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Inhibition of IGF-1R-dependent PI3K activation sensitizes colon cancer cells specifically to DR5-mediated apoptosis but not to rhTRAIL

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Abstract. *Background*: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) initiates apoptosis in tumor cells upon binding to its cognate agonistic receptors, death receptors 4 and 5 (DR4 and DR5). The activity of the insulin-like growth factor 1 (IGF-1) survival pathway is often increased in cancer, influencing both cell proliferation and apoptosis. We hypothesized that inhibiting the IGF-1 receptor (IGF-1R) using NVP-AEW541, a small molecular weight tyrosine kinase inhibitor of the IGF-1R, could increase death receptor (DR)-mediated apoptosis in colon cancer cells.

Methods: The analyses were performed by caspase assay, flow cytometry, Western blotting, immunoprecipitation and fluorescent microscopy.

Results: Preincubation with NVP-AEW541 surprisingly decreased apoptosis induced by recombinant human TRAIL (rhTRAIL) or an agonistic DR4 antibody while sensitivity to an agonistic DR5 antibody was increased. NVP-AEW541 could inhibit IGF-1-induced activation of the phosphatidylinositol 3-kinase (PI3K) pathway. The effects of the PI3K inhibitor LY294002 on TRAIL-induced apoptosis were similar to those of NVP-AEW541, further supporting a role for IGF-1R-mediated activation of PI3K. We show that PI3K inhibition enhances DR5-mediated caspase 8 processing but also lowers DR4 membrane expression and DR4-mediated caspase 8 processing. Inhibition of PI3K reduced rhTRAIL sensitivity independently of the cell line preference for either DR4- or DR5-mediated apoptosis signaling.

Conclusions: Our study indicates that individual effects on DR4 and DR5 apoptosis signaling should be taken into consideration when combining DR-ligands with PI3K inhibition.

Keywords: DR4, DR5, TRAIL, PI3K, colon cancer

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in western countries. Despite improvements in the area of prevention and treatment, mortality remains high because of the frequent presence of distant metastases at the time of diagnosis. Together with novel chemotherapies and liver surgery, it is estimated that targeted agents such as cetuximab and bevacizumab have improved the 5-year overall survival for patients with metastatic CRC to more than 30% [7, 25]. This supports the development of novel targeted treatment options, including agents that activate the TRAIL apoptotic pathway [12,17,39].

The TRAIL apoptotic pathway is initiated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein of the tumor necrosis factor superfamily. TRAIL binds to cell surface homo- and/or heterotrimers of death receptors 4 and 5 (DR4 and DR5), thus triggering the "caspase cascade" [23]. The extracellular domain of TRAIL can be

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processed proteolytically, leading to the release of a soluble trimeric form of TRAIL with similar proapoptotic properties [36,50]. The potential of the TRAIL apoptotic pathway as a target to treat cancer was first established when soluble recombinant human TRAIL (rhTRAIL) was found to selectively induce apoptosis in tumor cells without serious toxicity to normal tissues [1,45]. Several other agents that target the two death receptors have been engineered since the discovery of the TRAIL apoptotic pathway. So far, this pathway can be activated by rhTRAIL, by mutant forms of TRAIL which bind preferentially to either DR4 or DR5 homotrimers [22,28,42] and by agonistic monoclonal antibodies also designed to bind specifically DR4 or DR5 [4,13,20,37]. The potential of the TRAIL receptors as therapeutic targets is underscored by the fact that their expression and sensitivity have both been shown to increase during CRC tumor progression [15, 24]. Although the sensitivity of tumor cells to TRAIL-induced apoptosis is heterogeneous as a result of intrinsic or acquired resistance, combination therapies to overcome this phenomenon are already under investigation (for review see [21,35]).

Numerous growth factor receptor pathways are altered in CRC cancer, influencing gene expression, cell proliferation and apoptosis [5]. Some of these pathways constitute interesting targets for drugs that could be combined with rhTRAIL [6,30,34]. The insulin-like growth factor (IGF) signaling pathway is involved in the growth and development of many tissues, and plays a crucial role in the normal functioning of the organism [26]. IGF signaling cascades begin at the cell surface with IGF ligands (IGF-1 and -2) binding to several transmembrane receptors, namely IGF-1R, IGF-2R and the insulin receptor (IR). Downstream from these receptors, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are stimulated [26]. Diverse cellular responses such as gene expression, cell proliferation and apoptosis suppression are induced, thus promoting cell survival. Importantly, the IGF system is frequently deregulated in CRC cancers [2,26], more than 90% of which express IGF-1R while normal colonic mucosa does not show substantial expression levels [48].

The downstream PI3K/Akt receives signals not only from the IGF-1R but also from human epidermal growth factor receptor (HER) family receptors such as HER-2 and epidermal growth factor receptor (EGF-R) [19,53], which can also promote carcinogenesis when deregulated. Furthermore, alterations in the PI3K/Akt signaling itself have been found in many forms of cancer and can originate at multiple levels of the cascade [27,49]. For these reasons, the PI3K/Akt pathway holds promise as a target for cancer treatment. Clinical trials are being carried out with blocking antibodies that target IGF-1R, and also with kinase inhibitors targeting IGF-1R or PI3K [14,51]. In this context, we investigated whether inhibiting the IGF-1R survival pathway, and more specifically its downstream PI3K/Akt pathway, could functionally increase rhTRAIL-induced apoptosis.

