Cell Death and Differentiation (2000) 7, 1127–1136
© 2000 Macmillan Publishers Ltd All rights reserved 1350-9047/00 $15.00
www.nature.com/cdd

Sensitivity to TRAIL/APO-2L-mediated apoptosis in human renal cell carcinomas and its enhancement by topotecan

M Déjosez[1], U Ramp[1], C Mahotka[1], A Krieg[1], H Walczak[2], HE Gabbert[1] and CD Gerharz*[,1]

[1] Institute of Pathology, Heinrich Heine University, D-40225 Duesseldorf, Germany
[2] German Cancer Research Center (DKFZ), D-69120 Heidelberg, Germany
* Corresponding author: CD Gerharz, Institute of Pathology, Heinrich Heine University, PO Box 101 007, D-40225 Duesseldorf, Germany. Tel: +49 211 81 18348; Fax: +49 211 81 18353

Received 13.12.99; revised 20.6.00; accepted 12.7.00
Edited by S Nagata

Abstract

TRAIL (APO-2L) is a newly identified member of the TNF family and induces apoptosis in cancer cells without affecting most non-neoplastic cells, both *in vitro* and *in vivo*. Our study focused on the expression and function of TRAIL and its receptors in renal cell carcinoma (RCC) cell lines of all major histological types. Here, we demonstrate that all RCC cell lines express TRAIL as well as the death-inducing receptors TRAIL-R1 (DR4) and TRAIL-R2 (Killer/DR5). Exposure to TRAIL induced apoptosis in 10 of 16 RCC cell lines. Remarkably, five of six TRAIL-resistant RCC cell lines exhibited high levels of TRAIL expression. Topotecan, a novel topoisomerase I inhibitor, induced upregulation of TRAIL-R2 as well as downregulation of TRAIL. Neutralization of TRAIL with recombinant soluble TRAIL-R1-Fc and TRAIL-R2-Fc failed to inhibit topotecan-induced apoptosis indicating that topotecan-induced cell death can occur in a TRAIL-independent fashion. However, exposure to topotecan resulted in an enhancement of TRAIL-induced apoptosis in all primarily TRAIL-resistant RCC cell lines. This synergistic effect of cotreatment with Topotecan and TRAIL may provide the basis for a new therapeutic approach to induce apoptosis in otherwise unresponsive RCC. *Cell Death and Differentiation* (2000) **7,** 1127–1136.

Keywords: TRAIL; apoptosis; topotecan; topoisomerase I inhibitor; renal cell carcinoma

Abbreviations: RCC, renal cell carcinoma; RT, reverse transcription; TRAIL, TNF-related apoptosis-inducing ligand; HE, hematoxylin-eosin

Introduction

Metastasic renal cell carcinoma (RCC) is largely resistant to conventional chemotherapy and immunotherapy.[1,2] This resistance has been explained by various mechanisms, including the multidrug resistance phenotype due to P-glycoprotein over-expression,[3] different modes of immune evasion,[4–6] and defects of apoptotic pathways.[7] Recent evidence indicated that death receptors are directly involved in drug-induced apoptosis. CD95-mediated apoptosis has been implicated in the response of human cancer to cytotoxic drugs and defects of the CD95 signaling cascade were found to contribute to anticancer drug resistance.[8–11] In addition, one of the genes activated by p53 upon exposure to anticancer drugs is TRAIL-R2, one of the two apoptosis-inducing receptors for the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL).[12,13]

TRAIL (APO-2L) is a type II transmembrane protein that belongs to the TNF family[14,15] and selectively induces apoptosis in cancer cells but not in most non-neoplastic cells.[16–19] In contrast to other apoptosis-inducing TNF family members, such as CD95L, induction of apoptosis through TRAIL appears to be more complex, because four different TRAIL-binding surface-bound receptors have been identified so far.[20,21] TRAIL-R1 (DR4) and TRAIL-R2 (DR5/KILLER/TRICK2) are similar in structure, exhibiting a death domain in their cytoplasmatic regions. Overexpression of these receptors was shown to induce apoptotic cell death.[16,18,22,23] Besides these apoptosis-inducing TRAIL receptors, two additional non-apoptosis-inducing TRAIL receptors, TRAIL-R3 (DcR1/TRID)[16,23–26] and TRAIL-R4 (DcR2/TRUNDD),[27–29] have been identified. Both non-apoptosis-inducing receptors harbor an extracellular TRAIL-binding region but lack a functional cytoplasmatic death domain: TRAIL-R3 does not contain an intracellular signaling domain, whereas TRAIL-R4 exhibits a truncated death domain. Overexpression of these receptors after transfection did not trigger cell death but rather protected mammalian cells from TRAIL-induced apoptosis.[23,27,29] These data led to the suggestion that TRAIL-R3 and TRAIL-R4 may serve as apoptosis-inhibitory 'decoy' receptors for TRAIL. However, thus far, this hypothesis has not been verified in any cell type under non-overexpression conditions.[30] TRAIL and its receptors TRAIL-R1, -R2 and -R4 have been detected in a wide array of both non-neoplastic and neoplastic cells.[12,14–16,18,22,23,27–29] In contrast, the expression of TRAIL-R3 was found to be more restricted. However, the exact distribution of TRAIL-R3 mRNA remains controversial as it was detected either in peripheral blood mononuclear cells only[24] or in a wider range of non-neoplastic cells.[16,23] The precise physiological and pathological roles of TRAIL-mediated apoptosis are not well characterized so far and remain to be further elucidated. Recent observations, however, suggested that TRAIL constitutes an additional pathway of T-cell cytotoxicity directed against virus-infected cells and various tumor cells including human RCCs.[31]

Although TRAIL had been reported to induce apoptosis in many different cancer cells without affecting most non-neoplastic cells *in vitro*, it remained unclear whether this

selective apoptosis-inducing effect would be maintained *in vivo*. However, it was shown recently, that TRAIL exhibits potent anti-tumor activity upon systemic administration *in vivo* without any of the deleterious side effects on normal tissues known from TNF-α or CD95L.[19,32] Ashkenazi *et al*[19] also observed responsiveness to TRAIL/APO-2L-mediated apoptosis in some RCC cell lines. These observations raise the possibility that TRAIL/APO-2L, either alone or in combination with other strategies, might become a powerful cancer therapeutic for otherwise unresponsive RCCs.

Therefore, we investigated the expression and function of TRAIL and its corresponding receptors in a large panel of RCC cell lines of all major histological types. In addition, we used the topoisomerase I inhibitor topotecan (Hycamtin®), which is only a weak substrate for P-glycoprotein,[33] to evaluate the role of the TRAIL system for anticancer drug-induced apoptosis in human RCCs.

Results

Expression of TRAIL and its corresponding receptors in human RCCs

To get insight into the role of the TRAIL system in human RCCs of different histological types, we first analyzed the expression of TRAIL and its different receptors in 16 RCC cell lines using RT – PCR analysis and RNase protection assays. TRAIL expression was found in all RCC cell lines by RT – PCR (data not shown). In RNase protection assays, however, the extent of TRAIL expression markedly varied between the various RCC cell lines from weak to intensive signals (Figure 1A) and could be detected in some cell lines only after prolonged autoradiography (data not shown).

