Overview how adenocarcinoma cancer cells avoid immune- and chemotherapy-induced apoptosis

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Abstract

This review describes some aspects of uncontrolled tumor growth and development. In the past, it has been shown that colon adenocarcinomas use several tactics to avoid cell deletion and to maintain cell viability. In particular, colorectal cancer cells resist death ligands-induced apoptosis by expressing anti-apoptotic proteins. By direct interaction with FADD, the FLIP protein inhibits the signal transmission from death receptors to their cytoplasmic targets in COLO 205 cells. Colorectal cancer cells also stimulate own survival by inhibiting cytotoxic signals induced by interferons. Moreover, IFN-α increases immune-resistance of colon cancer cells by activation of NF-κB. Additionally, the cytoplasmic retention of proapoptotic protein clusterin also supports viability of cancer cells. Upon suitable stimulation normal cells are featured by clusterin translocation to the nucleus with concomitant cell death. We found that proapoptotic activity of clusterin is dependent on calcium ions, and depletion of intracellular calcium caused extensive death of COLO 205 cells. Other type of strategy to inhibit chemotherapy-dependent cell death is the activity of multidrug resistance proteins (MDR). These cell membrane efflux pumps actively expel the drugs from the cell interior to prevent their action on intracellular targets. The reversal of P-gp efflux pump in chemoresistant COLO 320 cell line was observed upon phenothiazine derivatives. The variety of antiapoptotic mechanisms in colorectal cancer cells makes anticancer therapy a great challenge but detailed knowledge of their complexicity gives promise to sensitize cancer cells to death stimuli.

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Introduction

Programmed cell death (apoptosis) assures every day elimination of the transformed cells to maintain regular functions of tissue organs. Apoptosis could be induced extrinsically by death ligands (TNFα, TRAIL, FasL) and/or other cytokines (IFNα, IFNβ, IFNγ) released by the immune system or intrinsically when cell death machinery is solely activated by mitochondria [1,2]. Additionally, sudden uncompensated intracellular changes in calcium homeostasis could also induce cell death [3,4]. During evolution, however, cancer cells developed and conserved several molecular mechanisms allowing them to avoid apoptotic signals. They either, gradually express less or modify membrane receptors (transmembrane ligands, soluble receptors) or they express constitutively active oncogenes. Furthermore, a plethora of antiapoptotic proteins, which inhibit death signals at different levels of signaling pathway, have been identified. Moreover, cancer cells resist chemotherapy producing a variety of proteins called multidrug resistance proteins (MDR), which act as an efflux pumps. Consequently, anticancer drugs are expelled extracellularly leading to low efficacy of the chemotherapy of cancer.

Colon cancer is one of the most common types of cancer and ranks third place in terms of occurrence and death rates among population in developed countries. The increasing number of people affected by colon adenocarcinomas in the most advanced metastatic forms prompted us to seek how this type of tumor develops to the stage where neither immune system nor chemotherapy could help. As an experimental model the human colon adenocarcinoma COLO 205 and COLO 320 cancer cell lines were used.

The resistance of colon adenocarcinoma cells to TNF-α-induced apoptosis

The TNF-α and its receptors are the best known group representing the TNF-α-superfamily death signals [5,6]. When TNF-α is released from macrophages it might interact with each of the two specific transmembrane receptors TNF-R1 and TNF-R2. However, it is TNF-R1 that plays the role to transduce the order from the immune system. In physiological conditions, if TNF-α trimeric ligand binds to its oligomerized receptor it enables to trigger few, sometimes opposite cellular responses. The response to die or to survive depends on the type of the adaptor proteins which subsequently assembly with TNF-R1 by homotypic interactions between death (DD) or death effector domains (DED) of respective proteins. Initially, TRADD (TNF-R1-associated death domain protein) is linked to intracellular domain of TNF-R1 (DD-DD) followed by assembly of FADD (Fas-associated death domain), RAIDD (RIP-associated ICE-homologous protein with death domain) or TRAF (TNF-R-associated factor) proteins to TRADD by DED-DED interactions.