We tested the effects of the IGF-1R inhibitor NVP-AEW541 [31] on TRAIL sensitivity in SW948, a colon cancer cell line and in SW948-TR, the TRAIL-resistant sub-line. Furthermore, we investigated whether sensitivity to agonistic DR4- or DR5-specific monoclonal antibodies was differentially affected by NVP-AEW541. We analyzed the effects of LY294002, an inhibitor of the downstream PI3K/Akt pathway [46], on the sensitivity to these various activators of TRAIL-mediated cell death. Finally, we validated these findings in another colon carcinoma cell line, Colo205.

2. Materials and methods

2.1. Reagents

rhTRAIL was produced non-commercially following a protocol described previously [1]. The agonistic anti-DR4 (HGS-ETR1) and anti-DR5 (HGS-TR2J) antibodies were a donation from Human Genome Science (HGS, Rockville, MD, USA). The TRAIL-receptor antibodies used for flow cytometry experiments were obtained from Immunex corporation (Seattle, WA, USA). NVP-AEW541 was kindly provided by Novartis Pharma AG, Basel, Switzerland. LY294002 was purchased from Cell Signaling Technology, Leiden, The Netherlands. The ZB ActiveFluor Caspase 3/7 kits were obtained from Zebra Bioscience, Enschede, The Netherlands. 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT)-solution was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands).

2.2. Cell lines

The TRAIL-sensitive colon carcinoma cell line SW948 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described previously [43]. The TRAIL-resistant sub-cell line SW948-TR was made as described previously [44].

Colo205 colon carcinoma and MCF7 breast carcinoma cell lines were purchased from the ATCC and grown in RPMI containing 10% FCS at 37°C in a humidified atmosphere with 5% CO_2.

2.3. Cytotoxicity assay

A microculture tetrazolium (MTT) assay was used to determine cytotoxicity. SW948 and SW948-TR cells were incubated in a total volume of 200 µl. Treatment consisted of continuous incubation with various concentrations of NVP-AEW541 for 96 h. Assays were performed as described before [43].

2.4. Flow cytometry

Cells were stained for TRAIL-receptor membrane expression as described previously [43] and analyzed with a Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA). Membrane expression was measured as the increase in mean fluorescence intensity of the whole analyzed cell population.

To determine surface expression of IGF-1R, a similar protocol was used. Cells were stained for 45 min on ice with phycoerythrin (PE)-conjugated anti-human IGF-1Rα or PE-conjugated mouse IgG1 as an isotype control (BD Pharmingen, Alphen aan den Rijn, The Netherlands). After two washes a minimum of 5,000 cells was analyzed by flow cytometry.

2.5. Caspase 3/7 activity assay

Caspase 3/7 activity was assayed using the caspase-specific fluorescence peptide substrate DEVD-MCA according to the manufacturer's instructions (Zebra Bioscience BV, Groningen, The Netherlands). A Bradford assay was used to normalize protein concentrations between samples. Fluorescence from free 7-amino-4-trifluoromethyl coumarin was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, The Netherlands) using 340 nm excitation and 460 nm emission filters.

2.6. SDS-polyacrylamide gel electrophoresis and Western blotting

Sample preparation and Western blot analysis were performed as described previously [43]. Proteins were detected with the following antibodies: mouse-anti-FADD from Transduction Laboratories (Lexington, KY, USA), mouse anti-caspase 8 from Cell Signaling Technology (Leusden, The Netherlands) and mouse anti-actin from ICN Biomedicals (Zoetermeer, The Netherlands). Mouse-anti-FLIP NF6 was kindly provided by Dr. M. Peter (University of Chicago, IL, USA). The secondary antibodies were labeled with horseradish peroxidase (HRP) (DAKO, Glostrup, Denmark), and chemiluminescence was detected using the BM-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Mannheim, Germany). A slightly modified lysis protocol was used to investigate Akt, $MAPK^{44/42}$, phospho-$MAPK^{44/42}$ and phospho-Akt (ser473) protein levels. Briefly, cells were seeded in 6-well plate and serum-starved for 24 h before 1 h treatment with NVP-AEW541 (500 nM). Cells were then stimulated for 15 min with recombinant human IGF-1 (R&D systems Europe Ltd, Abingdon, UK). Next, cells were washed once with cold PBS, lysed in 100 µl cold SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT) and boiled in a water bath for 5 min. Protein concentrations were determined using the DC protein assay (Biorad Laboratories BV, Veenendaal, The Netherlands). Western blot analysis was performed as described previously. A similar protocol was used to investigate the effects of LY294002 on Akt phosphorylation. Briefly, cells were cultured in medium with 10% FCS for 24 h before 1 h-treatment with 10 or 20 µM LY294002 and subsequent harvesting, using the methods described above. To detect Akt and phospho-Akt, the following antibodies were used: rabbit anti-Akt and rabbit anti-phospho-Akt (ser473) from Cell Signalling Technology, Leiden, The Netherlands. $MAPK^{44/42}$ and phospho-$MAPK^{44/42}$ (Thr202/Tyr204) were detected using rabbit anti-$MAPK^{44/42}$ from Santa Cruz and mouse anti-phospho-$MAPK^{44/42}$ from Cell Signalling Technology, Leiden, The Netherlands.