TRAIL-R1 mRNA was found in all RCC cell lines with the exception of chrompho-A, which was derived from a rare RCC variant, the chromophobe type of RCC (Figure 1A). ClearCa-5 cells exhibited TRAIL-R1 mRNA by RT – PCR analysis and by RNase protection assays after prolonged autoradiography (data not shown). TRAIL-R2 transcripts were found in all RCC cell lines and their expression levels uniformly exceeded those of TRAIL-R1 (Figure 1A). As shown in Figure 1B, two different specific amplification products (120 and 217 bp) of TRAIL-R2 were detected by RT – PCR and verified as TRICK2A and TRICK2B[34,35] by sequence analysis (data not shown). TRAIL-R3 was found in some RCC cell lines by RT – PCR only (Figure 1B), while it was not detectable by RNase protection assay. In contrast, TRAIL-R4 was detected in all RCC cell lines with the exception of chrompho-A. The expression levels, however, were rather low (Figure 1A) and signals could be detected in some RCC cell lines after prolonged autoradiography only (data not shown).

TRAIL-mediated apoptosis in human RCCs

To evaluate the functionality of the TRAIL-signaling pathway and the extent of TRAIL-mediated apoptosis, we used recombinant human soluble (rhs) TRAIL, thereby confronting our RCC cell lines with a uniform apoptotic signal. (rhs TRAIL is a soluble form of TRAIL tagged with a FLAG-epitope and its activity is enhanced by simultaneous incubation with a FLAG-antibody as described in Materials and Methods). In standard growth medium, tumor cells undergoing apoptosis were observed in all RCC cell lines, albeit with low frequencies ranging from 0.2 to 3.9%. After exposure to TRAIL (100 ng/ml) for 48 h, a markedly heterogeneous response was observed. A pronounced decrease of cell viability ($<$80% of the control) became evident in 10 out of 16 RCC cell lines (Figure 2) with reduction of cell viability to 10 ± 1% of the control in the most responsive cell line. In six cell lines we did not observe a reduction of cell number or only a modest response of doubtful biological significance (Figure 2).

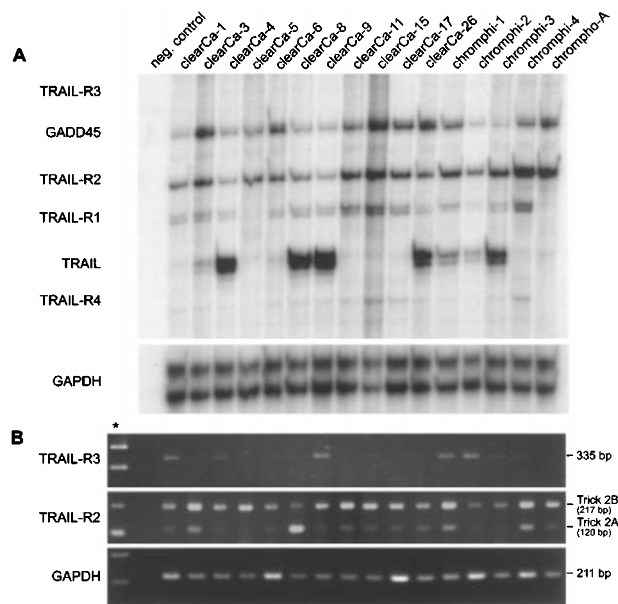

Figure 1 Expression of TRAIL and its receptors in 16 human RCC cell lines of different histological types. (**A**) 20 μg of total RNA were analyzed by RNase protection assay for expression of TRAIL, TRAIL-R1, -R2, -R3 and -R4. GAPDH served as control for equal RNA concentrations. (**B**) Two μg of total RNA was used in RT – PCR analysis for detecting TRAIL-R3 (upper panel) and TRAIL-R2 (lower panel). TRICK2A and TRICK2B are alternative splicing variants of TRAIL-R2. GAPDH detection was used as control for integrity of RNA and RT – PCR reactions. *100 bp ladder

The response to exogenous TRAIL could not be related to the expression levels of TRAIL receptors. However, five out of six cell lines that had been resistant to TRAIL-induced apoptosis (clearCa-4, -8, -9, -26 and chromphi-3) revealed the highest levels of TRAIL mRNA expression. This observation might indicate a process selecting for TRAIL resistance in RCC cells with high levels of TRAIL expression, thereby precluding fratricide of neighboring tumor cells.

Of note, five out of six RCC cell lines, which had been resistant to TRAIL-mediated apoptosis also proved to be resistant to CD95-mediated apoptosis, as indicated by the failure to induce apoptosis by the agonistic anti-CD95 antibody CH11 (Figure 2). Similar observations have previously been reported by Griffith *et al*.[36] and Thomas and Hersey [37] using melanoma cells.

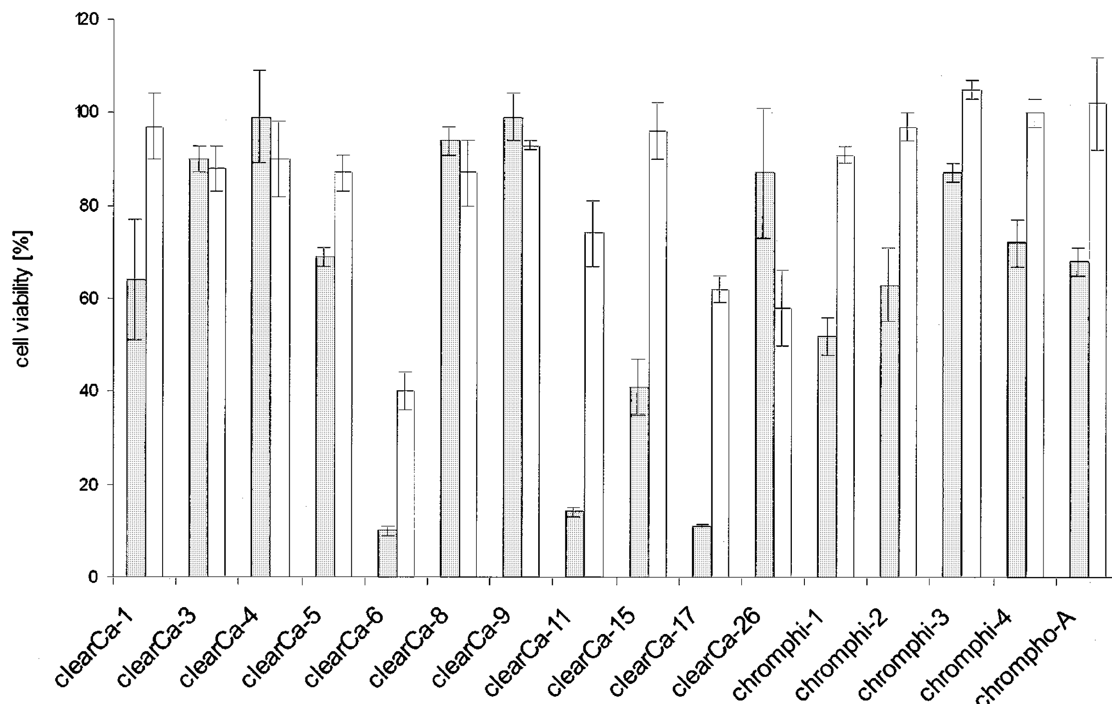

Figure 2 Sensitivity of human RCC cell lines to TRAIL- and CH11-induced cell death. Cells were cultured for 48 h in the presence of 100 ng/ml TRAIL (gray bars) or 500 ng/ml CH11 (white bars). Cell proliferation was measured by MTT assay

