of NF-kB signaling pathway, activation of p38 pathway and depletion of anti-apoptotic proteins. Importantly, use of luteolin in these analyses also identified specific molecular characteristics of NCI-ADR/RES and MCF-7/Mito^{R} cells that highlight their different tissue origins. These results suggest that luteolin possesses therapeutic potential to control the proliferation of MDR cancers without affecting the physiological function of drug transporters in the body tissues.

Keywords

Luteolin; multidrug resistance; drug transporters; P-glycoprotein; ABCG2

It is now well established that two mechanisms of apoptosis, termed intrinsic mitochondrion-initiated[1] and the death receptor-mediated pathways [2], induce cell death. Activation of the former pathway affects mitochondrial permeability, resulting in cytochrome c release, decrease in Bcl-2/Bax ratio and the activation of caspase-9. The latter pathway originates at the plasma membrane death receptors with their interaction with TNF-related proteins, resulting in the activation of downstream caspases [3]. Most cancers constitutively express inhibitors of apoptosis (IAP), a family of proteins that include c-IAP1,

[§]**Corresponding Author**: U.S. Rao, Ph.D., Department of Biomedical Sciences, 1109 Amarillo Research Building, 1406 S. Coulter St., Amarillo, TX 79106., Ph: 806-356-4750 x229, Fax: 806-356-4770, us.rao@ttuhsc.edu.

c-IAP2 and XIAP [4], some of which bind to caspases and inhibit their activation imparting resistance to apoptosis [5]. Other IAPs, through their ubiquitin ligase activity, regulate the proliferative pathways such as NF-kB, which in turn drive the expression of genes important for cell survival [6]. Cancer cells also express drug transporters that include P-glycoprotein (Pgp) and ABCG2, allowing them to survive chemotherapy, a phenomenon known as multidrug resistance (MDR) [7].

The long-term interest of our laboratory is to understand the mechanisms of MDR in cancers. Bioflavonoids possess anticancer activity [8, 9] as well as the ability to resensitize cancers to chemotherapeutic agents [10–13]. However, the usefulness of these agents against MDR cancers is unclear at present, as studies have shown that several bioflavonoids with clinical potential are in fact transport substrates of drug transporters, thus diminishing enthusiasm in bioflavonoids as therapeutic agents [14–16]. While compounds that inhibit drug transporters and thus resensitize cancers to anticancer drugs are desirable in the cancer treatment, one caveat of this concept is that such drugs would also inhibit drug transporters that are constitutively expressed in the body tissues such as brain, intestine and kidney, affecting their normal functions such as excretion of toxic byproducts of metabolism. Therefore, anticancer bioflavonoids that are not transport substrates of drug efflux transporters are desirable in the resensitization of MDR cancers to chemotherapy. The purpose of this work is to identify antiproliferative flavonoids that do not affect the function of drug transporters. This is achieved by screening several flavonoids for their ability to induce apoptosis in two well-characterized MDR cancer cell lines, NCI-ADR/RES and MCF-7/Mito^R, which respectively express high levels of Pgp and ABCG2 [17, 18]. These studies have identified luteolin as an effective agent in inhibiting cell proliferation of these MDR cancer cells. A detailed characterization of luteolin-induced apoptosis in these MDR cells is presented.

MATERIALS AND METHODS

Reagents

Luteolin, Rhodamine 123 (Rh123), verapamil, fumitremorgin and mitoxantrone were obtained from Sigma (St. Louis, MO). A771726 was purchased from Alexis Corp. (Lausen, Switzerland). Cyclosporine A (CsA) and Annexin V Apoptosis Detection kit were purchased from Calbiochem (Carlsbad, CA). Tetramethylrhodamine methyl ester (TMRM) and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCF-DA) were obtained from Invitrogen (Eugene, OR). Sources of antibodies and other reagents were reported previously [19].

Cell lines and culture conditions

Cell lines used in this study were authenticated by STR DNA profiling at Texas Cancer Cell Repository, Texas Tech University Health Sciences Center, Lubbock, under the direction of Dr. Patrick Reynolds. Pgp-expressing NCI-ADR/RES, and BCRP-expressing MCF-7/Mito^R cells were cultured and maintained, as described previously [20]. One day before testing the effects of test compounds, cells were grown in the absence of anticancer drug.

Drug uptake assay

All drug stocks (1000x) were prepared fresh in ethanol or DMSO. Drug uptake assays were carried out by flow cytometry as reported by others [21, 22] with modifications. NCI-ADR/RES cells ($1x10^6$) in logarithmic phase were resuspended in phosphate buffered saline (PBS) and incubated with 10 μM Rh123, 50 μM luteolin, 50 μM verapamil, 5 μM CsA or in various combinations, with continuous mixing at 37° C for 30 min. Similarly, MCF-7/Mito^R cells were incubated with 1 μM mitoxantrone, 50 μM luteolin, 1 μM fumitremorgin and 10

μM A771726 in various combinations. Cells were pelleted by centrifugation, rinsed twice with HEPES-Hanks buffer and fixed with 1% paraformaldehyde in HEPES-Hanks buffer for 30 min. Cells were washed with and resuspended in PBS and then analyzed by flow cytometry (FACScan flow cytometer equipped with CellQuest data acquisition software (Becton Dickinson, CA)). Data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Cell proliferation, cell cycle and apoptosis assays

The effect of bioflavonoids on cell proliferation, cell cycle and apoptosis were determined respectively, by MTS, nuclear staining with propidium iodide and annexin V-FITC staining of surface phosphatidylserine (PS) combined with DNA content by propidium iodide staining as described [19]. The mean value and S.E. for each treatment were determined, and expressed as percentage relative to control, where appropriate.

Mitochondrial membrane potential (MMP) assay

Cells were incubated with different concentrations of luteolin for 24 h, washed and resuspended in PBS containing 50 nM TMRM for 30 min in dark. MMP was measured by flow cytometry using FACScan flow cytometer and the percent of live cells was quantified using FlowJo software [19].

Measurement of intracellular ROS

Intracellular production of ROS was measured using DCF-DA reagent. Growth medium of cells growing in 24-well plates was changed to phenol red-free medium. Cells were loaded with 10 μM DCFH-DA for 30 min and washed with fresh medium and incubated with different concentrations of luteolin in the presence and absence of 0.5 mM glutathione (reduced form) and the ROS generated was continuously monitored over a 2 h time period by measuring the fluorescent DCF in a plate-reader at an excitation wavelength of 485 nm and emission wavelength of 525 nm.

DNA fragmentation analysis

Cells were incubated with DMSO, 1 μM camptothecin or 50 μM luteolin for 24 h and the DNAs were extracted and analyzed on agarose (1.5%) gel electrophoresis as described by Steinfelder, et al [23].

Apoptosis antibody array

Apoptosis antibody arrays were developed using the Human Apoptosis Array kit of R&D Systems, MN. Lysates were prepared from cells grown in the presence of 0 and 50 μM luteolin for 24 h as recommended by the supplier. Each antibody array membrane was incubated with 400 μg of total cellular protein and the blots were developed simultaneously.

SDS-PAGE, Western Blotting and protein estimation

Preparation of whole cell lysates, protein estimation, SDS-PAGE and western blotting were carried out as described previously [24].

Statistical analysis

Each experiment was carried out at least four different times, with essentially identical results. Apoptosis array analysis was carried out twice. Statistical analyses between different treatments or groups were determined using t-tests and ANOVA as appropriate, using GraphPad Prism Software. Each of the columns represent mean with s.e.m, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS

Luteolin inhibits cell growth of Pgp- and ABCG2-expressing cells

To identify phytochemicals with growth inhibitory effects, growth media of NCI-ADR/RES and MCF-7/Mito^R cells were supplemented with increasing concentrations of rutin, quercetin, catechin, bilobalide, myricetin, kaempferol and luteolin, and the extent of growth inhibition was monitored by MTS assays. These experiments identified luteolin with potent growth inhibitory effect on both of these cell lines in a concentration- and time-dependent manner in the absence of any anticancer drug (Fig.1A,B). The calculated IC_{50} of luteolin was approximately 45 μM at 24 h and, 35 μM at 48 h for both cell lines.