2.7. DR5-DISC isolation

Briefly, 50×10^6 cells per condition were grown in medium harvested and resuspended in fresh medium. Cells were stimulated with 5 µg/ml HGS-TR2J in a final volume of 2 ml. The antibodies were added after cell lysis for the control treatment. Cell suspensions were incubated for 15 min at 37°C and the reaction was stopped by the addition of 10 ml ice-cold phosphate buffered saline (PBS). The cells were immediately washed with ice-cold PBS and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) with complete protease inhibitors (Roche Diagnostics, Almere, The Nether-

lands) for 30 min on ice. After centrifugation (12,000× g) at 4°C for 10 min, the lysates were pre-cleared with 20 µl Sepharose-6B (Pharmacia, Uppsala, Sweden) for 2 h at 4°C and immunoprecipitated with 50 µl protein-G agarose beads (Roche Diagnostics, Mannheim, Germany) for 3 h at 4°C. Beads were washed twice with 1 ml lysis buffer and once with PBS before re-suspension in standard Western blot sample buffer and boiling for 5 min. Immunoprecipitated proteins were separated with SDS-PAGE. Western blot analysis for FADD and c-FLIP was performed as described above. DR5 was detected using rabbit anti-DR5 from ProSci Inc. (Poway, CA, USA). Caspase 8 was detected with rabbit anti-caspase 8 (Abcam plc, Cambridge, UK). Anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were used for signal detection (DAKO, Glostrup, Denmark).

2.8. Propidium iodide staining

Staining was performed according to Nicoletti's protocol [32]. Sub-G_1 DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Brussels, Belgium).

2.9. Fluorescent microscopy

SW948 cells were seeded in 6-well plate on poly-L-lysine coated glass cover slides (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Cells pretreated or not with LY294002 for 16 h were incubated for 45 min on ice with anti-DR5 antibody (eBiosciences, via ITK diagnostics BV, Uithoorn, The Netherlands), diluted 1:100 in ice-cold PBS. After two washing steps with ice-cold PBS, the cells were incubated with the secondary antibody Alexa Fluor 488-goat anti-mouse IgG1 (Invitrogen, Molecular probes, Breda, The Netherlands), diluted 1:200 in ice-cold PBS for 1 h on ice. Next, cells were either left on ice or stimulated with 200 nM HGS-TR2J diluted in medium for 30 min at 37°C. Cells were then fixated with ice-cold methanol-acetone (1:1) for 10 min at −20°C. After three washing steps with ice-cold PBS, the cells were stained for 5 min on ice with Hoechst 33258 (Invitrogen, Molecular Probes, Leiden, The Netherlands) diluted 1:10,000 in ice-cold PBS to visualize the nuclei. After mounting the slides in Vectashield Mounting medium H-1000 (Vector, via Brunschwig Chemie, Amsterdam, The Netherlands), analysis was performed using a Leica fluorescence microscope DM-RXA.

2.10. Statistical analysis

Data are represented as the mean ± SE. In all cases, statistical analyses were done using 2-tailed Student's t-test. p-Values < 0.05 were considered significant and are indicated with an asterisk.

3. Results

3.1. IGF-1R inhibition using NVP-AEW541 modulates sensitivity to rhTRAIL and DR-agonistic antibodies

We evaluated the role of IGF-1R expression and its inhibition on TRAIL sensitivity using the isogenic SW948/SW948-TR model. Membrane expression of IGF-1R was similar in the SW948 cell line and its TRAIL resistant sub-line SW948-TR (Fig. 1A, B). The surface levels were not as elevated as those seen in the MCF7 cells, known to express high levels of IGF-1R (Fig. 1B). We then incubated the cells with various concentrations of the IGF-1R inhibitor NVP-AEW541. Both cell lines showed nanomolar sensitivity to the compound, with a mean GI_{40} (concentration inhibiting cell growth by 40%) below 1 µM in a survival assay (Fig. 1C).