Topotecan-induced apoptosis and upregulation of TRAIL receptors in RCCs

To assess the role of the TRAIL system in anticancer drug-induced apoptosis of RCCs, four RCC cell lines (clearCa-6, -15, -17, chromphi-3) were exposed to topotecan (Hycamtin®), a novel topoisomerase I inhibitor with weak substrate affinity to P-glycoprotein.[33,38] Light microscopic evaluation revealed a dose-dependent increase of specific apoptotic death in three out of four cell lines (Figure 3). This induction of apoptosis was paralleled by a significant dose-dependent reduction of cell number with IC_{50} values between 0.3 μg/ml to 2.3 μg/ml (Figure 3). After exposure to topotecan, RNase protection assays demonstrated downregulation of TRAIL in two RCC cell lines (clearCa-17 and chromphi-3) which had shown high basic levels of TRAIL expression (Figure 4). In contrast, TRAIL-R1 and TRAIL-R2 mRNA expression increased in all those cell lines which had shown pronounced topotecan-induced apoptosis (i.e. clearCa-6, -17, and chromphi-3) (Figure 4). Flow cytometric analysis, however, revealed topotecan-induced upregulation of only TRAIL-R2 in clearCa-6 and chromphi-3, whereas TRAIL-R1 and TRAIL-R2 were not upregulated on the surface of the other two cell lines tested (Figure 5).

Interestingly, a weak upregulation of mRNA expression was also observed for TRAIL-R4 in those cell lines which had responded with induction of apoptosis upon topotecan treatment (Figure 4). TRAIL-R3 was not detectable by RNase protection assay even after prolonged topotecan treatment for 72 h.

Topotecan-induced upregulation of TRAIL-R2 despite p53 mutation

Because the expression of TRAIL-R2 had been observed to be upregulated by p53 after exposure to anticancer agents,[12,13] we additionally defined the mutational status of p53 in our RCC cell lines. Sequencing of p53 exons 5–8, which are the most commonly affected hot spot regions for p53 mutations in human cancer,[39,40] revealed to mutations in clearCa-15 and chromphi-3. In contrast, isolated point mutations were found in clearCa-6 and -17 (Table 1). Nevertheless, clearCa-6 and -17 responded with upregulation of TRAIL-R2 mRNA upon exposure to topotecan. These cell lines were also still able to transcriptionally activate GADD45 (Figure 4), known to be directly regulated by p53.[41] These data indicate that the p53 mutations observed in the RCC cell lines tested might not be functionally relevant for regulation of TRAIL-R2.

Topotecan-induced apoptosis is not mediated via the TRAIL pathway in human RCCs

The upregulation of TRAIL-R2 protein expression in clearCa-6 and chromphi-3 might contribute to topotecan-induced apoptosis via receptor activation by tumor cell-derived TRAIL. To test this hypothesis, we tried to block topotecan-induced apoptosis by neutralizing TRAIL ligands, using the recombinant human TRAIL-R1-Fc and TRAIL-R2-Fc chimeric receptors. (These chimeric receptors are fusion proteins of the extracellular regions of TRAIL-R1 (aa 1–239) and TRAIL-

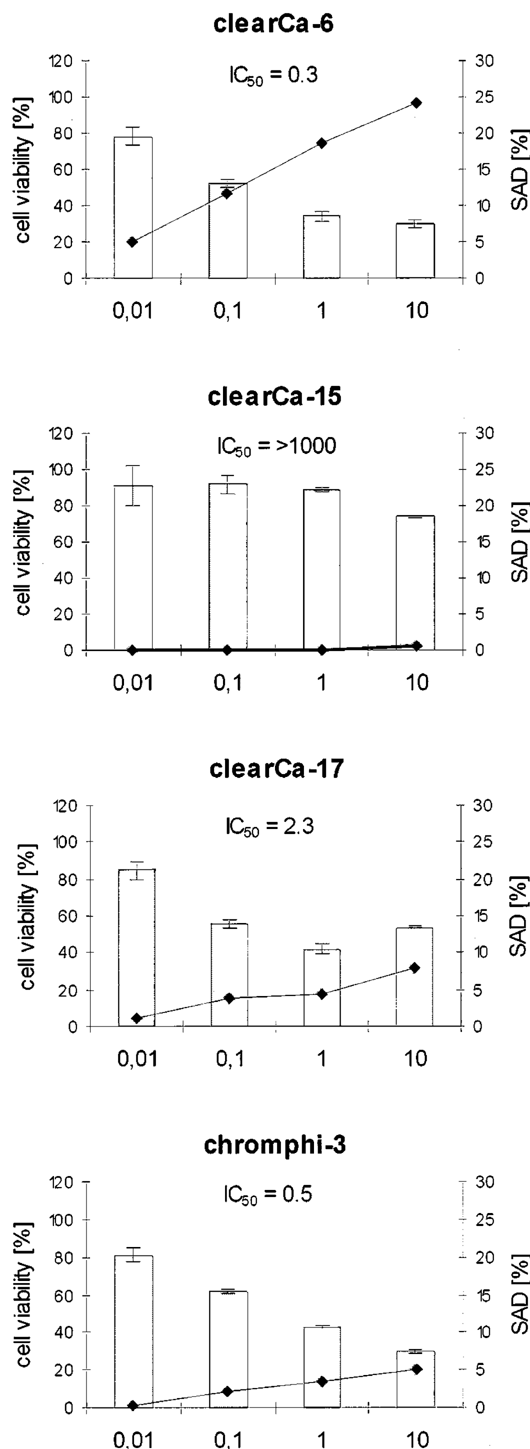

Figure 3 Topotecan-induced cell death in human RCC cell lines. Four RCC cell lines (clearCa-6, -15, -17 and chromphi-3) were cultured in the presence of the indicated concentrations of topotecan for 48 h. Cell viability was measured by MTT assay (bars) and specific apoptotic death (SAD) was determined by light microscopic counting after HE-staining

R2 (aa 1–182) with a carboxyterminal histidine-tagged FC portion of human IgG1.[18,22,42] If the apoptotic effects of topotecan were actually mediated via the TRAIL system, simultaneous incubation with these soluble chimeric receptors should block the effects of topotecan.

In these experiments, the reduction of cell number observed after exposure to topotecan as a single agent could not be inhibited by simultaneous exposure to the chimeric recombinant soluble TRAIL receptors in any cell line (Figure 6). This observation strongly argued against an involvement of TRAIL receptor activation by TRAIL binding in topotecan-induced apoptosis.

Topotecan synergistically enhances susceptibility for TRAIL-mediated apoptosis in primarily TRAIL-resistant RCCs

Topotecan-mediated upregulation of TRAIL-R2 in clearCa-6 and chromphi-3 obviously was not sufficient for an effective paracrine activation of the TRAIL system via tumor cell-derived TRAIL. Nevertheless, topotecan-induced upregulation of TRAIL-R2 might sensitize for TRAIL-mediated apoptosis. To test this hypothesis, TRAIL-resistant RCC cell lines were simultaneously exposed to both topotecan (1 μg/ml) and TRAIL (100 ng/ml) for 48 h. As shown in Figure 7, all primarily TRAIL-resistant RCC cell lines responded with a striking reduction in cell viability. Fractional inhibition analysis revealed that the effects of TRAIL and topotecan in combination were synergistic compared with either agent alone.