Luteolin is not a transport substrate or an inhibitor of Pgp

The effect of luteolin on Pgp function was measured by flow cytometry, and the results were presented in Fig.1C. Upper panel summarizes the transport activity of Pgp in NCI-ADR/RES cells as measured by the intracellular accumulation of Rh123, a fluorescent transport substrate of Pgp. Incubation of cells with Rh123 yielded a fluorescence peak, right to the cell auto fluorescence peak. This shift was due to intracellular accumulation of Rh123 in equilibrium between its diffusion into cells and efflux by Pgp. In the presence of CsA, a potent Pgp inhibitor [25], the Rh123 fluorescence peak was shifted farther to the right, suggesting further increase in Rh123 accumulation due to inhibition of Pgp. We also measured changes in Rh123 accumulation in the presence of verapamil, a well-established transport substrate of Pgp [26–28]. Cells incubated with Rh123 in the presence of verapamil exhibited a peak farther right to that of cells incubated with Rh123 alone, suggesting that Rh123 efflux was competitively inhibited by the transporting verapamil. Although not shown, the autofluorescence peak of cells was unaltered either by CsA or verapamil.

Together, these data suggested that flow cytometry allows in the identification of transport substrates and inhibitors of P g p by measuring changes in the intracellular accumulation of Rh123.

NCI-ADR/RES cells were incubated with Rh123 in the presence and absence of luteolin and changes in the intracellular Rh123 level were measured. Fig.1C, middle panel, shows that the intensity of Rh123 fluorescence was essentially unaltered in the presence of luteolin, suggesting that luteolin did not inhibit Pgp or compete with the Rh123 transport.

Changes in the intracellular luteolin accumulation in the presence of verapamil and CsA, were measured by flow cytometry. Fig.1C, lower panel, shows that the luteolin [29] fluorescence peak was unaffected when cells were co-incubated with either verapamil or CsA. These results together suggested that luteolin accumulates intracellularly, and this process is unaffected either by a transport substrate or an inhibitor of Pgp.

Luteolin is not a transport substrate or an inhibitor of ABCG2

Using the above assay, we measured the transport activity of ABCG2 in MCF-7/Mito^R cells using mitoxantrone. Fig.1D, upper panel, shows that the cell auto fluorescence peak was shifted to the right upon incubation with mitoxantrone, suggesting its intracellular accumulation. The intracellular mitoxantrone level was further increased when cells were co-incubated with ABCG2-specific inhibitors, fumitremorgin or A771726.

Fig.1D, middle panel, shows that when cells were co-incubated with mitoxantrone and luteolin, the mitoxantrone peak shifted slightly to left, which could be either due to stimulation of ABCG2-mediated efflux of mitoxantrone, decreased diffusion of mitoxantrone into cells, or quenching of mitoxantrone fluorescence by luteolin. Fig.1D,

lower panel, shows that cells incubated with luteolin alone exhibited fluorescence peak, which was however unaffected in the presence of fumitremorgin or A771726. Thus, the absence of any significant effect on the intracellular accumulation of luteolin upon ABCG2 inhibition suggested that luteolin is not a transport substrate of ABCG2.

Luteolin induces cell cycle arrest, apoptosis, and inhibits cell migration

To investigate the anti-proliferative effects of luteolin, cells grown in growth medium containing 50 μM luteolin for 18 h were subjected to cell cycle analysis by flow cytometry. Fig.2A shows that luteolin treatment significantly increased the S-phase cell population in both cell lines, with simultaneous decrease in G_1 phase cell population, suggesting that luteolin induced cell cycle arrest at S-phase.

To determine whether luteolin treatment induced apoptosis, cells incubated with increasing concentrations of luteolin for 24 h were analyzed by flow cytometry for the diploid DNA content and for PS exposure to the cell surface. Percent live, early- and late-apoptotic and necrotic cells were calculated, using FlowJo software. Fig.2B (and supplemental Figure 1) shows that luteolin treatment decreased the live cell population in both cell lines in a concentration-dependent manner. Apoptosis in MCF-7/Mito^R cells was evident as the percent of cells progressively increased from early-apoptosis through late- apoptosis and finally to necrotic stage. On the other hand, majority of NCI-ADR/RES cells were found to accumulate in pre-apoptotic stage and did not progress to apoptosis. Thus, the apparent absence of progression to apoptosis in NCI-ADR/RES cells when compared to MCF-7/Mito^R cells suggested that additional mechanisms needed to be activated for these cells to enter apoptosis. Cell migration experiments indicated that luteolin at sub-lethal concentration (10 μM) significantly decreased cell migration of both of these cell lines (Fig. 2C).

Luteolin promotes ROS production, DNA fragmentation and MMP depolarization

The effect of luteolin on the ROS generation was monitored by measuring the production of fluorescent DCF from DCF-DA hydrolysis and its subsequent oxidation by ROS. Fig. 3A and B show that both cells continuously generated ROS (open circles) as a consequence of normal metabolic activity, which was quenched to basal level by glutathione (filled circles). In the presence of 50 μM luteolin, the amount of ROS accumulated was decreased (open triangles), suggesting luteolin quenched the ROS, thus functioning as an antioxidant. The ROS produced in the presence of luteolin could be quenched further by glutathione (filled triangles). In the presence of 100 μM luteolin, ROS production was however increased (open squares) to near control level (open circles), which could be quenched by glutathione to basal level (filled squares), This observation suggests that luteolin at high concentrations is an ROS generating agent.

To determine whether the antioxidant property of luteolin progressively transitions to ROS producing agent, cells were incubated with 50 μM luteolin for 24 h and the ROS was measured by flow cytometry. Fig.3C shows a net accumulation of ROS suggesting that luteolin-generated ROS accumulates in cells upon prolonged incubation.

Cells treated with 50 μM luteolin for 24 h were analyzed for DNA fragmentation. Fig.3D shows that luteolin-treatment induced DNA fragmentation with a characteristic pattern of DNA cleavage into ~180 bp integer fragments, a pattern similar to that induced by camptothecin. Luteolin-induced DNA fragmentation was also observed by others [30].

We investigated the effects of luteolin on mitochondrial membrane potential (MMP) in both cell lines treated with increasing concentrations of luteolin using TMRM. TMRM is a cationic, lipophilic dye that accumulates in the negatively charged mitochondrial matrix in

healthy cells with intact mitochondria. As the MMP collapses during apoptosis, TMRM is dispersed throughout the cell and fluoresces less intensely. The reduction in live cell population can therefore be quantified by measuring the decrease in TMRM fluorescence. Fig.3E (and Supplemental Figure 2) shows that the live cell population of MCF-7/Mito^R cells decreased proportionately with luteolin concentration, suggesting luteolin induces apoptosis through induction of MMP collapse. However, luteolin treatment marginally decreased the live cell population in NCI-ADR/RES cells, suggesting that these cells are resistant to MMP collapse. These data also suggest that NCI-ADR/RES and MCF-7/Mito^R cells differ in the mitochondrial functional characteristics.

Changes in Bcl-2 and Bax levels were determined by western blotting. Fig.3F shows a gradual loss of Bcl-2 and a slight increase in Bax levels in both cell lines treated with luteolin, indicating that luteolin treatment decreases Bcl-2/Bax ratio. Since a decrease in Bcl-2/Bax ratio is an indicator of cells undergoing apoptosis[31], these results suggested that luteolin induces apoptosis in MDR cells.

Luteolin induces phosphorylation of ATR, H2AX, Chk2 proteins and cleavage of poly (ADP-ribose) polymerase (PARP)

We investigated phosphorylation of H2AX and ATR, and PARP cleavage, which reflect on the activation of DNA damage response by Western blotting. Fig.4 shows that exposure to luteolin resulted in ^{428}S-phosphorylation of ATR, ^{139}S-phosphorylation of H2AX and cleavage of PARP in both cell lines. Since phospho-ATR phosphorylates Chk1, Chk2, cdc25C and cdc2 leading to cell cycle arrest [32], we tested this possibility by Western blotting. Fig.4 shows that luteolin treatment resulted in increased phosphorylation of Chk2, but not Chk1. While cdc25C was phosphorylated in NCI-ADR/RES cells, this phosphatase stayed constitutively phosphorylated in MCF-7/Mito^R cells. Finally, cdc2 was slightly dephosphorylated in the presence of luteolin. Together, these results suggested that luteolin induces cell growth arrest through the activation of ATR→Chk2 pathway.