Cytotoxicity assays with TRAIL or the agonistic antibodies in combination with NVP-AEW541 were complicated by the interference of growth inhibitory effects versus apoptosis-inducing effects. We therefore focused on the apoptosis-inducing effects of TRAIL and the agonistic antibodies, using a shorter incubation time. To compensate for the shorter incubation, NVP-AEW541 was combined at a concentration corresponding to the GI_{60} with rhTRAIL or the DR-agonistic antibodies to stimulate apoptosis. Cleavage of poly-ADP ribose phosphate (PARP) was used to assess apoptosis in SW948 (Fig. 1D). The untreated cells and the cells incubated with NVP-AEW541 alone only exhibited the full form of PARP, indicating that NVP-AEW541 does not induce apoptosis at the concentration used. Upon treatment with rhTRAIL PARP was fully cleaved into its 89 kDa fragment, reflecting high levels of apoptosis. To study DR4 and DR5-mediated apoptosis individually, cells were stimulated with agonistic DR4 (HGS-ETR1) or DR5 antibody (HGS-TR2J). Similarly to rhTRAIL, HGS-ETR1 was a very effective inducer of PARP cleavage. HGS-TR2J also induced PARP cleavage, but only partially, indicating that DR5 was less potent than DR4 in trans-

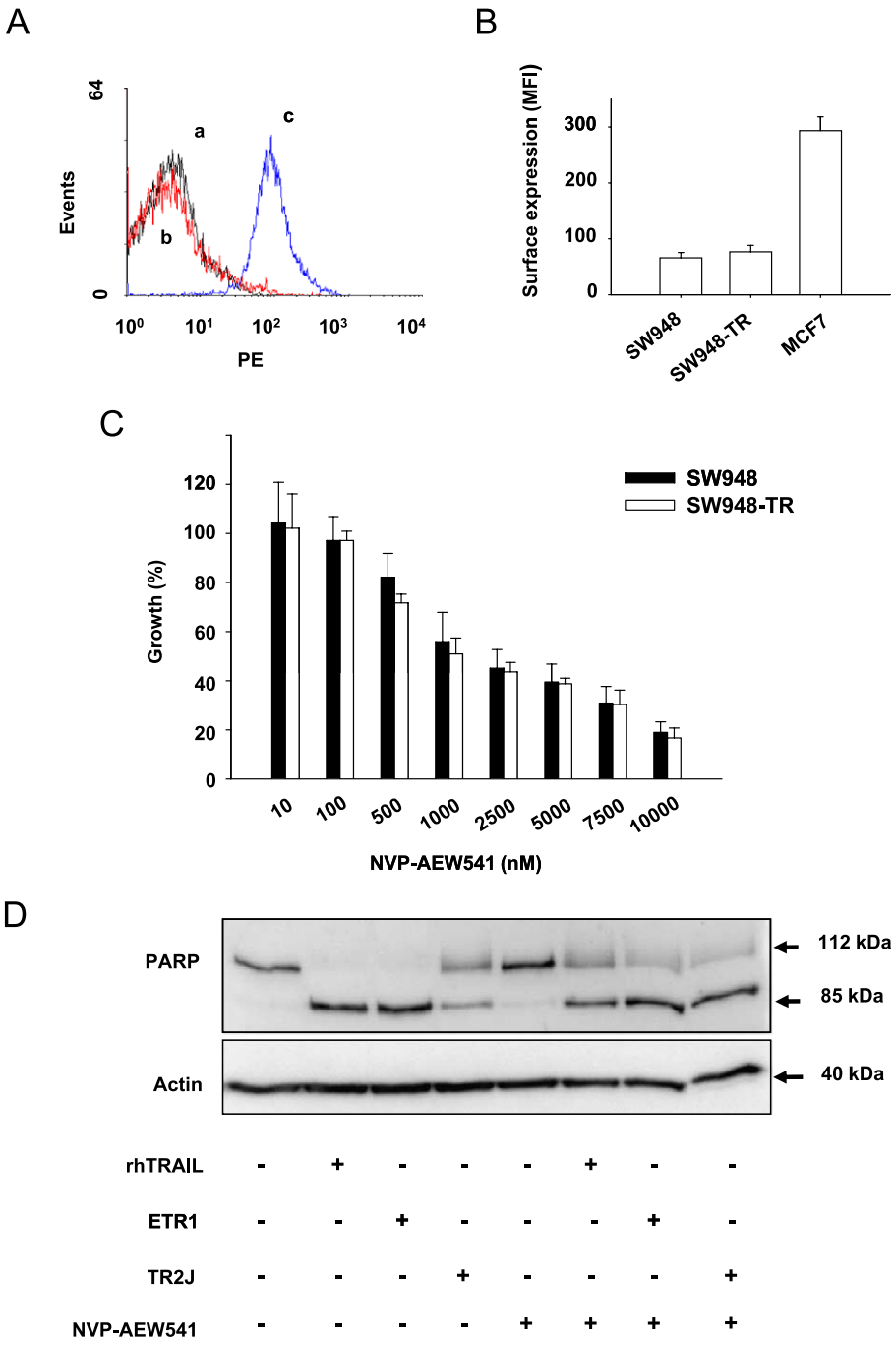

Fig. 1. SW948 and SW948-TR cells express surface IGF-1R and inhibition of this receptor modifies sensitivity to death receptor agonists. (A) Representative example of IGF-1R expression in SW948. Positive receptor expression was detected as an increased fluorescence intensity of the whole cell population and resulted in a peak-shift to the right (a – control; b – IgG1 and c – IGF-1R). (B) Surface expression of IGF-1R in SW948, SW948-TR and MCF7 cells (positive control for IGF-1R expression), as determined by flow cytometry. IGF-1R expression is given as Mean Fluorescent Intensity (MFI), normalized to 100. Values are mean ± SE of at least three independent experiments. (C) Survival (%) of SW948 and SW948-TR cells after continuous incubation with NVP-AEW541 for 96 h, as measured by cytotoxicity assays. Values are mean ± SD of at least three independent experiments. (D) Western blot analysis of PARP cleavage in SW948. The cells were pre-incubated for 15–18 h with 5 μM NVP-AEW541, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/ACP-CLO-2010-0549.)