Discussion

TRAIL has recently been shown to induce apoptosis of tumor cells *in vivo* without deleterious side effects on normal tissues.[19,32] Because cancer therapy most potently operates through induction of apoptosis, this newly recognized apoptosis-inducing receptor-ligand system could provide powerful therapeutic options for the treatment of human RCCs.

The data presented here demonstrate TRAIL expression in human RCCs of all major histological types. Moreover, RCCs exhibited expression of TRAIL-R1 as well as TRAIL-R2, but only low mRNA expression levels of TRAIL-R4. In contrast, TRAIL-R3 could be detected by RT–PCR analysis in some RCCs yet, by RNase protection assay, it was not detectable in any of the RCCs tested. This observation is consistent with previous studies which demonstrated TRAIL-R3 mRNA expression in non-neoplastic tissues, but not in most cancer cell lines examined so far.[16,24,43]

Despite the constitutive coexpression of TRAIL and its apoptosis-inducing receptors TRAIL-R1 and -R2, spontaneous apoptosis was quite infrequent in our RCC cell lines. These observations suggested some degree of resistance to TRAIL-mediated apoptosis in RCCs. Nevertheless, the essential components of the machinery involved in the execution of TRAIL-triggered cell death were present in most RCC cell lines, as TRAIL was capable of inducing apoptosis in 10 out of 16 (63%) RCC cell lines, irrespective of their histological types. Similar observations on TRAIL sensitivity have recently been reported by Kayagaki *et al*[31] and Ashkenazi *et al*[19] for a small number of RCC cell lines,

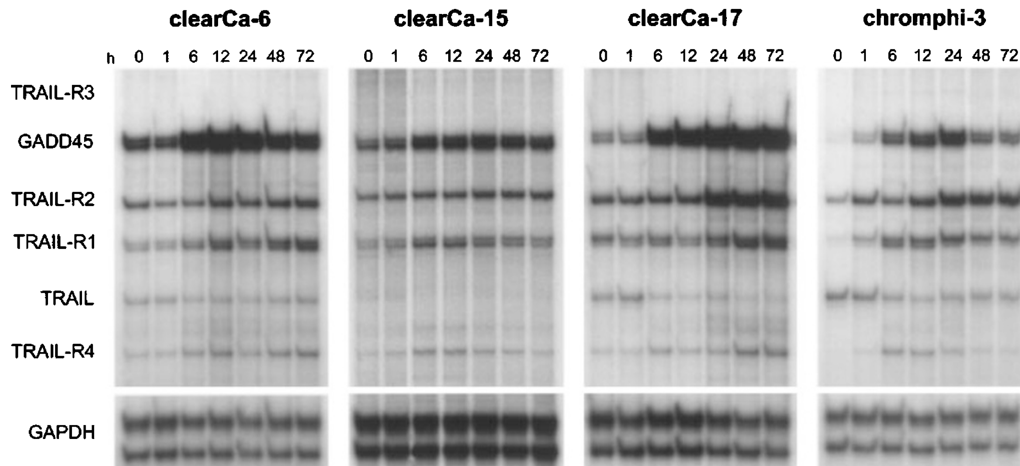

Figure 4 Expression of TRAIL and its receptors in human RCC cell lines after topotecan treatment. Cells were cultured for the indicated time period in the presence of topotecan (1 μg/ml). Total RNA was extracted and 20 μg were analyzed by RNase protection assay. GAPDH signal served as control for equal RNA concentrations

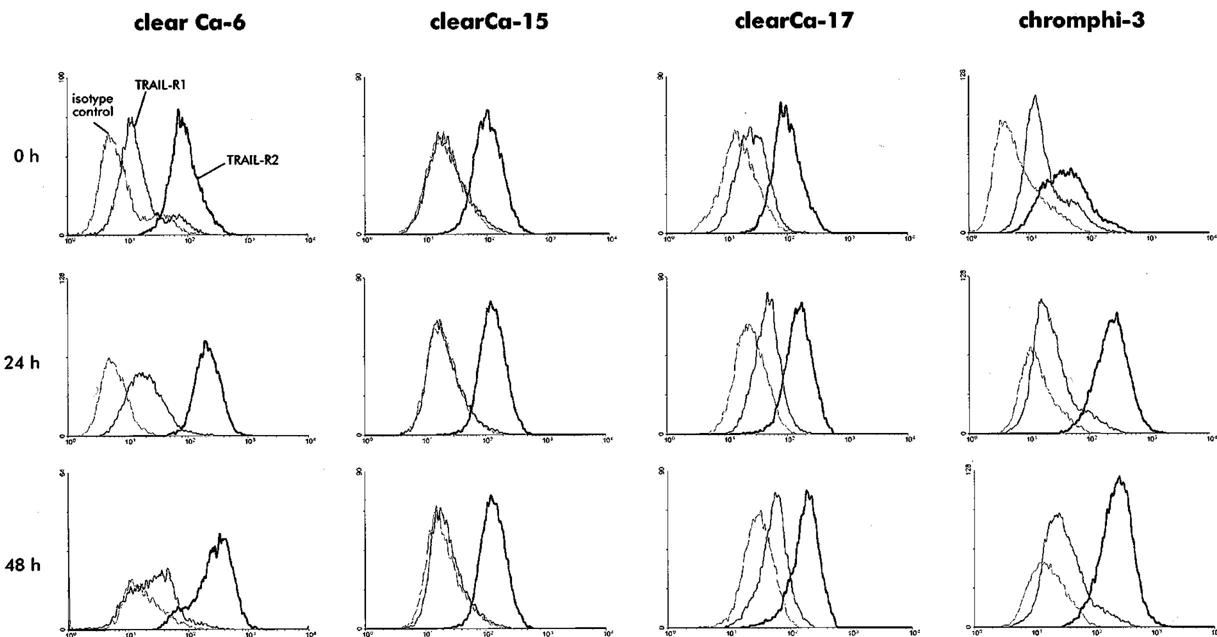

Figure 5 Surface expression of TRAIL-R1 and TRAIL-R2 before and after topotecan treatment. 1×10^5 cells were analyzed by flow cytometry with antibodies specific for TRAIL-R1 and TRAIL-R2. Analysis was performed before (0 h) and after treatment with 1 μg/ml topotecan for 24 and 48 h

Table 1 p53 mutational status of clearCa-6, -15, -17 and chromphi-3

Cell line	p53 status
clearCa-6	$Arg^{290} \rightarrow His$
clearCa-15	wt
clearCa-17	$Arg^{213} \rightarrow Leu$
chromphi-3	wt

Two of four RCC cell lines showed substitution of Arg at position 213 and 290 to Leu or His. wt=wildtype

although the proportion of non-responders was larger in our panel of RCC cell lines. The extent of response in our RCC cell lines closely corresponded to that described in hematological malignancies[44] and tended to exceed that reported for breast cancer cell lines.[45] The demonstration of TRAIL-mediated apoptosis in human RCCs was important, because it proved the existence of a potent mechanism of self-destruction in most human RCCs, provided that appropriate stimuli can be applied or sensitivity to weak stimuli can be raised.