Analysis of cell proliferative pathways affected by luteolin

To determine whether luteolin inhibited NF-kB activation, ^{536}S-phospho-p65 level was analyzed. Fig.4 shows that p65 was constitutively phosphorylated in both cell lines. In NCI-ADR/RES cells, the levels of phospho-p65 and total p65 were decreased. Although there was no change in the total p65 level in MCF-7/Mito^R cells, a small loss of phospho-p65 was evident after prolonged incubation with luteolin. Luteolin induced a rapid decrease in cyclin D1 level in both cell lines. These data together suggested that luteolin treatment inhibited the activation of p65, leading to the loss of cyclin D1, an NF-kB target gene.

Fig.4 also shows the effects of luteolin on p38 phosphorylation at ^{180}T/^{182}Y. NCI-ADR/RES cells contained constitutively phosphorylated p38, which decreased to minimum with luteolin exposure. On the other hand, MCF-7/Mito^R cells were devoid of phospho-p38. Importantly, exposure to luteolin acutely increased phospho-38 level in MCF-7/Mito^R cells.

p38 [33] and ATR [34] and Chk2 [35], are known to phosphorylate p53 at ^{15}S resulting in its increased ability to bind DNA and promote the expression of p53-dependent genes such as *p21*. These events lead to the inhibition of cyclinD1-cdk2 or cyclin-CDK4 complexes, which together regulate cell cycle progression. We therefore analyzed these changes in luteolin-treated cells. Fig.5, upper left panel shows that p53 was constitutively phosphorylated at ^{15}S, ^{46}S and ^{392}S in NCI-ADR/RES cells, and these phosphorylations were unaffected by luteolin treatment. p53 in MCF-7/Mito^R cells was constitutively phosphorylated only at ^{392}S, which was unaffected by luteolin. Importantly, luteolin treatment selectively induced phosphorylation of p53 at ^{15}S in MCF-7/Mito^R cells. These

results suggested that phosphorylation of p53 is involved in cell cycle arrest in MCF-7/Mito^R cells.

Fig.5, upper right panel, shows that p21 was absent in both cell lines, which was induced in MCF-7/Mito^R cells but not NCI-ADR/RES cells upon exposure to luteolin. These results clearly suggested that *de novo* phosphorylation of p53, which occurred in MCF-7/Mito^R cells but not in NCI-ADR/RES cells, is necessary for the increased expression of p53-dependent gene expression.

Luteolin activates caspase 7

To further establish the luteolin-induced apoptosis, activation of caspases was analyzed in cells treated with 50 μM luteolin by Western blotting and antibody arrays. Fig. 5 shows that NCI-ADR/RES cells while contained pro-caspase 3, did not contain its activated cleaved form. These analyses clearly indicated the absence of procaspase 3 and its cleaved form in MCF-7/Mito^R cells, which are known to be procaspase 3 negative [36]. Activation of other caspases including caspase 8 or caspase 9 could not be established conclusively in our repeated experiments. However, luteolin treatment resulted in the activation of caspase 7, as judged by the appearance of ~20 kDa caspase 7 fragment in both cell lines.

Luteolin promotes depletion of anti-apoptotic proteins

Fig.5 shows that treatment of cells with luteolin resulted in the depletion of antiapoptotic proteins, c-IAP1, claspin, survivin and XIAP in both cell lines. On the other hand, heat shock proteins Hsp27 and Hsp90, and heme oxygenase-2 (HO-2), which are essential for cell survival, were down-regulated in MCF-7/Mito^R but not NCI-ADR/RES cells. These data suggested that depletion of antiapoptotic proteins in general contribute to the luteolin-induced apoptosis.

DISCUSSION

The goal of this work is to identify antiproliferative phytochemicals that are not transport substrates or inhibitors of drug transporters. Results presented in this paper provide clear evidence that luteolin exhibits antiproliferative effects on MDR cancer cells without affecting the function of Pgp and ABCG2. We used 50 μM luteolin in the transport assays, since cell proliferation was inhibited significantly at this concentration. The results that luteolin does not affect the function of these drug transporters even at this concentration, suggest that luteolin is potentially a safe bioflavonoid to achieve inhibition of MDR cancer cell proliferation *in vivo* without affecting the function of drug transporters in normal body tissues.

Several laboratories have found that flavonoids are effective as antiproliferative agents at relatively higher concentration. For example, phytochemicals including, resveratrol [32], quercetin [37], myricetin [38] and luteolin [39], were found to inhibit cancer cell proliferation at ~50 μM. Thus, 50 μM luteolin used here is reasonable to gain meaningful insights into its mechanisms in MDR cancer cells. Intracellular accumulation of luteolin shown here by FACS analysis was further confirmed by paper chromatography of the cytosolic fractions prepared from cells (data not shown). Although bioflavonoids are remarkably safe nutrients and common constituents in fruits and vegetables, these agents are less efficiently absorbed by the gastrointestinal tract and a significant portion of the absorbed flavonoids undergo modification either by microflora in the intestine and/or intestinal wall and liver as glucuronidate, sulfate, methyl conjugates [40]. Thus, achieving a plasma level of ~50 μM luteolin may not be possible if not impossible via oral supplementation due to the low oral bioavailability. However, it is achievable via repeated intravenous injections of luteolin, as

suggested by van Zanden et al [38]. In support of this view, pharmacokinetic studies have shown that serum concentrations of quercetin, a structurally related flavonoid, reach up to 400 μM after intravenous injection [41]. Alternately, high serum levels of luteolin could also be achieved through appropriately designed drug formulations or structural variations introduced in its backbone.

Previous studies have shown that luteolin is an antiproliferative agent [42, 43], which elicits its effects through activation of multiple mechanisms [30, 39, 43, 44]. Cheng, et al [30] have shown that luteolin induces apoptosis in human leukemia cells through activation of mitochondrial apoptotic pathway. Horinaka et al [39] have observed that luteolin induces apoptosis in HeLa cells through up-regulation of death receptor 5. Luteolin was also shown to exhibit its apoptotic effects by promoting XIAP degradation [11].

Fig.6 summarizes major mechanisms by which luteolin induces cell cycle arrest and apoptosis. Luteolin-induced apoptosis is evident from the activation of a variety of processes including ROS generation, DNA fragmentation, phosphorylations of ATR, Chk2 and H2AX, cleavage of PARP, loss of cyclin D1, activation of caspase 7, decrease in Bcl-2/Bax ratio and loss of anti-apoptotic proteins, c-IAP1, XIAP, survivin and claspin. However, the analysis of PS exposure and MMP do not clearly suggest that NCI-ADR/RES cells undergo apoptosis. Although the reasons for the lack of PS exposure or MMP collapse are unclear, the works of Mehta and his coworkers, demonstrated that NCI-ADR/RES cells contain deficient levels of intracellular calcium pool [45]. Since PS exposure and MMP collapse are regulated by intracellular calcium level, it is therefore likely that measurements of PS exposure or MMP collapse in NCI-ADR/RES cells may fail to indicate the evidence for apoptosis.

The activation of p38 signaling pathway is characterized by the phosphorylation of p38. The phosphorylated (activated) p38 is known to phosphorylate p53 [46]. Phosphorylation of either p38, p53 or both are known to contribute to cyclin D1 degradation [47]. Thus, the acute phosphorylations of both p38 and p53 as well as the acute loss of cyclin D1 in MCF-7/Mito^R cells suggest that luteolin activated the p38 signaling pathway, leading to cell cycle arrest.

In contrast, NCI-ADR/RES cells contain high levels of constitutively phosphorylated p38 and p53. The loss of cyclin D1 in these cells was rather slow. These data together suggest that p38 pathway is constitutively activated in these cells, perhaps functioning as pro-proliferative pathway in this cell line. Luteolin appears to antagonize this pro-proliferative pathway by promoting the cyclin D1 degradation.

One of the important signaling pathways constitutively activated in many cancers is the NF-kB signaling [48]. The presence of phospho-p65 in both NCI-ADR/RES and MCF-7/Mito^R cells and its loss upon exposure to luteolin (Fig.4) suggests that this flavonoid inhibits the NF-kB signaling pathway. In support of this, the NF-kB target genes, cyclin D1, Bcl-2, XIAP, cIAP1 and survivin, were all depleted in both cell lines.