ducing the apoptotic signal. When NVP-AEW541 was combined with rhTRAIL or HGS-ETR1, the cleavage of PARP was unexpectedly lower compared to rhTRAIL or HGS-ETR1 alone, which suggested that NVP-AEW541 decreased the sensitivity to these ligands. In contrast, the amount of cleaved PARP was higher when HGS-TR2J was combined with NVP-AEW541 in comparison to HGS-TR2J alone, signifying an increase in DR5-mediated apoptosis. Next, we performed caspase 3/7 enzyme activity assay as an additional method to assess apoptosis in SW948 and also in the TRAIL-resistant SW948-TR cells. Incubation with NVP-AEW541 alone had no effect on caspase 3/7 activation in either cell line (Fig. 2A and B). Upon inhibition of IGF-1R using NVP-AEW541, the sensitivity to rhTRAIL decreased by 46.2 ± 11.5% (mean ± SE) in SW948. Similar findings were done in SW948-TR cells, which became even more resistant to rhTRAIL (42.6 ± 2% decrease in caspase 3/7 activity after rhTRAIL treatment). IGF-1R inhibition effectively decreased DR4-mediated caspase 3/7 activity by 39.8 ± 10.8% in SW948 cells, while SW948-TR cells followed a similar trend (19.3% decrease ±15.2). In contrast, both cell lines exhibited an increase in DR5-mediated caspase 3/7 activity (an increase of 78.2 ± 5.9% in SW948 and of 92.7 ± 26.7% in SW948-TR).

Although we do not show a formal concentration-dependent analysis here, the effects of NVP-AEW541 on DR-mediated apoptosis proved to be concentration dependent. Concentrations below the GI_{60} showed similar, although less pronounced effects, and higher concentrations of NVP-AEW541 (up to 10 μM) had even more dramatic effects on DR-mediated apoptosis, in absence of additional cell death as a single agent.[1]

3.2. IGF-1R inhibition can modulate processing and expression of DISC components

The effects of NVP-AEW541 on the protein levels of the components of the death-inducing signaling complex (DISC) were analyzed by Western blotting. NVP-AEW541 did not decrease the basal expression of procaspase 8, nor did it affect the expression of fas-associated death domain (FADD) or cellular Flice inhibitory protein (c-FLIP), in SW948 (Fig. 2C). Following treatment with rhTRAIL, procaspase 8 was cleaved into its intermediate (p43/41) and ultimately active forms (p18). However, after pretreatment with NVP-AEW541, rhTRAIL failed to cause a complete cleavage of procaspase 8. As expected, c-FLIP was cleaved after treatment with rhTRAIL. NVP-AEW541 pretreatment did not induce any noticeable change in the processing of c-FLIP induced by rhTRAIL. Following pretreatment with NVP-AEW541, HGS-ETR1 induced the same pattern of caspase 8 and c-FLIP cleavage as found with the combination of rhTRAIL and NVP-AEW541. In contrast, DR5 targeting using HGS-TR2J alone failed to fully cleave procaspase 8 and c-FLIP. NVP-AEW541 pretreatment enhanced the responsiveness to HGS-TR2J, triggering almost full cleavage of both proteins.

Such changes in the apoptotic response could be due to modulation of the surface expression of DR4 and DR5. We assessed the effects of NVP-AEW541 on cell membrane DR4 and DR5 expression in both SW948 and SW948-TR (Fig. 2D). Incubating the cells with NVP-AEW541 indeed resulted in a downregulation of membrane DR4. On the other hand, no changes were observed in DR5 membrane level.

3.3. IGF-1R exerts its effects on TRAIL sensitivity via the PI3K pathway

Because we expected NVP-AEW541 to influence DR-mediated apoptosis by inhibiting pathways downstream of IGF-1R, we tested whether IGF-1 could induce Akt phosphorylation, and whether NVP-AEW541 was able to inhibit this phosphorylation. Akt is one of the key proteins phosphorylated by PI3K downstream of IGF-1R [26]. Cells were serum-starved for 24 h, pretreated or not with NVP-AEW541 for one hour at a concentration neighboring the GI_{20} of both cell lines, and finally either left untreated or treated for 15 min with recombinant human (rh)IGF-1. Concentrations of rhIGF-1 (10–50 ng/ml) within the physiological range induced phosphorylation of Akt in both cell lines (Fig. 3A). Pretreatment with NVP-AEW541 dramatically reduced IGF-1-induced phosphorylation of Akt in both cell lines. Total Akt levels were similar in the two cell lines and were not affected by NVP-AEW541 or rhIGF-1. Phospho-$MAPK^{44/42}$ ($pMAPK^{44/42}$) levels were studied as this protein can also be activated by phosphorylation in response to IGF-1. The levels of $MAPK^{44/42}$ and $pMAPK^{44/42}$ were similar with and without NVP-AEW541 treatment, suggesting that the activity of this pathway might be independent of IGF-1 in these cells.[2] We then decided to investigate whether

[1] Data not shown.