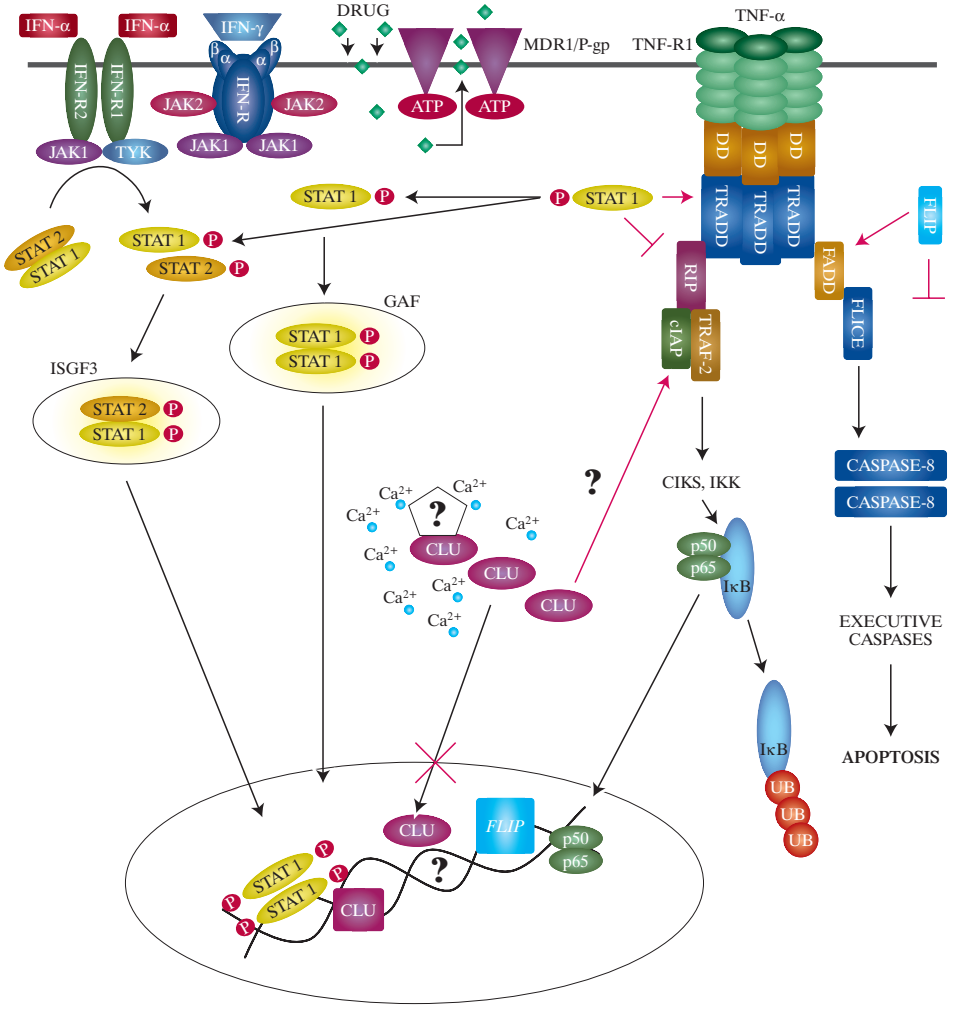
Preferentially, TRAF2 (TNF-R-associated factor-2) and RIP (receptor interacting protein) interact with TRADD (complex I) [7] to form the so-called complex I. Consequently, other important functional proteins, CIKS (NF-κB inducing kinase, NIK), are sequentially recruited to activate JNK (c-jun N-terminal kinase) or survival-signalosome by IκBα (inhibitory subunit of NF-κB) phosphorylation and subsequent NF-κB (nuclear factor kappa B) activation [8]. The latter is the transcriptional factor crucial for cell survival since it transactivates several antiapoptotic genes *A20* [9], *cIAP1/2* [10], *TRAF1/2* [11], *Bfl-1/A1* [12], *IEX-1L* [13] and *Bcl-x_L* [14]. On the other hand, if the synthesis of antiapoptotic proteins is not sufficient for the protection from apoptosis, the complex I dissociates and complex II called DISC (death-initiating signaling complex) is recruited [7]. In complex II, TRADD associates with FADD to allow FLICE (FADD-like interleukin converting enzyme) dimerization. FLICE is autocatalytically activated to caspase-8 [15] and at that moment other downstream caspases could be activated (caspase-3, -6, -7) and programmed cell death (PCD) is executed. Additionally, if RAIDD and RIP are recruited to TRADD, then procaspase-2 is activated [16]. However, the final cellular effect of TNF-α depends on the quantitative input of the particular transmission pathway. Recently, it was found, that additional interactions at the level of adaptor proteins with STAT 1 (signal transducer and activator of transcription 1) kinase or clusterin render TNF-α signal more complex and hard to predict [17,18].