The net ROS generated contributes to the MMP dissipation leading to the activation of the mitochondrion-mediated apoptotic pathway. Although luteolin functions both as antioxidant and ROS-generating agent (Fig.3), the molecular changes, such as phosphorylation of ATR, histone H2A and Chk2 (Fig.4), which are known to occur as a cellular response to DNA damage, occurred soon after luteolin exposure to cells, pointing out that luteolin-induced DNA damage may not be related directly to ROS generation, but occurring as an independent mechanism. This DNA damage response activates p53 in MCF-7/Mito^R cells, either by the phosphorylated ATR and/or Chk2 leading to the activation of G1/S checkpoint mediated p21 inhibition of cdc2 [32]. This pathway provides a mechanism of cell cycle arrest induced by luteolin in MCF-7/Mito^R cells. Neither the ATR-p53 nor Chk2-p53 pathway

appear to contribute to the cell cycle arrest in NCI-ADR/RES cells, as there was no further increase in phospho-p53 level or synthesis of p21 upon exposure to luteolin. Thus, the mechanism of cell cycle arrest in NCI-ADR/RES cells is unclear. It is possible that the above-mentioned molecular alterations including the activations of NF-kB, p38, depletion of antiapoptotic proteins and DNA damage response together contribute to cell cycle arrest and apoptosis.

Although NCI-ADR/RES cell line was once thought to have originated from breast cancer MCF-7 cells, the presence of procaspase 3 [49] and mutated p53 [50], has clarified that this cell line is closely related to that of an ovarian cancer cell line. Importantly, the use of luteolin has unraveled several hereto-unknown important molecular characteristics of NCI-ADR/RES cell line, which distinguish it from MCF-7/Mito^R cells. In addition, the data presented in this paper confirms that NCI-ADR/RES cells do contain procaspase 3, which was absent in MCF-7/Mito^R cells. NCI-ADR/RES cells contain p53 which is constitutively phosphorylated at ^{15}S and ^{46}S, whereas These sites in p53 are not phosphorylated in MCF-7/Mito^R cells. Luteolin induces p21 expression in MCF-7/Mito^R cells but not in NCI-ADR/RES cells. Another distinguishing feature between these two cell lines is the activation of p38 (Fig.4). p38 is constitutively activated in NCI-ADR/RES cells whereas; it is activated only upon luteolin treatment in MCF-7/Mito^R cells. Finally, these two cell lines also differ in their response to luteolin treatment in the PS exposure and MMP collapse. These differences will serve as reference in the future studies aimed at further understanding of apoptosis in MDR cancers.

In conclusion, this work demonstrates that luteolin is an antiproliferative flavonoid, which induces apoptosis in cancer cells expressing drug transporters, without affecting the function of drug transporters.

Novelty and Impact

Drug resistance is a major obstacle in the treatment of cancers. Several mechanisms contribute to drug resistance that include the drug transporter-mediated anticancer drug efflux. Inhibition of drug transporters is not a better choice in the treatment of drug resistant cancers as these transporters carry out important physiological functions in the body. Here we demonstrate that luteolin, a safe bioflavonoid commonly found in fruits and vegetables, induce apoptosis in model multidrug resistant cancer cells, without affecting the function of P-glycoprotein and ABCG2. This paper identifies specific molecular mechanisms of luteolin-induced apoptosis. The mechanism-based apoptosis suggests that luteolin has potential to control MDR cancer proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by Funds from NIH RO1 CA 106625 and Laura Bush Institute of Women's Health grant to U.S. Rao.

We thank Dr. Patrick Reynolds and Texas Cancer Cell Repository team for STR DNA profiling the cell lines.

REFERENCES

1. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010; 11:621–632. [PubMed: 20683470]

2. Thorburn A. Death receptor-induced cell killing. Cell Signal. 2004; 16:139–144. [PubMed: 14636884]
3. MacFarlane M. TRAIL-induced signalling and apoptosis. Toxicol Lett. 2003; 139:89–97. [PubMed: 12628743]
4. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010; 10:561–574. [PubMed: 20651737]
5. Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis. 2007; 12:1543–1568. [PubMed: 17573556]
6. Varfolomeev E, Vucic D. (Un)expected roles of c-IAPs in apoptotic and NFkappaB signaling pathways. Cell Cycle. 2008; 7:1511–1521. [PubMed: 18469528]
7. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998; 95:15665–15670. [PubMed: 9861027]
8. van Zanden JJ, Wortelboer HM, Bijlsma S, Punt A, Usta M, Bladeren PJ, Rietjens IM, Cnubben NH. Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2. Biochem Pharmacol. 2005; 69:699–708. [PubMed: 15670588]
9. Kandaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, Lee MT. The antitumor activities of flavonoids. In Vivo. 2005; 19:895–909. [PubMed: 16097445]
10. Imai Y, Tsukahara S, Asada S, Sugimoto Y. Phytoestrogens/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance. Cancer Res. 2004; 64:4346–4352. [PubMed: 15205350]
11. Shi RX, Ong CN, Shen HM. Protein kinase C inhibition and x-linked inhibitor of apoptosis protein degradation contribute to the sensitization effect of luteolin on tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in cancer cells. Cancer Res. 2005; 65:7815–7823. [PubMed: 16140950]
12. Horinaka M, Yoshida T, Shiraishi T, Nakata S, Wakada M, Nakanishi R, Nishino H, Sakai T. The combination of TRAIL and luteolin enhances apoptosis in human cervical cancer HeLa cells. Biochem Biophys Res Commun. 2005; 333:833–838. [PubMed: 15963948]
13. Amin AR, Wang D, Zhang H, Peng S, Shin HJ, Brandes JC, Tighiouart M, Khuri FR, Chen ZG, Shin DM. Enhanced anti-tumor activity by the combination of the natural compounds (−)-epigallocatechin-3-gallate and luteolin: potential role of p53. J Biol Chem. 2010; 285:34557–34565. [PubMed: 20826787]
14. Alvarez AI, Real R, Perez M, Mendoza G, Prieto JG, Merino G. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. J Pharm Sci. 2009; 99:598–617. [PubMed: 19544374]
15. Brand W, Schutte ME, Williamson G, van Zanden JJ, Cnubben NH, Groten JP, van Bladeren PJ, Rietjens IM. Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. Biomed Pharmacother. 2006; 60:508–519. [PubMed: 16978825]
16. Morris ME, Zhang S. Flavonoid-drug interactions: effects of flavonoids on ABC transporters. Life Sci. 2006; 78:2116–2130. [PubMed: 16455109]
17. Nakagawa M, Schneider E, Dixon KH, Horton J, Kelley K, Morrow C, Cowan KH. Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells. Cancer Res. 1992; 52:6175–6181. [PubMed: 1358431]
18. Fairchild CR, Moscow JA, O'Brien EE, Cowan KH. Multidrug resistance in cells transfected with human genes encoding a variant P-glycoprotein and glutathione S-transferase-pi. Mol Pharmacol. 1990; 37:801–809. [PubMed: 1972772]
19. Satelli A, Rao PS, Thirumala S, Rao US. Galectin-4 functions as a tumor suppressor of human colorectal cancer. Int J Cancer. 2010 [Epub ahead of print].
20. Rao PS, Govindarajan R, Mallya KB, West W, Rao US. Characterization of a new antibody raised against the NH2 terminus of P-glycoprotein. Clin Cancer Res. 2005; 11:5833–5839. [PubMed: 16115923]