[2] Data not shown.

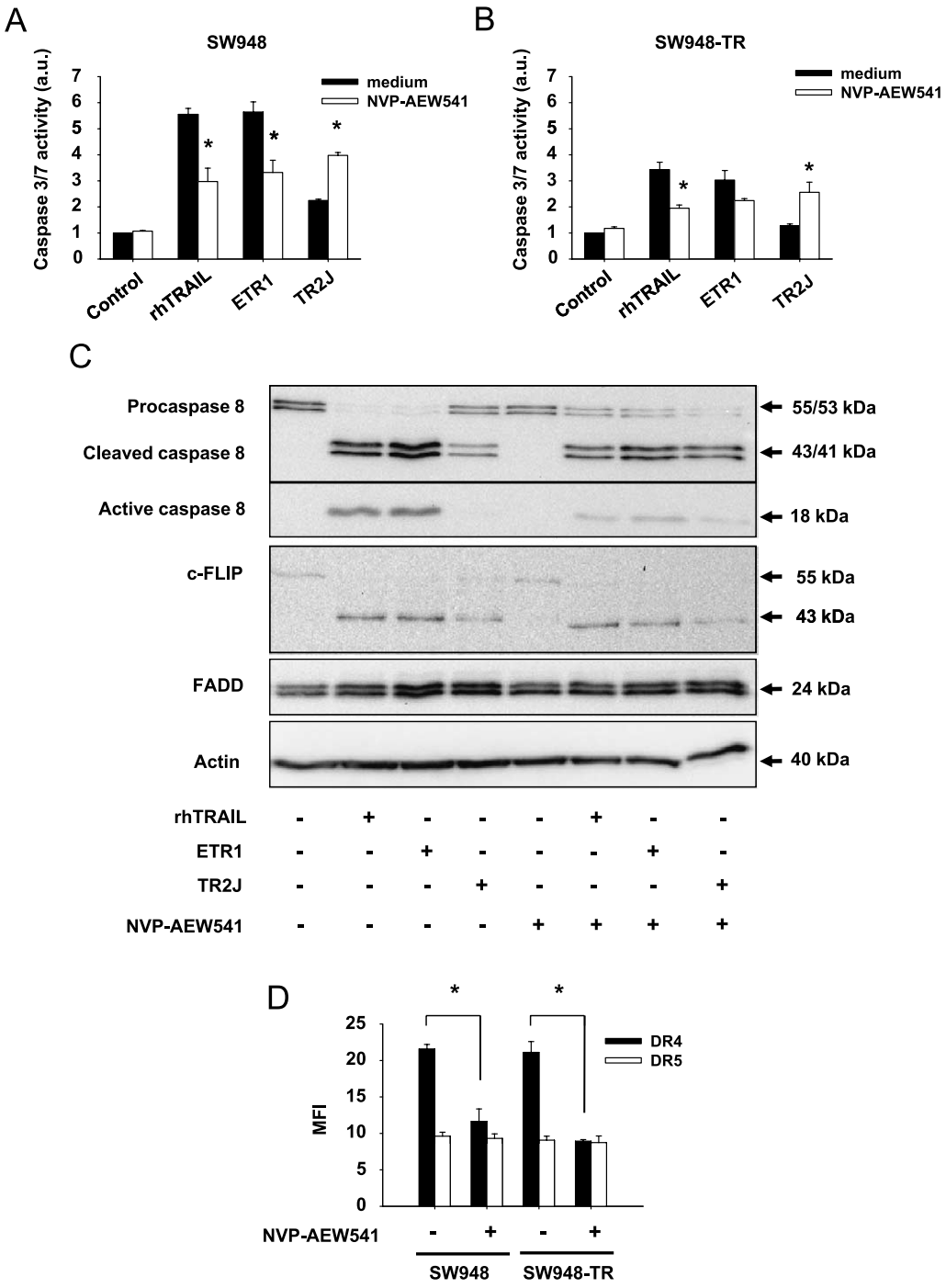

Fig. 2. IGF-1R inhibition modulates DR-mediated apoptosis and downregulates surface DR4 expression. Modulation of rhTRAIL or agonistic antibody induced apoptosis by NVP-AEW541 in SW948 (A) and SW948-TR (B), as assessed by caspase 3/7 activity assay. Cells were pre-incubated for 15–18 h with 5 μM NVP-AEW541, then either left untreated or exposed to rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. Caspase 3/7 activity is expressed in arbitrary units (a.u.), as the ratio of caspase 3/7 activity in treated cells to untreated cells. Values are mean ± SE of at least three independent experiments. (C) Western blot analysis of the expression levels of several proteins located downstream from the TRAIL receptors, after IGF-1R inhibition and treatment with rhTRAIL or agonistic DR antibody, in SW948. The cells were pre-incubated for 15–18 h with 5 μM NVP-AEW541, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. (D) Surface expression of TRAIL receptors in SW948 and SW948-TR, in control cells and after 15–18 h exposure to 5 μM NVP-AEW541.