In COLO 205 cells the TNF-α-induced death signal is limited to the receptor type. Irrespective to the expression of either type of receptor, solely TNF-R1 is able to interact with soluble TNF-α ligand (sTNF-α). The TNF-RII is activated by the direct interaction with anchored transmembrane TNF-α of the activated macrophages [19] but do not respond to sTNF-α. However, in our study we found, that the TNF-α does not induce apoptosis in COLO 205 cells, even though these cells express the functional TNF-RI receptor [20]. We hypothesized that some antiapoptotic protein(s) had to inhibit TNF-α-dependent death signal. The potent candidate is cFLIP (FLICE-inhibitory protein) protein, which competes with FLICE for a binding in the DISC complex. There are known two isoforms of cFLIP: $cFLIP_S$ and $cFLIP_L$. On one hand, $cFLIP_L$ is structurally similar to procaspase-8, since it contains two DEDs and a caspase-like domain (CASP). However, the latter domain lacks residues that are important for the catalytic activity of caspase-8 [21,22]. On the other hand, $cFLIP_S$ possesses only two DEDs. Via DED--DED interaction cFLIP is recruited to FADD protein and activation of caspase-8 is blocked. As a regulator of lymphocytes T and B maturation and embryonic cells development also positive effects of cFLIP were reported (for details see the review article by Pajak et al. [23]).

Importantly, the presence of cFLIP has been identified in many types of cancer cells and is proposed to cause the resistance to death ligand-induced apoptosis [24-27]. To verify the role of cFLIP in cancer cell apoptosis the mouse lymphoma MBL2-Fas cancer cells, which express the high level of Fas protein and tumor embryonic AR6 cell, with low level of Fas, were both transfected with cFLIP protein [28]. Transfected and mock-transfected cells were injected into mice, however, exclusively cFLIP-transfected cells (MBL2-Fas and AR6) developed into cancer. Interestingly, the presence of cFLIP (antiapoptotic) but not Fas protein (proapoptotic) appeared crucial for cancer development. Importantly, the overexpression of $cFLIP_L$ was found in colon carcinoma cells isolated from surgical specimens [29]. The promising approach is limiting cFLIP protein level, once achieved it could sensitize cancer cells to death ligand-induced cell death. Actually, it has been shown that the use of metabolic inhibitors, such as anisomycin, emetine, harringtonine or puromycin [26] sensitize prostate cancer cells to TRAIL-induced apoptosis. Similarly, the treatment with actinomycin D (transcription inhibitor) and/or cycloheximide (CHX) (non-specific translation inhibitor) of immune resistant cancer cells render them sensitive to FasL [30,31].