21. Polgar O, Robey RW, Morisaki K, Dean M, Michejda C, Sauna ZE, Ambudkar SV, Tarasova N, Bates SE. Mutational analysis of ABCG2: role of the GXXXG motif. Biochemistry. 2004; 43:9448–9456. [PubMed: 15260487]
22. Kim M, Turnquist H, Jackson J, Sgagias M, Yan Y, Gong M, Dean M, Sharp JG, Cowan K. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin Cancer Res. 2002; 8:22–28. [PubMed: 11801536]
23. Steinfelder HJ, Quentin I, Ritz V. A fast and sensitive technique to study the kinetics and the concentration dependencies of DNA fragmentation during drug-induced apoptosis. J Pharmacol Toxicol Methods. 2000; 43:79–84. [PubMed: 11091133]
24. Rao PS, Mallya KB, Srivenugopal KS, Balaji KC, Rao US. RNF2 interacts with the linker region of the human P-glycoprotein. Int J Oncol. 2006; 29:1413–1419. [PubMed: 17088979]
25. Rao US, Scarborough GA. Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. Mol Pharmacol. 1994; 45:773–776. [PubMed: 7514263]
26. Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci U S A. 1992; 89:8472–8476. [PubMed: 1356264]
27. Rao US. Mutation of glycine 185 to valine alters the ATPase function of the human P-glycoprotein expressed in Sf9 cells. J Biol Chem. 1995; 270:6686–6690. [PubMed: 7896810]
28. Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem. 1992; 267:4854–4858. [PubMed: 1347044]
29. Favaro G, Clementi C, Romani A, Vickackaite V. Acidichromism and ionochromism of luteolin and apigenin, the main components of the naturally occurring yellow weld: a spectrophotometric and fluorimetric study. J Fluoresc. 2007; 17:707–714. [PubMed: 17710519]
30. Cheng AC, Huang TC, Lai CS, Pan MH. Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. Eur J Pharmacol. 2005; 509:1–10. [PubMed: 15713423]
31. Farrow SN, Brown R. New members of the Bcl-2 family and their protein partners. Curr Opin Genet Dev. 1996; 6:45–49. [PubMed: 8791486]
32. Tyagi A, Singh RP, Agarwal C, Siriwardana S, Sclafani RA, Agarwal R. Resveratrol causes Cdc2-tyr15 phosphorylation via ATM/ATR-Chk1/2-Cdc25C pathway as a central mechanism for S phase arrest in human ovarian carcinoma Ovcar-3 cells. Carcinogenesis. 2005; 26:1978–1987. [PubMed: 15975956]
33. Kim SJ, Hwang SG, Shin DY, Kang SS, Chun JS. p38 kinase regulates nitric oxide-induced apoptosis of articular chondrocytes by accumulating p53 via NFkappa B-dependent transcription and stabilization by serine 15 phosphorylation. J Biol Chem. 2002; 277:33501–33508. [PubMed: 12091386]
34. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. 2001; 15:2177–2196. [PubMed: 11544175]
35. Shieh SY, Ahn J, Tamai K, Taya Y, Prives C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev. 2000; 14:289–300. [PubMed: 10673501]
36. Janicke RU. MCF-7 breast carcinoma cells do not express caspase-3. Breast Cancer Res Treat. 2009; 117:219–221. [PubMed: 18853248]
37. Priyadarsini RV, Vinothini G, Murugan RS, Manikandan P, Nagini S. The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors. Nutr Cancer. 2011; 63:218–226. [PubMed: 21294050]
38. van Zanden JJ, de Mul A, Wortelboer HM, Usta M, van Bladeren PJ, Rietjens IM, Cnubben NH. Reversal of in vitro cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin. Biochem Pharmacol. 2005; 69:1657–1665. [PubMed: 15896345]

39. Horinaka M, Yoshida T, Shiraishi T, Nakata S, Wakada M, Nakanishi R, Nishino H, Matsui H, Sakai T. Luteolin induces apoptosis via death receptor 5 upregulation in human malignant tumor cells. Oncogene. 2005; 24:7180–7189. [PubMed: 16007131]
40. Spencer JP, Chowrimootoo G, Choudhury R, Debnam ES, Srai SK, Rice-Evans C. The small intestine can both absorb and glucuronidate luminal flavonoids. FEBS Lett. 1999; 458:224–230. [PubMed: 10481070]
41. Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, Baker J, Kerr DJ. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. Clin Cancer Res. 1996; 2:659–668. [PubMed: 9816216]
42. Ju W, Wang X, Shi H, Chen W, Belinsky SA, Lin Y. A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor-kappaB pathway and sensitization of apoptosis in lung cancer cells. Mol Pharmacol. 2007; 71:1381–1388. [PubMed: 17296806]
43. Fang J, Zhou Q, Shi XL, Jiang BH. Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells. Carcinogenesis. 2007; 28:713–723. [PubMed: 17065200]
44. Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T, Yano H, Kojiro M, Sata M. Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. Cancer Res. 2006; 66:4826–4834. [PubMed: 16651438]
45. Chen JS, Agarwal N, Mehta K. Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools. Breast Cancer Res Treat. 2002; 71:237–247. [PubMed: 12002342]
46. Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010; 429:403–417. [PubMed: 20626350]
47. Casanovas O, Miro F, Estanyol JM, Itarte E, Agell N, Bachs O. Osmotic stress regulates the stability of cyclin D1 in a p38SAPK2-dependent manner. J Biol Chem. 2000; 275:35091–35097. [PubMed: 10952989]
48. Sethi G, Sung B, Aggarwal BB. Nuclear factor-kappaB activation: from bench to bedside. Exp Biol Med (Maywood). 2008; 233:21–31. [PubMed: 18156302]
49. Pirnia F, Breuleux M, Schneider E, Hochmeister M, Bates SE, Marti A, Hotz MA, Betticher DC, Borner MM. Uncertain identity of doxorubicin-resistant MCF-7 cell lines expressing mutated p53. J Natl Cancer Inst. 2000; 92:1535–1536. [PubMed: 10995814]
50. Ogretmen B, Safa AR. Expression of the mutated p53 tumor suppressor protein and its molecular and biochemical characterization in multidrug resistant MCF-7/Adr human breast cancer cells. Oncogene. 1997; 14:499–506. [PubMed: 9053847]

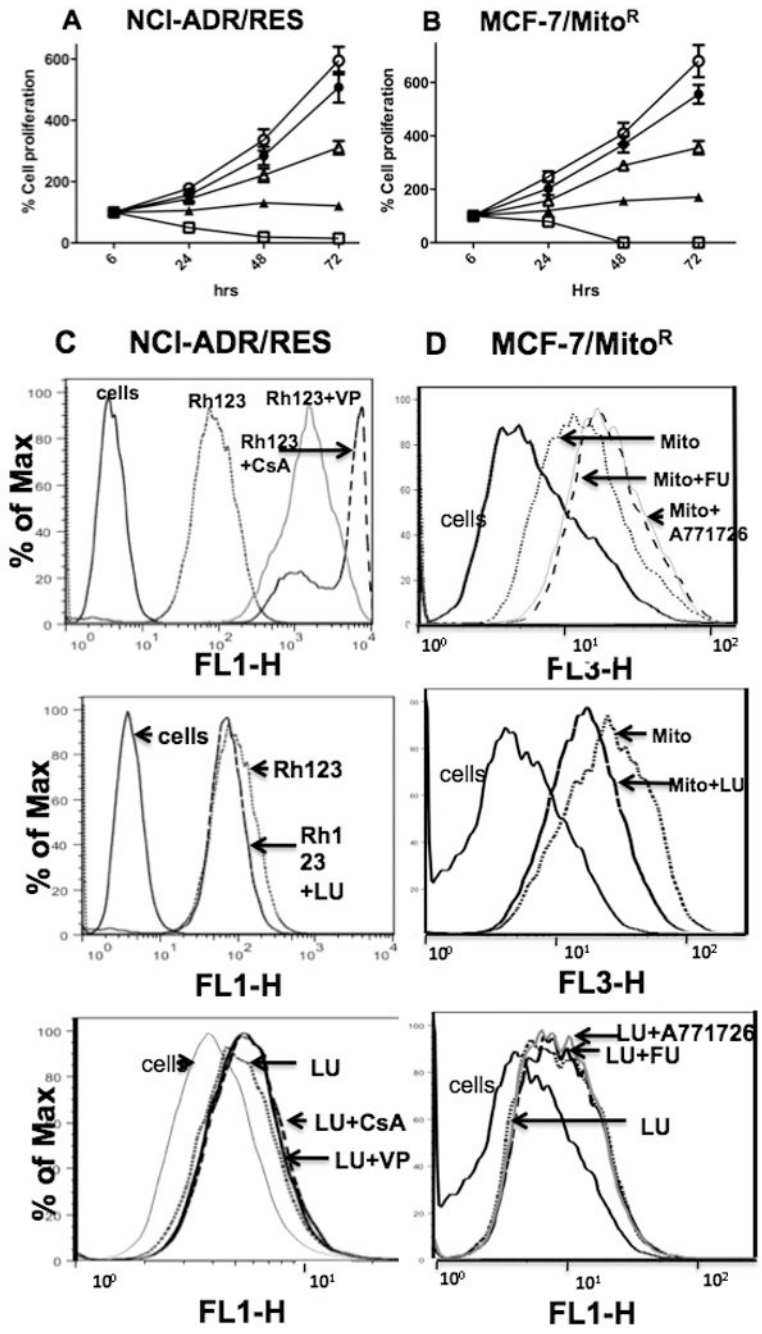

Figure 1.