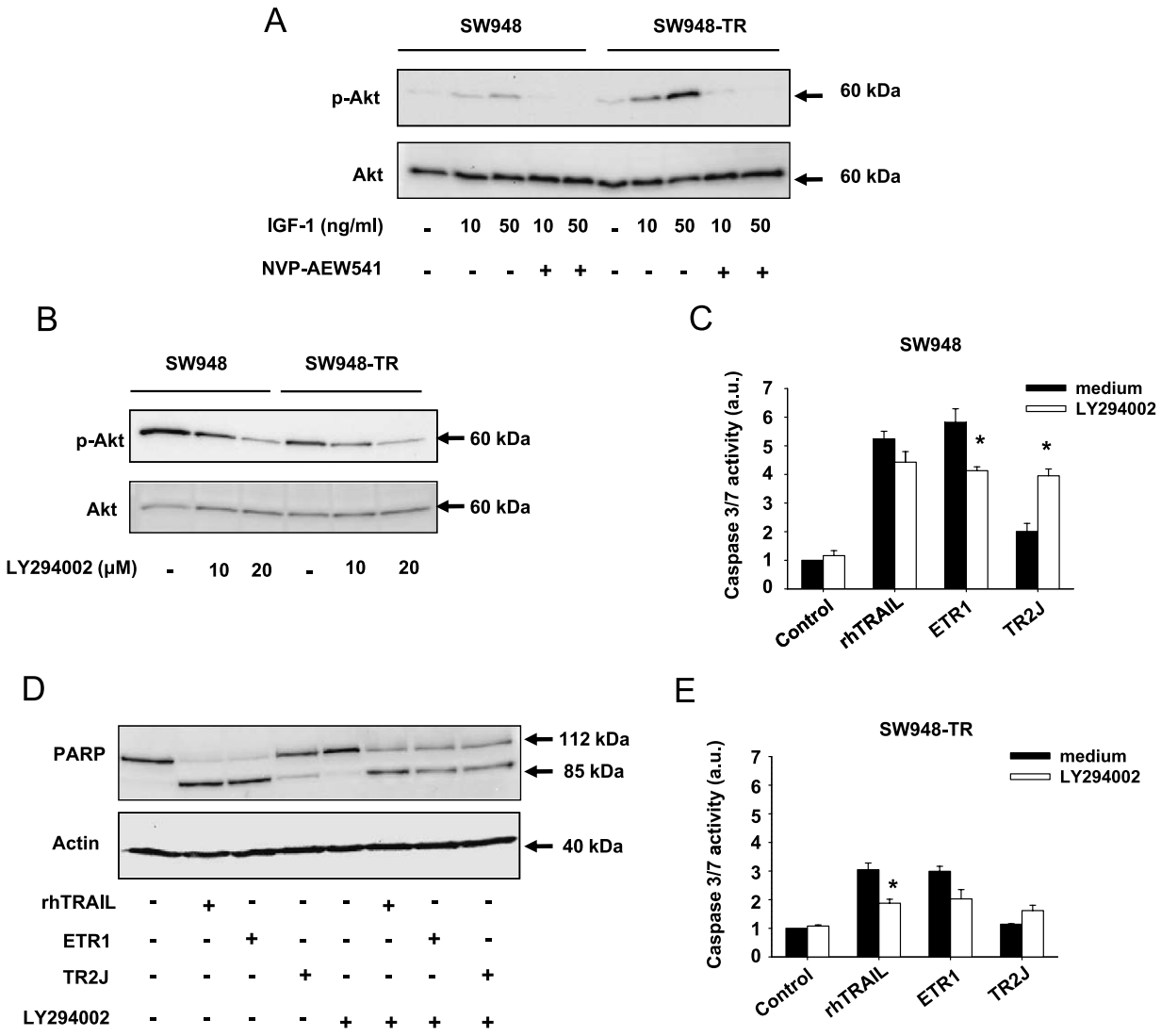

Fig. 3. PI3K mediates the effects of IGF-1R inhibition on DR-mediated apoptosis. (A) Western blot analysis showing inhibition of IGF-1-induced Akt phosphorylation by NVP-AEW541 in SW948 and SW948-TR. Cells were pretreated for 1 h with 5 μM NVP-AEW541 before treatment with increasing concentrations of rhIGF-1 and subsequent harvesting. (B) Western blot analysis showing inhibition of Akt phosphorylation by LY294002 in SW948 and SW948-TR. Cells grown in medium with FCS were treated or not for 1 h with increasing concentrations of LY294002 before harvest. (C) Modulation of rhTRAIL or agonistic antibody induced apoptosis by PI3K inhibition in SW948, as assessed by caspase 3/7 activity assay in SW948. Cells were pre-incubated for 15–18 h with 20 μM LY294002, then either left untreated or exposed to rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. (C) Caspase 3/7 activity assay in SW948 cells. Cells were pre-incubated for 15–18 h with 20 μM LY294002, then either left untreated or exposed to rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. (D) Western blot analysis of PARP cleavage in SW948. The cells were pre-incubated for 15–18 h with 20 μM LY294002, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. (E) Caspase 3/7 activity assay in SW948-TR. Cells were treated as described in (C).

a specific PI3K inhibitor could also inhibit Akt phosphorylation in SW948 and SW948-TR cells. As seen in Fig. 3B, upon treatment with 10–20 μM LY294002 for 1 h, Akt phosphorylation in both cell lines was inhibited in a concentration-dependent manner. LY294002 had no effects on total Akt expression levels.