Thus far, little is known about the mechanisms that confer responsiveness or resistance to TRAIL-mediated apoptosis in human RCCs. The propagation of TRAIL-mediated cell death, however, is regulated at different levels along the TRAIL-dependent signal transduction

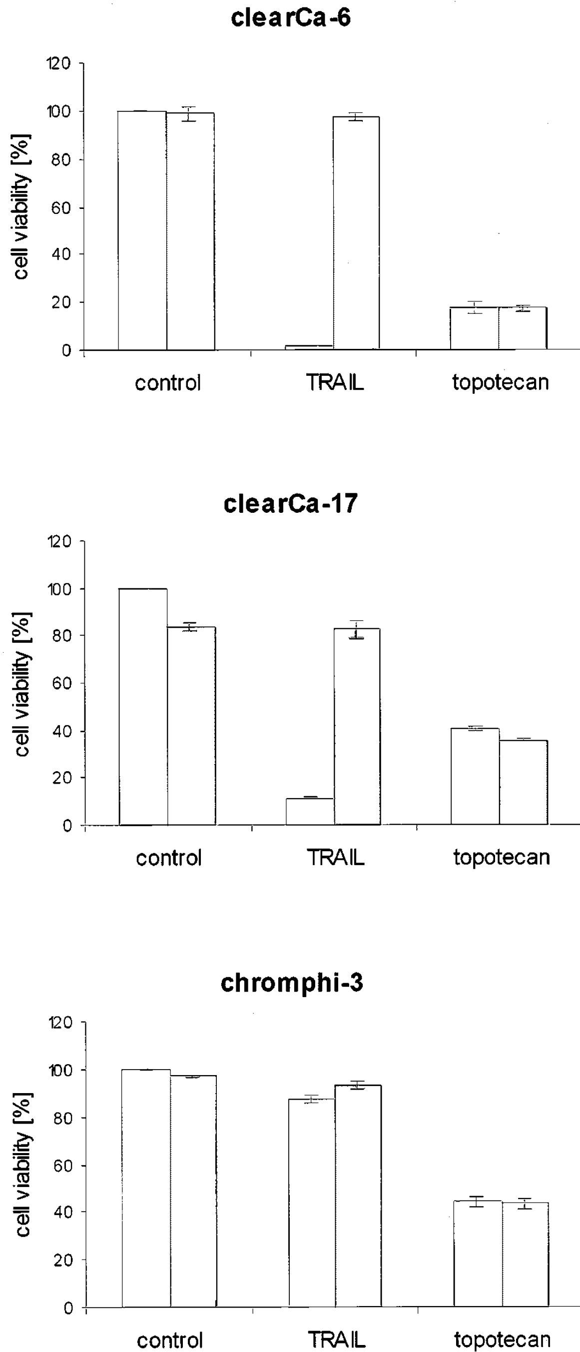

Figure 6 Topotecan-induced apoptosis in human RCCs is independent of the TRAIL system. The topotecan-sensitive cell lines clearCa-6, -17 and chromphi-3 were cultured for 48 h without (white bars) or with (gray bars) recombinant human TRAIL-R1-Fc and TRAIL-R2-Fc in standard growth medium ('control') or in presence of 100 ng/ml TRAIL ('TRAIL') or 1 μg/ml topotecan ('topotecan'). Cell viability was measured by MTT assay

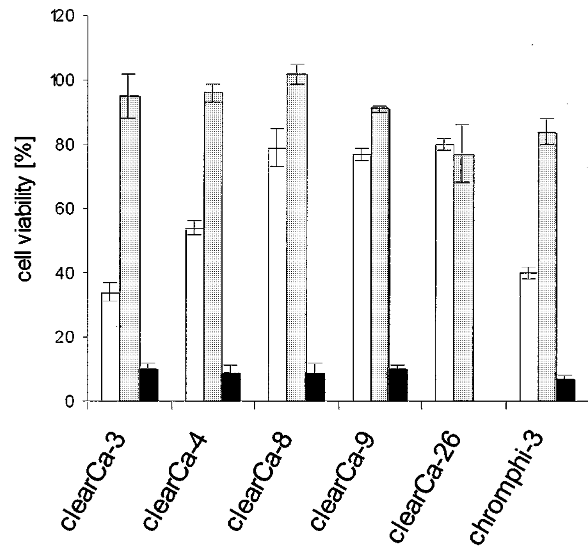

Figure 7 Synergistic enhancement of TRAIL-induced apoptosis in human RCCs by topotecan. Primarily TRAIL-resistant RCC cell lines were cultured for 48 h in the presence of 1 μg/ml topotecan (white bars), 100 ng/ml TRAIL (gray bars) alone or combined (dark gray bars). Cell viability was determined by MTT assays

cascade. The function of the TRAIL system does not only depend on the effective interaction between TRAIL and its death-inducing receptors TRAIL-R1 and TRAIL-R2, but is additionally determined by the balance of other pro- and anti-apoptotic regulator proteins. These may include – inter alia – osteoprotegerin (OPG), FLICE-inhibitory proteins (FLIPs) as well as members of the Bcl-2 and IAP (inhibitor of apoptosis protein) families.[30,46] Therefore, it was not surprising that the extent of TRAIL-induced apoptosis observed in our RCC cell lines could not be correlated to the expression levels of the apoptosis-inducing receptors TRAIL-R1 and TRAIL-R2.

Of note, five out of six RCC cell lines with remarkably high levels of TRAIL transcripts proved to be resistant to apoptosis triggered by TRAIL. This observation might indicate a process selecting for TRAIL resistance in RCC cell lines with high levels of TRAIL expression, thereby precluding fratricide between neighboring tumor cells. Although we cannot exclude that this selective process took place during *in vitro* cultivation of our RCC cell lines, RCC cells might have benefited from this selective process already under *in vivo* conditions. In close analogy to the CD95 system,[47,48] high levels of TRAIL and simultaneous resistance to TRAIL-mediated apoptosis could facilitate immune evasion of RCC cells *in vivo*. These RCC cells could ward off the attack of TRAIL-bearing immunocompetent cells.[31] Thus, the observed correlation between high levels of TRAIL expression and resistance to TRAIL-induced apoptosis may prove to be clinically relevant. Thereby, the level of TRAIL expression in human RCCs might provide a prognostic indicator for the responsiveness to TRAIL treatment.