A and B: Inhibition of MDR cancer cell proliferation by luteolin. NCI-ADR/RES and MCF-7/Mito^R cells were incubated with DMSO (○), 12.5 μM (●), 25 μM (△), 50 μM (▲), and 100 μM (□) luteolin. At indicated time points, cell growth was quantitated by MTS assay. Growth determined at 4 h after incubation was considered 100%, and the growth observed in the subsequent time points was expressed as percent of growth observed at 4 h. Data points are means of octaplicates. Bars ± SD.

C and D. Drug uptake assays by flow cytometry. *C. NCI-ADR/RES cells. Upper panel: Effect of verapamil and CsA on Rh123 accumulation.* Cells were incubated with 10 μM

Rh123, 5 μM CsA and 50 μM verapamil in combinations as indicated in the figure and analyzed.

Middle panel. Effect of luteolin on Rh123 accumulation. Cells were incubated with 10 μM Rh123 and 50 μM luteolin in combinations as indicated in the figure and analyzed.

Lower panel. Effect of verapamil and CsA on luteolin accumulation. Cells were incubated with 50 μM luteolin, 5 μM CsA and 50 μM verapamil in combinations as indicated in the figure and analyzed

D. MCF-7/Mito^R cells. Upper panel. Effect of fumitremorgin and A771726 on mitoxantrone accumulation. Cells were incubated with 1 μM mitoxantrone, 1 μM fumitremorgin and 10 μM A771726 in combinations as indicated in the figure and analyzed.

Middle panel. Effect of luteolin on mitoxantrone accumulation in MCF-7/Mito^R cells. Cells were incubated with 1 μM mitoxantrone and 50 μM luteolin in combinations as indicated in the figure and analyzed.

Lower panel. Effect of fumitremorgin and A771726 on luteolin accumulation. Cells were incubated with 50 μM luteolin, 1 μM fumitremorgin and 10 μM A771726 in combinations as indicated in the figure and analyzed.

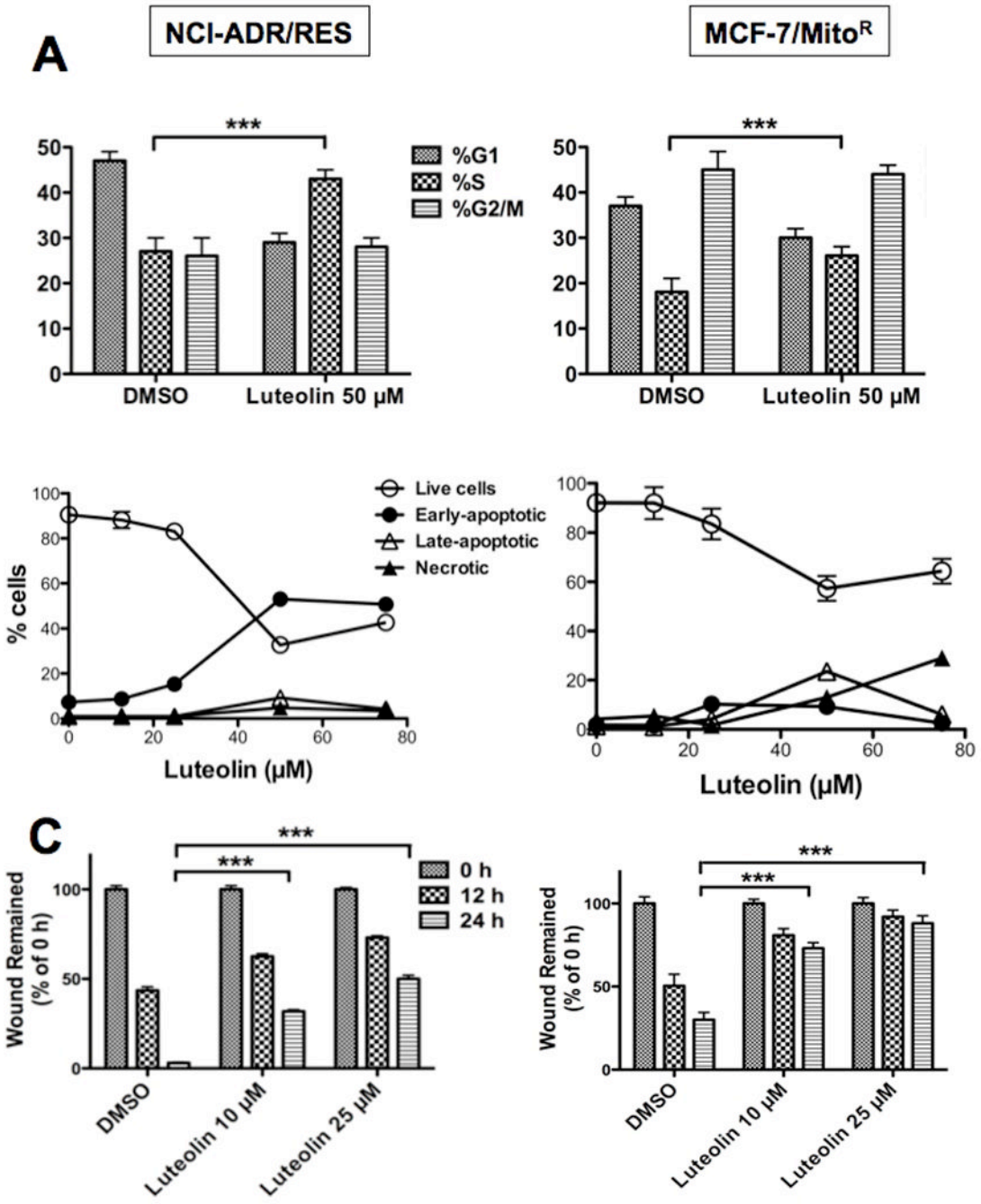

Figure 2.

Effect of luteolin on cellular processes. *Left panel*: NCI-ADR/RES cells; *right panel*: MCF-7/Mito^R cells.

A. *Effect of luteolin on cell cycle*. Cells were treated with 50 μM luteolin or DMSO for 18 h and the percent cells in the G_1, G_2/M, and S phases of the cell cycle were analyzed by flow cytometry.

B. *Luteolin induces apoptosis*. Cells were incubated with increasing concentrations of luteolin or ethanol (control) for 18 h and stained with annexin V-FITC reagent and propidium iodide and analyzed by flow cytometry. Percent live, early- and late-apoptotic and necrotic cells were calculated using FlowJo software (Supplemental Figure 1).

C. *Effect of luteolin on cell migration*. Cells were incubated with indicated concentrations of luteolin and were subjected to wound healing assay. The average initial wound width measured at 0 h was considered 100%. The scratch widths at 12 and 24 h were expressed as percent of 0 h time point. A P value <0.001 was considered significant.