In our studies cycloheximide, when used alone, did not cause cell death at least during initial 12 hours, however, when cells were treated with TNF-α in the presence of CHX, the viability of COLO 205 adenocarcinoma cells progressively and inevitably fall down. It was confirmed by electron microscopy analysis that COLO 205 cells died from the extensive apoptosis [20]. The Western blot and immunocytochemistry analyses showed that the cFLIP protein level decreased although it was not the case when TNF-α was used alone. Moreover, the immunoprecipitation studies confirmed that the cFLIP level dropped in DISC complex, and that this reduction was accompanied by the higher caspase-3 activity. The results seem to support the hypothesis, that COLO 205 cancer cells could no longer avoid TNF-α--induced apoptosis if protein synthesis is inhibited. To clarify the exact role of cFLIP in TNF-α signaling pathway we used siRNA oligonucleotides to knockdown FLIP gene. It was supposed that silencing FLIP gene would resemble CHX co-treatment. In fact, western blot analyses demonstrated decreasing levels of cFLIP in siRNA transfected cells (in comparison to mock-transfected cells). Additionally, TNF-α treatment activated procaspase-3 in siRNA transfected COLO 205 cells (unpublished data). These results confirm the statement that cFLIP retards

Figure 1. **Schematic representation of the molecular mechanisms involved in "immune escape" and chemoresistance of colon adenocarcinoma cells. STAT 1 kinase plays significant role by inhibiting both the formation of TNF-R1 complex I and subsequent NF-κB activation. IFNAR and IFNGR compete with TNF-R1 for STAT 1. Thus, IFNs release TNF-R1 complex I from STAT 1 kinase inhibition. Calcium ions (Ca^{2+}) sequester clusterin (CLU) in the cytoplasm and by this route COLO 205 cells become resistant to apoptogenic stimuli. Lastly, high expression of P-gp efflux pump renders COLO 320 cells unsusceptible to anticancer drugs. Black arrows indicate the direction of the respective signaling pathways. Red arrows indicate targets for possible interactions whereas dashed stops show subsequent blockages**



TNF-α-induced death signal. Because TNF-R1 DISC complex is similar to DISC complexes found at DR 4 and DR 5 receptors activated by TRAIL (TNF-α-related apoptosis inducing ligand), it is assumed that cFLIP expression might also help COLO 205 cells to avoid TRAIL-induced apoptosis [32]. However, hitherto no available data exist that confirm the role of cFLIP in TRAIL-induced apoptosis in COLO 205 cell line.

The crosstalk between interferons and TNF-R1 signaling pathways in COLO 205 cells

Interferons (IFNs) are a family of cytokines of multifunctional activity. They block viral infections, inhibit cell proliferation and modulate cell differentiation [33]. These cytokines also play important roles in immunosurveillance for malignant cells. The IFNs family includes two main classes of related cytokines (type I and type II). There is a variety of type I interferons, all of which have considerably similar structure. In humans these group include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω [34,35]. All type I IFNs assembly with a common cell surface receptor, which is known as the type I IFN receptor (IFNAR) [34]. By contrast, there is only one type II interferon called IFN-γ that binds another cell surface receptor known as the type II IFN receptor (IFNGR) [35]. The type I receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus activated kinases (JAKs): tyrosine kinase 2 (TYK2) and JAK1, respectively. The type II receptor is also composed of two subunits: IFNGR1 and IFNGR2, which are in turn associated with JAK1 and JAK2, respectively. Activation of JAKs by type I IFNs results in tyrosine phosphorylation of STAT 2 and STAT 1; this leads to the formation of STAT 2-STAT 1-IRF9 (IFN-regulatory factor 9) complexes, which are also known as ISGF3

(IFN-stimulated gene factor 3) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA strand to initiate gene transcription. Both types of IFNs also induce the formation of STAT 1-STAT 1 homodimers that translocate to the nucleus and bind GAS (IFNγ-activated site) elements that are present in the promoter regions of certain ISGs, thereby initiating the transcription of these genes [36-39].