Only recently, CD95-triggered apoptosis has been identified as a key mediator of chemotherapy in various leukemias and some solid tumor types. Anticancer drugs were shown to induce upregulation of CD95 receptor and ligand, the interaction of which resulted in fratricide between neighboring tumor cells.[8,10,11] *Vice versa*, resistance to anticancer drugs has been shown to be associated

with defects of the CD95 system.[9] Therefore, we asked whether the TRAIL system might also be directly involved in anticancer drug-induced apoptosis of human RCCs. Interestingly in this context, TRAIL-R2 had been identified by subtractive hybridization screening of a library obtained from teratocarcinoma cells after treatment with anticancer drugs.[12,13]

Our data demonstrate that therapeutically relevant concentrations of topotecan[38] induced a marked dose-dependent increase of apoptosis and a significant reduction of cell number in three out of four RCC cell lines. The response to topotecan was paralleled by an increase in the surface expression of TRAIL-R2 in two out of three topotecan-responsive RCC cell lines. This observation suggested that the TRAIL system might actually be involved in chemotherapy-induced apoptosis, as had previously been demonstrated for the CD95 system.[9,11] To further test this hypothesis, we utilized recombinant human TRAIL-R1-Fc and TRAIL-R2-Fc proteins. These agents competitively block the binding of TRAIL to its receptors, thereby preventing signal transduction via the TRAIL system. However, both TRAIL-R1-Fc and TRAIL-R2-Fc failed to inhibit topotecan-induced apoptosis. These data indicate that the cytotoxicity of topotecan can occur via TRAIL-independent mechanisms in human RCCs.

As demonstrated in our study, topotecan-induced upregulation of TRAIL-R2 did not result in effective fratricide via paracrine activation of the TRAIL system between neighboring RCC tumor cells. Nevertheless, this upregulation of TRAIL-R2 might be exploited for tumor treatment by administration of exogenous TRAIL in combination with topotecan. In fact, all primarily TRAIL-resistant RCC cell lines of our study responded with a striking reduction in cell viability when exposed to both topotecan and exogenous TRAIL. Fractional inhibition analysis revealed that the effect observed upon combination of TRAIL and topotecan was synergistic compared with either agent alone. Similar observations have recently been reported for breast carcinoma cell lines[44] and colon carcinoma transplants in SCID mice[49] after combined exposure to doxorubicin and TRAIL[44] or CPT-11 and TRAIL,[49] respectively. However, doxorubicin did not affect the expression of any TRAIL receptor in these breast carcinoma cell lines. Thus, the mechanisms underlying the synergism between chemotherapeutic drugs and TRAIL in terms of apoptosis induction in tumor cells appear to vary between different tumors and anticancer drugs.

In summary, our data demonstrate a marked heterogeneity in the responsiveness of human RCC cell lines to TRAIL-mediated cell death. Importantly, although topotecan-induced cytotoxicity can occur independent of the TRAIL system, this anticancer drug synergistically enhanced susceptibility to TRAIL-mediated apoptosis in primarily TRAIL-resistant human RCCs. The synergistic effect described here suggests that activation of the TRAIL pathway, either alone or in combination with topotecan or similar chemotherapeutic drugs, may become an attractive therapeutic strategy in different types of human RCC.

Materials and Methods

Cell lines and cell culture

All cell lines used in this study were derived from typical representatives of the clear cell, chromophilic/papillary, and chromophobe types of RCC, established in our laboratory as previously described.[50–53] The cell lines were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin as well as 100 μg/ml streptomycin and cultivated at 37°C in an atmosphere with 5% CO_2.

RNA purification from cultured cells

Total cellular RNA was isolated from RCC cell lines using either the RNeasy kit (Qiagen, Germany) for RT–PCR analysis, or the cesium chloride ultracentrifugation method[54] for RNase protection assays. RNA concentration was measured by photometry at 260 nm. The quality of total cellular RNA was verified by the integrity of 18 S/28 S ribosomal RNA in ethidium bromide-stained agarose gels.

RNase protection assays

Cells (3×10^6 to 10×10^6) were treated with 1 μg/ml topotecan for 1, 6, 12, 24, 48 or 72 h and RNA was subsequently isolated. Twenty μg of total RNA were analyzed for the presence of TRAIL, TRAIL-R1, -R2, -R3 and -R4 as well as GADD45 transcripts by RNase protection assays. These were performed with the RiboQuant Multi-Probe RNase protection assay system (Pharmingen, USA) according to the manufacturer's recommendations using the RiboQuant custom template set #559129. GAPDH was included as internal control. The protected probes were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography.

RT–PCR analysis

For monitoring TRAIL-R2 and TRAIL-R3 on mRNA level, reverse transcription was performed using 2 μg of total cellular RNA, 25 nmol dNTPs (Stratagene, USA), 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega, Germany), 100 pmol random primer (in the case of TRAIL-R2; Stratagene, USA) or 10 pmol of the gene-specific 3′-primer (in the case of TRAIL-R3) as well as 5 U AMV reverse transcriptase (Promega, USA) with the corresponding RT-buffer in a final volume of 30 μl. The reactions were incubated at 55°C for 1 h.

The PCR mixture for TRAIL-R2 and GAPDH was composed as follows: 1.5 μl of the cDNA template, 25 pmol of each gene-specific primer, 12.5 nmol dNTPs (Stratagene, USA) and 1.5 U Taq polymerase (Sigma, Germany) with the corresponding buffer in a final volume of 50 μl. The mixture for amplification of TRAIL-R3 was similar but 2.5 U AmpliTaq Gold and the recommended buffer supplemented with 1.2 mM $MgCl_2$ (Perkin Elmer, USA) were applied. The following primer sequences were used: TRAIL-R2-forward: 5′>GGGAAGAAGATTCTCCTGAGATGTG<3′; TRAIL-R2-reverse: 5′>ACATTGTCCTCAGCCCCAGGTCG<3′[85]; TRAIL-R3-forward: 5′>CTGCCAGTCC TAGCTTACTCTG<3′; TRAIL-R3-reverse: 5′>CTGCTACACTTCC GGCACATCT<3′; GAPDH-forward: 5′>ACGGATTTGGTCGTATTGGGCG<3′; GAPD H-reverse: 5′>CTCCTGGAAGATGGTGATGG<3′.

The initial denaturation step at 94°C for 2 min was followed by 35 cycles (or 27 cycles for GAPDH) of denaturation for 30 s (GAPDH) or 1 min (TRAIL-R2, -R3), annealing for 1 min at 64°C (GAPDH) or 55°C (TRAIL-R2, -R3), extension at 72°C for 1 min and a final extension

step at 72°C for 5 min. PCR products were separated on 3% agarose gels and the identity of the amplification products was confirmed by direct sequencing.

Flow cytometric analysis

The expression of TRAIL-R1 and TRAIL-R2 was assessed by fluorescence-activated cytometry in a FACScan flow cytometer (Becton-Dickinson, Germany), using the CellQest software system. After culturing RCC cells for 24 and 48 h in the absence or presence of 1 μg/ml topotecan, the cells were harvested by incubation for 5 min with 0.05% (w/v) EDTA in PBS. A total of 1×10^5 cells was transferred into polystyrene tubes, placed on ice, and washed twice with washing buffer (3% FCS in PBS). Afterwards, the cells were incubated for 30 min at 4°C with 1 μg/ml of the primary antibodies TRAIL-R1 (clone M272), TRAIL-R2 (clone M413) or mouse-IgG_1, 2.5 μg/ml of the secondary biotinylated-goat anti-mouse antibody and 2.5 μg/ml PE-labeled streptavidine. Between each incubation step the cells were washed twice with washing buffer and after the last washing procedure, the cells were measured. TRAIL-R1- and TRAIL-R2-specific monoclonal antibodies were kindly provided by Immunex Corp. (Seattle, WA, USA). Isotype-matched mouse-IgG1, biotinylated goat anti-mouse IgG as well as PE-labeled streptavidine were obtained from PharMingen (Germany).