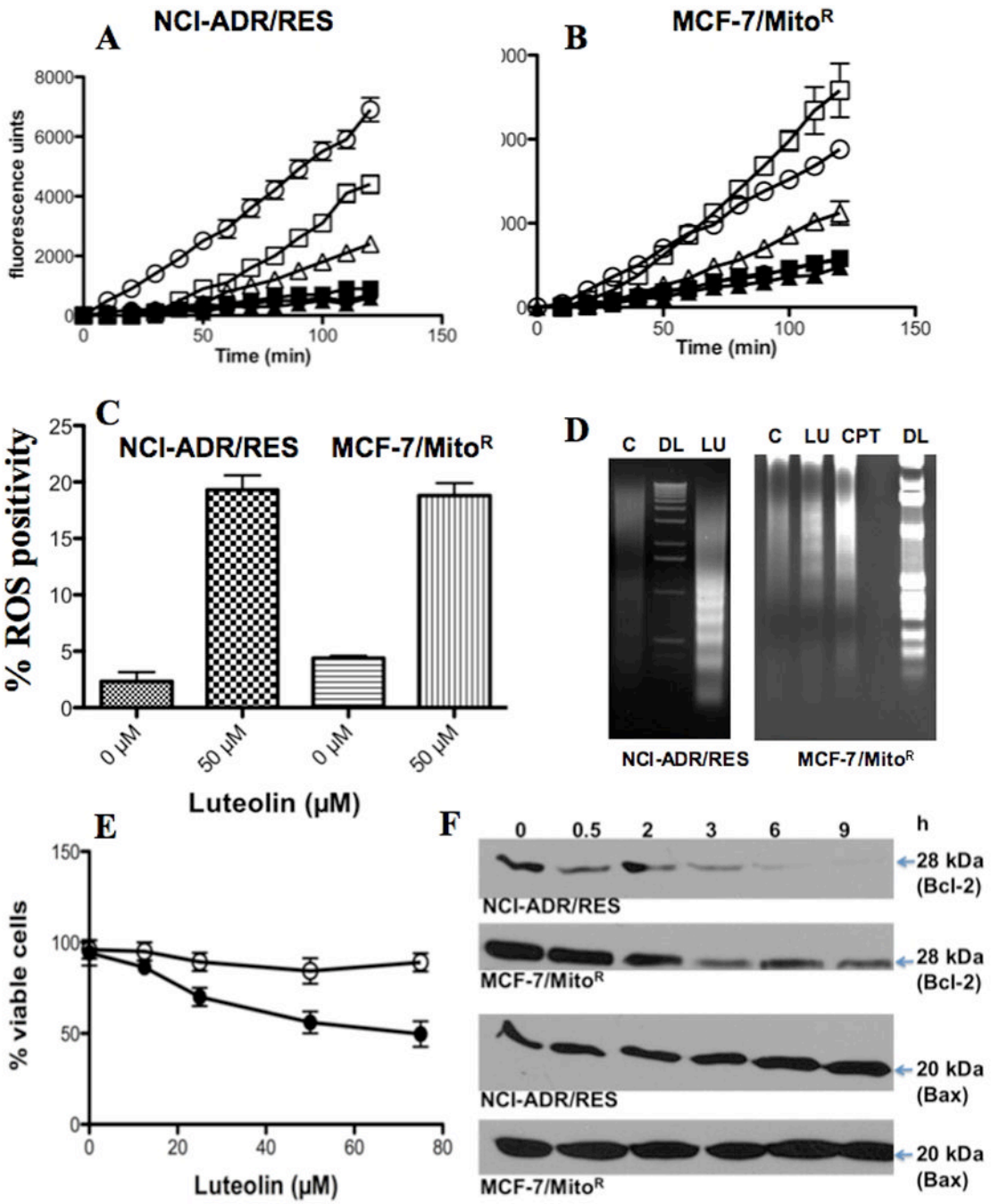

Figure 3.

Effect of luteolin on ROS production, mitochondrial function and genomic DNA fragmentation. *On ROS production*. NCI-ADR/RES (**A**) and MCF-7/Mito^{R} (**B**) were incubated with control (○), 50 μM (△) and 100 μM (□) luteolin and the ROS was measured as described under Materials and Methods. ROS was also measured in cells (●), cells incubated with 50 μM (▲) and 100 μM (■) luteolin in the presence of 0.5 mM glutathione (reduced form). Points, mean; bars, ± SD. (**C**). Cells were incubated with 50 μM luteolin for 24 h and ROS was measured by flow cytometry as described [19]. Columns, mean; bar, SE.

D. *Analysis of DNA fragmentation*. Cells were treated with DMSO (*Lane:* C), 25 μM luteolin (*Lane:* LU) and 1 μM camptothecin (*Lane:* CPT) for 24 h and DNA fragmentation was analyzed. Standard DNA ladder (*Lane*: DL).
E. *On MMP*. NCI-ADR/RES (○) and MCF-7/Mito^{R} (●) cells were incubated with increasing concentrations of luteolin for 24 h and the percent viable cells were measured by TMRM assay as described. (Supplemental Figure 2). Points, mean; bars, ± SD.
F. *Analysis of Bcl-2 and Bax*. Cells were incubated with 50 μM luteolin for indicated time periods, and 30 μg protein from each sample was analyzed by western blotting using specific antibodies.

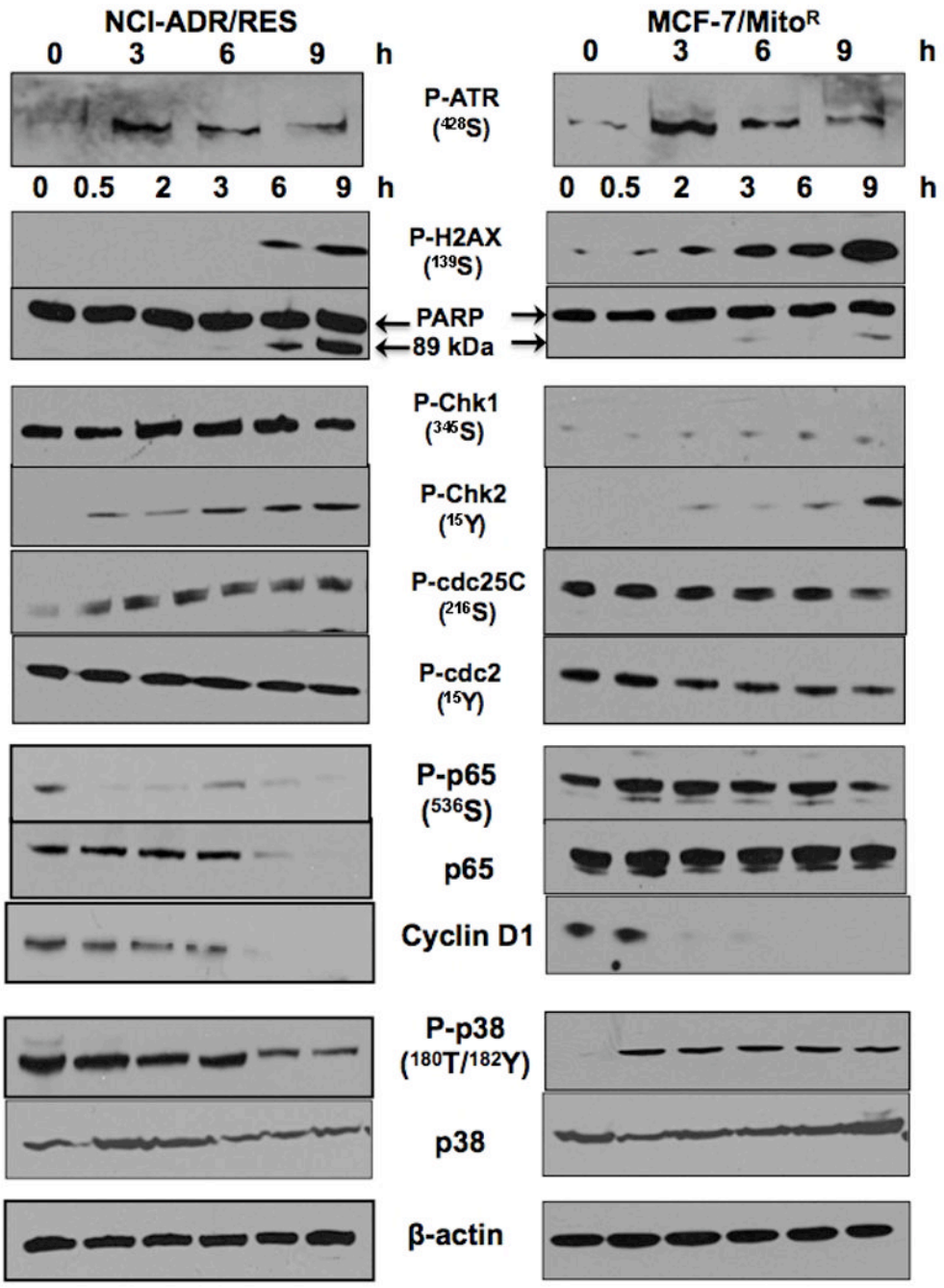

Figure 4.

Effect of luteolin on cellular proteins. Cells treated with 50 μM luteolin and 30 μg protein from each sample collected at indicated times was analyzed by western blotting using specific antibodies.