It is well established that IFNs induce the expression of hundreds of genes, which mediate various biological responses. In any case, it has been apparent that the activation of JAK--STAT pathways alone is not sufficient for the generation of all of the biological activities of IFNs. There is accumulating evidence that several other IFN-regulated signaling elements and cascades are required for the generation of many cellular responses to IFNs. Some of these pathways co-operate with JAK--STAT pathway, whereas others only with STATs. For example, type I IFNs were shown to activate the mitogen-activated protein kinase (MAPKK) p38 and its downstream effectors [40]. Moreover, also the activity of PI3-K signaling pathway could be modulated by type I IFNs [41,42]. In cancer cells IFNs have been examined as potential synergistic agents in death ligands-induced apoptosis [43,44]. STAT 1 kinase was also described as a component of TNF-R1 signaling complex. According to Wang et al. [45] in HeLa cell line stimulated with TNF-α the STAT 1 kinase binds to TRADD protein and attenuates the interaction of TRADD with TRAF2 and RIP. As a consequence the NF-κB activation is blocked. The authors claimed that the TRADD-STAT 1 complex promotes DISC formation and TNF--α-dependent apoptosis. In our studies performed on COLO 205 cells we found that neither IFN-α nor IFN-γ, and not even IFN-α combined with IFN-γ treatment affect cell survival (unpublished data). Interestingly, also NF-κB activation in the presence of TNF-α was not detectable [18].

We hypothesized, that the lack of NF-κB activation results from the presence of STAT 1 kinase in TNF-R1 complex. The immunoprecipitation analysis confirmed this interaction. Moreover, the level of STAT 1 kinase in TNF-R1 signalosome was TNF-α-dose-dependent [18]. We also concluded that inefficiency of IFNs to amplify death signal might be a consequence of the competition for STAT 1 kinase between IFNs and TNF-α signaling pathway. Again, the immunoprecipitation studies showed that in the absence of activation of TNF-R1, STAT 1 kinase is bound to TRADD protein. It seems likely, that in COLO 205 cells, STAT 1 kinase preferentially binds TNF-R1 complex. Consequently, the decreased level of STAT 1 in the cytoplasm limits the transduction of IFNs signal. The pretreatment of COLO 205 cells with IFN-α or IFN-γ reduced the STAT 1 kinase level in TNF-R1 complex. IFN-α also stimulated Y701 phosphorylation and increased the expression of STAT 1. Regardless of the reduced level of STAT 1 complexed with TRADD, only IFN-α pretreatment combined with subsequent administration of TNF-α subsequently induced NF-κB (unpublished data). Lack of STAT 1 phosphorylation by IFN-γ suggests the disturbances in the upstream kinases activity, probably JAK2. It is also not clear whether NF-κB activation was induced by TNF-α or whether it resulted from the higher PI3-K activity stimulated by type I IFNs (at the moment this aspect is under scrutiny). It is clear from this examination that administration of IFN-α could stimulate colon cancer cell survival. In the past, the conflicting results led to the concern whether there is sufficient evidence to make use of IFN-α in cancer therapy. There are known positive effects of type I IFNs action against hematopoietic neoplasms [46,47] and some vascular tumors, such as pulmonary hemangiomatosis, infantile hemangiomas, Kaposi's sarcoma and others [48]. IFN-α was also effective in some groups of patients suffering from renal cancer [49] and melanoma. At the same time, there are reports indicated negative effects of type I IFNs in anticancer treatment [50,51]. Our observations are in favour of the opinion stressing possible negative effect of IFN-α therapy in colon cancer therapy.