Sequence analysis of RT–PCR products

Bands of interest were eluted from agarose gels using the QIA quick gel extraction kit (Qiagen, Germany), ligated into the pGEM-T-cloning vector (Promega, Germany) and cloned in accordance with standard protocols. Plasmid DNA was recovered employing the Plasmid Mini Kit (Qiagen, Germany) and analyzed with an ABI Prism 310 sequencing apparatus (Applied Biosystems, USA) using T7 or SP6 site specific primers.

Quantification of apoptosis

1×10^4 cells were seeded in 8-chamber slides (Nunc, Germany). After 24 h, the cells were treated with 1 μg/ml topotecan for 48 h. The frequency of apoptotic cells per 10^3 cells was determined by light microscopical counting of hematoxylin-eosin (HE)-stained cells that showed the typical morphological hallmarks of apoptosis, i.e. chromatin condensation and/or cell fragmentation into apoptotic bodies. Counting of apoptotic cells was performed in two independent experiments and the data presented are the mean out of these experiments. Specific apoptotic death (SAD) was calculated as the frequency of apoptotic cells after exposure to topotecan minus the frequency of apoptotic cells in standard growth medium.

Assessment of cell number after induction or inhibition of apoptosis

Tumor cells in the exponential growth phase were transferred to microwell plates (Gibco, Germany) at 1×10^4 cells per well in 0.1 ml standard growth medium. After 24 h the tumor cells were exposed to topotecan (0.01–10 μg/ml; kindly provided by Smith Kline Beachem, Germany) or to the CD95 receptor-activating antibody CH11 (500 ng/ml; Immunotech, Germany) or to recombinant human soluble (rhs) TRAIL (100 ng/ml) in combination with the recommended enhancer (1 μg/ml; Alexis, Germany) for another 48 h. rhs TRAIL is a soluble form of TRAIL tagged with the FLAG epitope and used in conjunction with anti-FLAG antibodies as enhancer. Inhibitory recombinant human TRAIL-R1-Fc and TRAIL-R2-Fc proteins (R&D Systems, Germany) were used at 500 ng/ml in order to block induction of apoptosis by anticancer drugs. These chimeric proteins are fusion proteins of the extracellular regions of TRAIL-R1 and TRAIL-R2 with the Fc portion of human IgG_1.[18,22,42] The number of viable cells was estimated using the colorimetric MTT assay described by Mosman[55] and measured on a spectrophotometric plate reader (Titertek Multiskan, Germany) at 570 nm. The percentage of viable cells in each well was calculated from the following:

$$\text{percent viability} = \frac{\text{absorbance of test} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}}$$

The data presented are the mean $\pm$ SD from eight replicate wells per microtiter plate and three plates per cell line. Similar experiments were obtained in two independent experiments.

Analysis of p53 mutations

Extraction of genomic DNA was performed using the QIAmp Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. For amplification of p53 exons 5 to 8 following oligonucleotide primers were used: exons 5 and 6: forward: 5′-TTCCTCTTCCTGCAGTACTC-3′, reverse: 5′-ATGTGCAAACC AGACCTCAG-3′, exons 7 and 8: forward: 5′-GTGTTGTCTCCTAGGTTGGC-3′, reverse: 5′-AAGT-GAATCTGAGGC ATAAC-3′. Each amplification reaction was carried out in a total volume of 50 μl containing 200 ng of genomic DNA 10 pmol of reverse/forward primer, 10 nmol dNTPs, 2 U Taq polymerase and PCR reaction buffer (Sigma, Germany). After an initial denaturation step at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, and extension at 72°C for 1 min as well as a last delay at 72°C for 10 min. The PCR products were purified from surplus oligonucleotides using Microspin S-300 columns (Pharmacia, Germany). Sequence analysis was carried out using the forward/reverse primers as described.

Statistical analysis

Interactions between TRAIL and topotecan were analyzed by the fractional inhibition method as follows: when expressed as the fractional inhibition cell viability, additive inhibition produced by both inhibitors (i) occurs when $i_{1,2}=i_1+i_2$; synergism when $i_{1,2}>i_1+i_2$; and antagonism when $i_{1,2}<i_1+i_2$.[56] The concentrations of topotecan that induced a 50% reduction in cell viability (IC_{50}) were statistically determined by SSPS (probit analysis).

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Anja Florange-Heinrichs, Martina Bellak, Heiko Stahl, and Michael Ringler. The results are part of the PhD thesis of Marion Déjosez and the medical thesis of Andreas Krieg. This work was supported by a grant from the Mildred Scheel-Stiftung.

References

1. Krigel RL, Padavic-Shaller KA, Rudolph AR, Konrad M, Bradley EC and Comis RL (1990) Renal cell carcinomas: treatment with recombinant interleukin-2 plus beta-interferon. J. Clin. Oncol. 8: 460–467