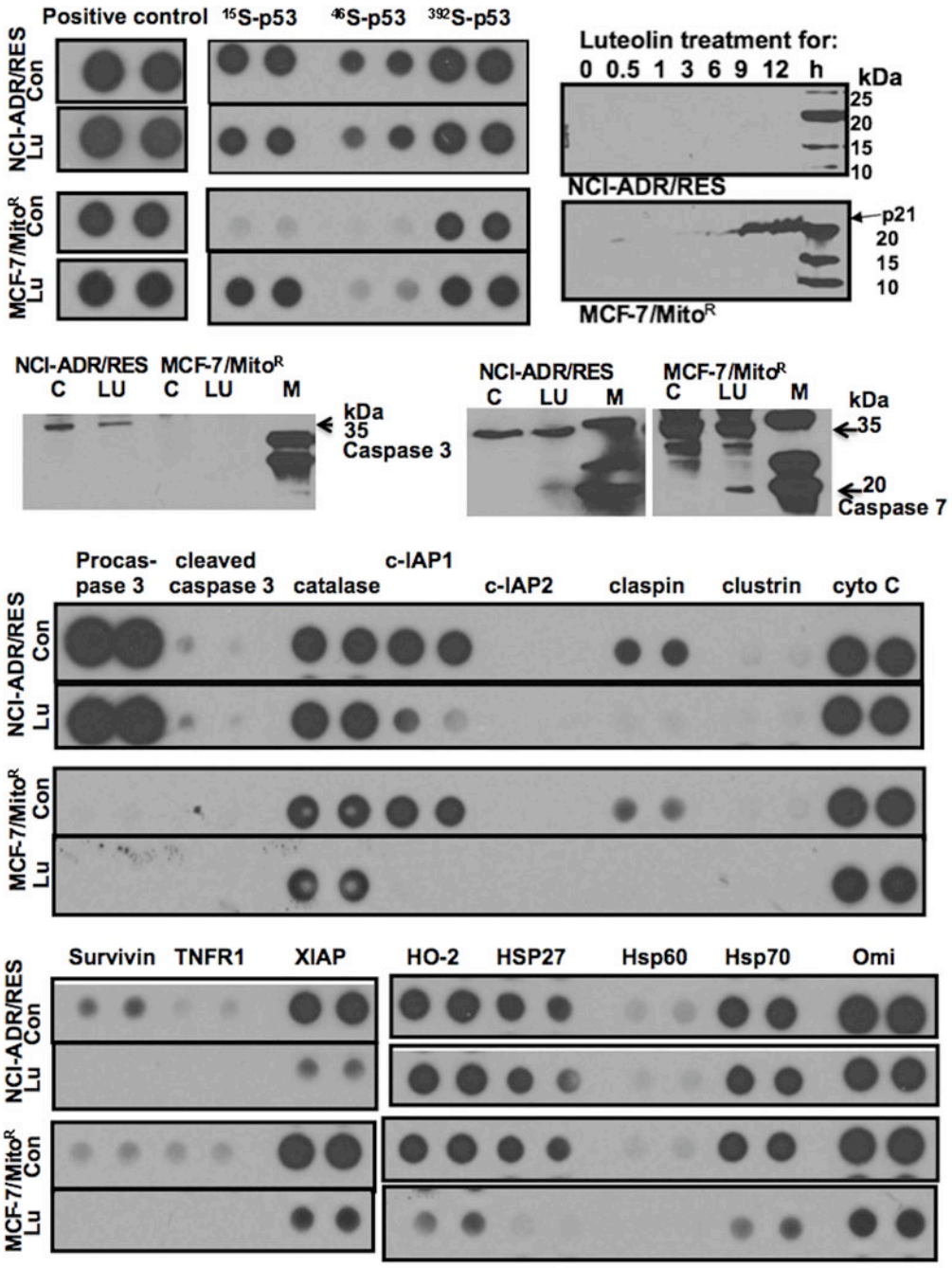

Figure 5.

Effect of luteolin on proteins involved in apoptosis. Cells were treated with DMSO (control) and 50 μM luteolin for 24 h and analyzed either by Western blotting or Apoptosis Antibody Array, as described under Materials and Methods. Positive controls in the Apoptosis Antibody Arrays were shown to indicate that the paired blots were incubated with equal amounts of protein.

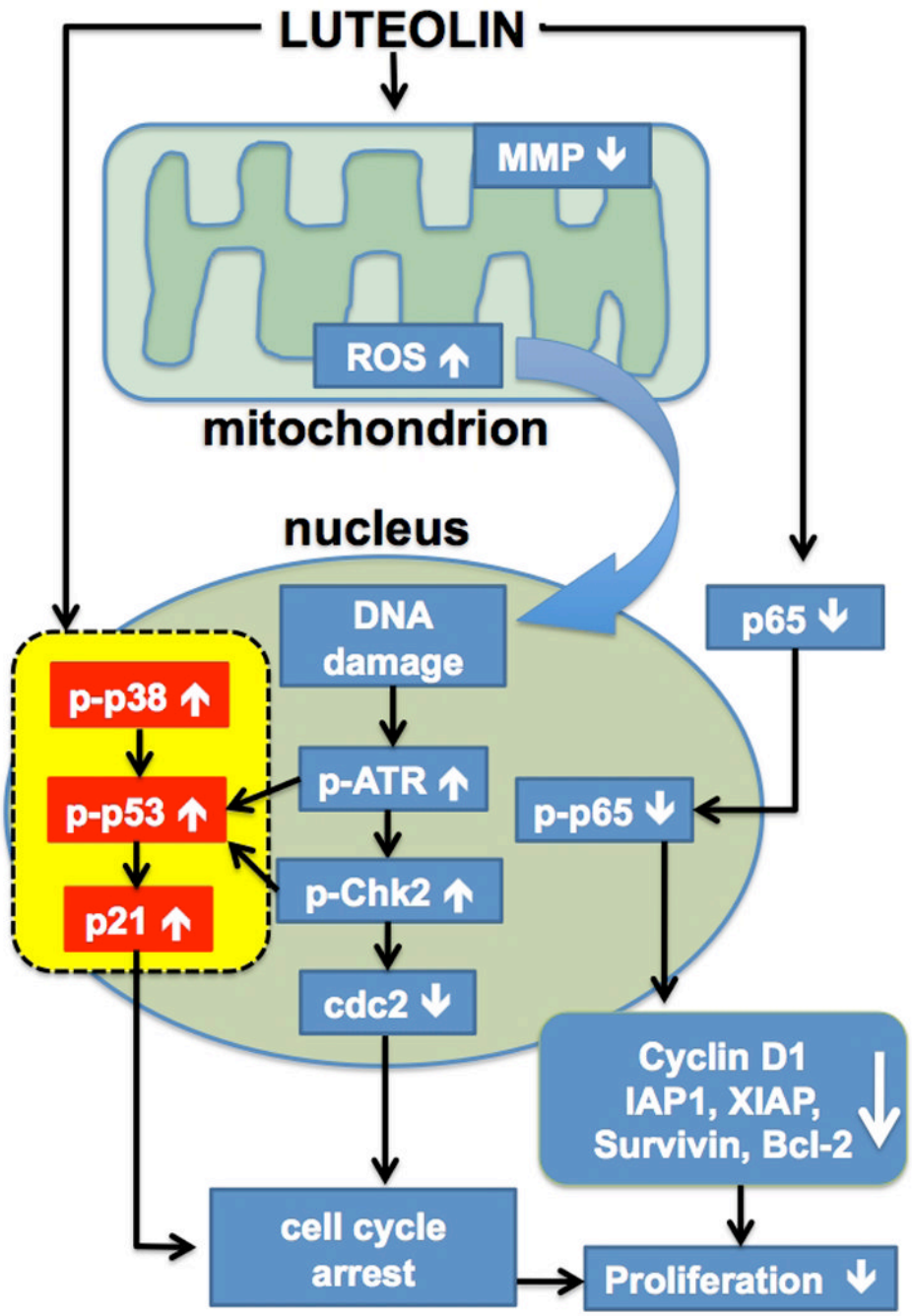

Figure 6.

Schematic overview of the mechanism of luteolin in NCI-ADR/RES and MCF-7/Mito^R cells. Mechanisms that are commonly affected in both MDR cancer cell lines are highlighted in blue, whereas mechanism that is unique to MCF-7/Mito^R cells is highlighted in yellow.