The calcium-dependent regulation of apoptotic activity of clusterin

As indicated in the first section of the article, apoptosis is induced by extrinsic signals but could be also activated by intrinsic pathway. One of the most important modifiers of apoptosis is calcium homeostasis. Changes in calcium ion concentration within different cell compartments activate pro- or antiapoptotic proteins. It is known, that there are some types of cancers which have increased intracellular calcium level in comparison to normal cells. According to our results, the reduction of intra- but not extracellular calcium concentration reduces COLO 205 cell survival [20]. Among the antiapoptotic proteins blamed for this phenomenon, clusterin (CLU) is a potent candidate. The exact function of clusterin in the regulation of cell death remains unclear, especially in cancer cells, although, according to the latest reports, clusterin could play significant role in the failures of chemotherapy and/or modulation of immune system to delete cancer cells. Clusterin has a wide range of physiological functions (for details see the review by Pajak et al. [52]), however, clusterin has been described also as both a pro- and as an antiapoptotic protein. The discrepancy in function of CLU could be related to the specific proteomic profile, which results from the cell type specific routes of expression CLU. The main product of clusterin gene expression is 60 kDa precursor form which is glycosylated and cleaved to α- and β-subunits, held together by five disulfide bonds [53]. The mature, 80 kDa protein (sCLU) could be secreted to the extracellular space and body fluids [54]. The second 50 kDa isoform of clusterin (intracellular iCLU) is localized in the cytoplasm. Upon some cytotoxic stimuli, including ionizing radiation (IR) [55], transforming growth factor β [56], phorbol ester (TPA) [57], or cytokines, such as TNF-α [58] iCLU is activated and translocates to the nucleus. It is also indicated that clusterin activity is dependent on the type of chemotherapeutic agent. According to Scaltriti et al. [59] and Caccamo et al. [60] the administration of topoisomerase II inhibitor – etoposide, elevated the level of clusterin within the nucleus with the concomitant reduction of iCLU pool in the cytoplasm of the PC-3 prostate cancer cells. Interestingly, in adenocarcinoma COLO 205 cancer cells, etoposide induced the nuclear localization of iCLU, but the Western blot analysis did not show the decreased level of 50 kDa clusterin in the cytoplasm (unpublished data). The presence of clusterin in the COLO 205 cells

nuclei could result from changes in gene transcription profile as an alternative to CLU translocation.

In 2005, Caccamo et al. [61] presented interesting observations pointing to the role of calcium ions in iCLU activity. The use of BAPTA-AM (intracellular calcium ions chelator) in PC3 prostate cancer cells resulted in a highly significantly inhibition of both cell growth and viability. Cell death was associated with characteristic hallmarks of anoikis. The accumulation of iCLU in the nuclei was accompanied by the activation of execution caspases and progression to apoptosis. Our results obtained from COLO 205 cells are similar, although we independently used other calcium chelators. Diminishing extracellular calcium concentration by EGTA in COLO 205 cells did not induce changes in iCLU localization, whereas EDTA, which chelates both extra- and intracellular calcium, promoted nuclear iCLU expression with concomitant caspase-3 activation. These results suggest that the lack of iCLU in the nuclei of colon adenocarcinoma cells promotes cell survival, whereas its translocation/expression within nucleus correlates with cell death (article in press). It is not clear, however, what are the details of proapoptotic activity of CLU. Keeping in mind the presence of coiled-coil domain in the clusterin sequence (domain characteristic for some transcription factors) we suppose that CLU is able to interact with DNA to control the expression of proapoptotic genes. The *in vitro* observations have been confirmed by the *ex vivo* studies, which also demonstrated the increased level of nuclear localization of iCLU in tumor-bearing gut compared with normal subjects [62]. The analogous results were obtained from prostate, breast and colon cancer specimens suggesting the existence of a common molecular mechanism of CLU action in cancer cells. The knowledge about clusterin activity and its possible regulators, such as calcium channel modifiers, could be a promising prospect in future cancer therapy. The latter studies are also needed to explain the connections between clusterin and the TNF-α signaling pathway, especially if the effect of calcium chelators does not oppose cell viability affected by cycloheximide and TNF-α [18].