2. Palmer PA, Vinke I, Evers P, Pourreau C, Oskam R, Roest G, Vlems F, Becker L, Loriaux E and Franks CR (1992) Continuous infusion of recombinant interleukin-2 with or without autologous activated killer cells for the treatment of advanced renal cell carcinoma. Eur. J. Cancer. 28A: 1038 – 1044
3. Mickisch G, Röhrich K, Kössig J, Forster S, Tschada R and Alken P (1990) Mechanisms and modulation of multidrug-resistance in primary human renal cell carcinoma. J. Urol. 144: 755 – 759
4. Cordon-Cardo C, Fuks Z, Drobnjak M, Moreno C, Eisenbach L and Feldmann M (1991) Expression of HLA-A, B, C antigens on primary and metastasic tumor cell populations of human carcinomas. Cancer Res. 51: 6372 – 6380
5. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mulé JJ, Rosenberg SA and Bennink JR (1993) Identification of human cancers deficient in antigen processing. J. Exp. Med. 177: 265 – 272
6. Ramp U, Jaquet K, Reinecke P, Nitsch T, Gabbert HE and Gerharz CD (1997) Acquisition of TGF-β1 resistance: An important progression factor in human renal cell carcinoma. Lab. Invest. 76: 739 – 749
7. Hetts SW (1998) To die or not to die. An overview of apoptosis and its role in disease. JAMA. 279: 300 – 307
8. Friesen C, Herr I, Krammer PH and Debatin KM (1996) Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nature Med. 2: 574 – 577
9. Friesen C, Fulda S and Debatin KM (1997) Deficient activation of the CD95 (APO-1/ Fas) system in drug-resistant cells. Leukemia 11: 1833 – 1841
10. Fulda S, Sieverts H, Friesen C, Herr I and Debatin KM (1997) The CD95 (APO-1/ Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res. 57: 3823 – 3829
11. Müller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH and Galle PR (1997) Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) system and involves activation of wild-type p53. J. Clin. Invest. 99: 403 – 413
12. Wu GS, Bums TF, McDonald 3rd ER, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R, Hamilton SR, Spinner NB, Markowitz S, Wu G and El-Deiry WS (1997) KILLER/DR5 is a DNA damage inducible p53-regulated death receptor gene. Nat. Genet. 17: 141 – 143
13. Sheikh MS, Bums TF, Huang Y, Wu GS, Amundson S, Brooks KS, Fomace Jr AJ and El-Deiry WS (1998) p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. Cancer Res. 58: 1593 – 1598
14. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C and Smith CA *et al* (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673 – 682
15. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashekenazi A (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 271: 12687 – 12690
16. Sheridan JP, Marsters SA, Pitti RM, Gumey A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P and Ashkenazi A (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277: 818 – 821
17. Gura T (1997) How TRAIL kills cancer cells, but not normal cells. Science. 277: 68
18. Walczak H, Degli-Esposti A, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, Goodwin RG and Rauch CT (1997) TRAIL-R2: a novel apoptosis mediating receptor for TRAIL. EMBO J. 16: 5386 – 5397
19. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z and Schwall RH (1999) Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. 104: 155 – 162
20. Griffith TS and Lynch DH (1998) TRAIL: a molecule with multiple receptors and control mechanisms. Curr. Opin. Immunol. 10: 559 – 563
21. Degli-Esposti MA (1999) To die or not to die - the quest of the TRAIL receptors. J. Leuk. Biol. 65: 535 – 542
22. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J and Dixit VM (1997) The receptor for the cytotoxic ligand TRAIL. Science 276: 111 – 113
23. Pan G, Ni JN, Wie YF, Yu GL, Gentz R and Dixit VM (1997) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277: 815 – 818
24. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Haung CP, DuBose RF, Goodwin RG and Smith CA (1997) Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J. Exp. Med. 186: 1165 – 1170
25. MacFarlane M, Ahmad M, Srinivasula SM, Femandes-Alnemri T, Cohen GM and Alnemri ES (1997) Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J. Biol. Chem. 272: 25417 – 25420
26. Mongkolsapaya J, Cowper AE, Xu XN, Morris G, McMichael AJ, Bell JI and Screaton GR (1998) Lymphocyte inhibitor of TRAIL (TNF-related apoptosis-inducing ligand): a new receptor protecting lymphocytes from the death ligand TRAIL. J. Immunol. 160: 3 – 6
27. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA and Goodwin RG (1997) The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 7: 813 – 820
28. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P and Ashkenazi A (1997) A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr. Biol. 7: 1003 – 1006
29. Pan G, Ni JN, Yu GL, Wie YF and Dixit VM (1998) TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signaling. FEBS Lett. 424: 41 – 45
30. Walczak H and Krammer PH (2000) The CD95 (APO-1/Fas) and the TRAIL apoptosis systems. Exp. Cell Res. 256: 58 – 66
31. Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K and Yagita H (1999) Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing Ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of Type 1 IFNs. J. Exp. Med. 189: 1451 – 1460
32. Walczak H, Miller RE, Ariail K, Gliniak B and Griffith TS (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. 5: 157 – 163
33. Hendricks LB, Rowinsky EK, Grochow LB, Donehower RC and Kaufmann SC (1992) Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F104864), a new camptothecin analogue. Cancer Res. 52: 2268 – 2278
34. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ and Bell JI (1997) TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr. Biol. 7: 693 – 696
35. Arai T, Akiyama Y, Okabe S, Saito K, Iwai T and Yuasa Y (1998) Genomic organization and mutation analyses of the DR5/TRAIL receptor 2 gene in colorectal carcinomas. Cancer Lett. 133: 197 – 204
36. Griffith TS, Chin WA, Jackson GC, Lynch DH and Kubin MZ (1998) Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J. Immunol. 161: 2833 – 2840
37. Thomas WD and Hersey P (1998) TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in FAS ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. J. Immunol. 161: 2195 – 2200
38. Kollmannsberger C, Mross K, Jakob A, Kanz L and Bokemeyer C (1999) Topotecan - A novel topoisomerase I-Inhibitor: Pharmacology and clinical experience. Oncology 56: 1 – 12
39. Harris CC and Hollstein M (1993) Clinical implications of the p53 tumor-suppressor gene. New Engl. J. Med. 329: 1318 – 1327
40. Reiter RE, Anglard P, Liu S, Gnarra JR and Linnehan WM (1993) Chromosome 17p deletions and p53 mutations in renal cell carcinoma. Cancer Res. 53: 3092 – 3097
41. Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fomace Jr AJ (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71: 587 – 597
42. Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J and Hood L (1997) Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. Immunity 7: 821 – 830
43. Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N and Tschopp J (1997) Characterization of two receptors for TRAIL. FEBS Lett. 416: 329 – 334
44. Snell V, Clodi K, Zhao S, Goodwin R, Thomas EK, Morris SW, Kadin ME, Cabanillas F, Anreeff M and Younes A (1997) Activity of TNF-related apoptosis-inducing ligand (TRAIL) in haematological malignancies. Br. J. Haematol. 99: 618 – 624

45. Keane MM, Ettenberg SS, Nau MM, Russel EK and Lipkowitz S (1999) Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res. 59: 734 – 741
46. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S and Peter ME (1998) Apoptosis signaling by death receptors. Eur. J. Biochem. 254: 439 – 459
47. Strand S, Hofmann WJ, Hug H, Müller M, Otto G, Strand D, Mariani SM, Stremmel W, Krammer PH and Galle PR (1996) Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand expressing tumor cells - A mechanism of immune evasion? Nature Med. 2: 1361 – 1366
48. Krammer PH (1997) The tumor strikes back. New data on expression of the CD95 (APO-1/Fas) receptor/ligand system may cause paradigm changes in our view on drug treatment and tumor immunology. Cell Death Differ. 4: 362 – 364
49. Gliniak B and Le T (1999) Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11. Cancer Res. 59: 6153 – 6158
50. Gerharz CD, Moll R, Störkel S, Ramp U, Thoenes W and Gabbert HE (1993) Ultrastructural apperance and cytoskeletal architecture of the clear, chromophilic, and chromophobe types of human renal carcinoma in vitro. Am. J. Path. 142: 851 – 858
51. Gerharz CD, Moll R, Störkel S, Ramp U, Hildebrandt B, Molsberger G, Koldovsky P and Gabbert HE (1995) Establishment and characterization of two divergent cell lines derived from a human chromophobe renal cell carcinoma. Am. J. Path. 146: 953 – 961
52. Gerharz CD, Hildebrandt B, Moll R, Ramp U, Sarbia M, Störkel S, Koldovsky P and Gabbert HE (1996) Chromophilic renal cell carcinoma: cytomorphological and cytogenetic characterisation of four permanent cell lines. Br. J. Cancer. 74: 1605 – 1614
53. Gerharz CD, Ramp U, Déjosez M, Mahotka C, Czamotta B, Bretschneider S, Lorenz I, Müller M, Krammer PH and Gabbert HE (1999) Resistance to CD95 (APO-1/Fas)-mediated apoptosis in human renal cell carcinomas: An important factor for evasion from negative growth control. Lab. Invest. 79: 1521 – 1534
54. Mahotka C, Wenzel M, Springer E, Gabbert HE and Gerharz CD (1999) Survivin-ΔEx3 and survivin-2B: Two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer Res. 59: 6097 – 6102
55. Mosman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxity assays. J. Immunol. Meth. 65: 55 – 64
56. Webb JL (1963) Effects of more than one inhibitor. In *Enzymes and Metabolic Inhibitors. (New York: Academic Press)* Vol 1: pp. 487 – 512