MDR proteins and the resistance to chemotherapy-induced cell death

Since cancer cells avoid immune system-induced apoptosis, the presently used cancer treatments are often based on administration of compounds which promote physical or chemical stress in order to evoke apoptosis through the mitochondrial or intrinsic pathway. However, chemotherapy is effective only in some cancer types. In addition, even if tumors initially are not resistant to a specific anticancer treatment, the genetic and epigenetic heterogeneity in the face of the powerful selection imposed by potent anticancer drugs results in overgrowth of drug-resistant variants and the rapid acquisition of drug resistance by many cancers. A great deal of colon cancers confers simultaneous resistance to many different structurally and functionally unrelated drugs. This phenomenon, known as multidrug resistance [63], can results from changes that limit accumulation of drugs within cells by limiting uptake, enhancing efflux, or affecting membrane lipids [64]. Such changes unable the execution of programmed cell death previously activated by anticancer drugs administration. It came as something of a surprise that the major mechanism of multidrug resistance in cultured cancer cells was the expression of an energy-dependent drug efflux pump, known alternatively as P-glycoprotein (P-gp) or the multidrug transporter [65]. The P-gp, the human *MDR1* gene product is one of the 48 known ABC transporters in human cells. It is also highly expressed in cancers of gastrointestinal origin (small and large intestine, liver cancer and pancreatic cancer), cancers of hematopoietic system (myeloma, lymphoma, leukemia), cancers of genitourinary system (kidney, ovary, testicle), and childhood cancers (neuroblastoma, fibrosarcoma). P-gp is a protein that can detect and bind a large variety of hydrophobic natural-product drugs as they enter the plasma membrane. These drugs include many commonly used anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol, as well as many pharmaceuticals. Binding of these drugs results in activation of one of the ATP-binding domains, and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in release of the drug into the extracellular space [66].

The current efforts are focused on identification of new compounds, which by direct interaction with P-pg could inhibit its drug-transport activity *in vitro* in experimental models [67]. Hitherto, wide ranges of natural and newly synthesized compounds have been described as potent inhibitors of P-gp activity. As an effective in inhibition of drug efflux carotenoids, flavonoids and other natural plant derivatives were described [68]. Phenothiazines are also among the compounds known to modify MDR mediated by P-gp in various cancers. In our studies on MDR/COLO 320 cell line the perphenazine and prochlorperazine, the new phenothiazine derivatives, appeared as potential inhibitors of drug efflux [69]. The combined treatment of cytotoxic drugs with concomitant administration of MDR inhibitors seems to be a potential approach in the future cancer therapy.

Conclusions

Antiapoptotic strategies developed by colon cancer cells prevent their proliferation and lead to cancer progression. The first level of cancer resistance is the "immune escape" – the inhibition of death signals induced by natural killers, such as death ligands and interferons. The insufficiency of TNF-α to induce extrinsic apoptosis is caused by the overexpression of the antiapoptotic proteins, such as FLIP, which inhibit the death signal transduction. Therefore, the administration of metabolic inhibitors, which limit the level of antiapoptotic proteins, could sensitize cancer cells to natural apoptogenic signals. Unfortunately, the clinical use of metabolic inhibitors has been limited because of their non-specificic action and toxic side effects. The new natural, non-toxic compounds are intensively tested including modulators of P-gp transport proteins. On the other hand, the interferons, which were sometimes reported as promising in anticancer therapy, appeared totally inefficient to potentiate TNF-α-dependent death of COLO 205 cells. Moreover, the IFN-α treatment might activate antiapoptosis by NF-κB-dependent genes and by the involvement of STAT 1 kinase

in both signaling pathways. So far, the crosstalk between TNF-α and IFNs transduction pathways has not been described in details, but it seems to be one of the most significant achievements of molecular evolution in colon cancers. Clarification the role of clusterin role in TNF-α-dependent signaling pathways as well as possible function of STAT 1 kinase in transcription of antiapoptotic genes in cancer cells could widen the perspectives for search of a new anticancer strategies. New findings related to the molecular mechanisms of antiapoptosis could show new targets for future anticancer therapy. Moreover, the sequential treatment with metabolic inhibitors, death ligands (TRAIL) and modulators of calcium homeostasis (such as calcium channels modifiers or calcium chelators) might be a promising tool to delete cancer cells whereas the inhibitors of drug efflux might be beneficial to minimize the losses of active substances in anticancer therapy.

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