Next, LY294002 was combined with rhTRAIL or death receptor-specific antibodies. Treatment of SW948 cells with LY294002 alone did not affect apoptosis, as measured by caspase 3/7 activity (Fig. 3C). HGS-ETR1-induced caspase 3/7 activity was reduced by 27.6 ± 8.2%. Although there was only a non-

significant trend, rhTRAIL-induced caspase 3/7 activity decreased when the cells were pretreated with LY294002 (by 14.7 ± 9.8%). In contrast, sensitivity through DR5 was clearly increased by 107.8 ± 38.5%. Analysis of PARP cleavage confirmed the changes in susceptibility to these agents in SW948 (Fig. 3D).

In SW948-TR (Fig. 3E), LY294002 similarly decreased sensitivity to rhTRAIL (by 38.3 ± 0.6%), as assessed by caspase 3/7 activity measurement. Although these differences were not statistically significant, pretreatment with LY294002 lowered sensitivity to HGS-ETR1 (by 32.3 ± 10.3%) but increased HGS-TR2J sensitivity (41.2 ± 14.7% increase).

3.4. PI3K inhibition modulates caspase 8 and c-FLIP cleavage

We examined whether LY294002 induced similar effects to NVP-AEW541 at the level of the DISC proteins. The results for caspase 8 were similar to those previously seen using NVP-AEW541, albeit the inhibition of procaspase 8 processing to the intermediate and active forms upon rhTRAIL and HGS-ETR1 seemed less strong (Fig. 4A). Basal c-FLIP and FADD levels were not affected by LY294002. The changes in c-FLIP cleavage after LY294002 exposure followed the pattern seen after NVP-AEW541 treatment, although the loss of c-FLIP through cleavage upon HGS-ETR1 or HGS-TR2J treatment after LY294002 was slightly higher.

Next, we studied whether inhibiting PI3K would modulate death receptor expression at the cell surface, as seen with NVP-AEW541. As expected, LY294002 downregulated DR4 to the level of DR5 in SW948 and SW948-TR, providing an explanation for the reduction in rhTRAIL sensitivity (Fig. 4B).

Localization of the death receptors is known to be modulated by certain drugs which cause them to aggregate and thereby increase their sensitivity to death inducing ligands [35]. This prompted us to examine whether the distribution of DR5 at the cell surface was affected by PI3K inhibition. Fluorescent microscopy imaging of membrane DR5 in SW948 cells did not show any change in DR5 distribution at the cell surface following treatment with 20 μM LY294002

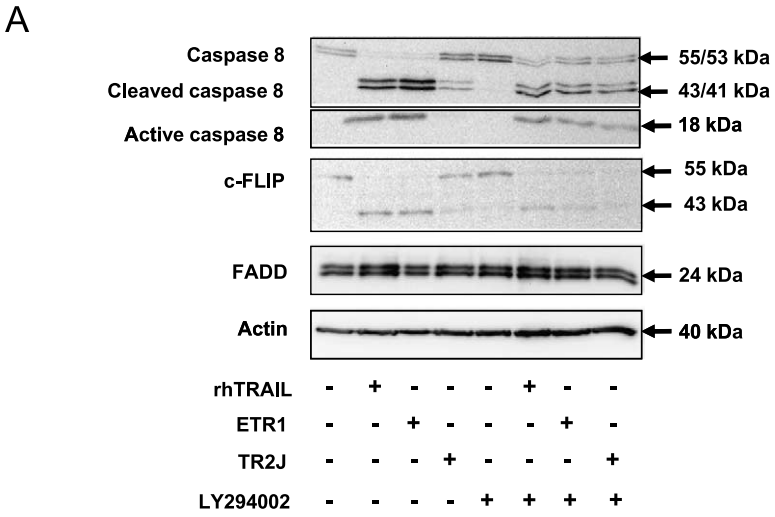

Fig. 4. PI3K inhibition modulates DISC proteins cleavage and surface DR4 levels, but not cell membrane localization of DR5 or initial DR5-DISC formation. (A) Expression levels of several proteins located downstream of the TRAIL receptors were determined by Western blotting in SW948. The cells were pre-incubated for 15–18 h with 20 μM LY294002, then either left untreated or exposed for 3 additional hours with rhTRAIL (0.1 μg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) before harvest. (B) Surface expression of TRAIL receptors in SW948 and SW948-TR, in control cells and after 15–18 h exposure to 20 μM LY294002. Receptor expression was detected as the average antigenic density of the whole cell population (Black line histograms: fluorescence of the untreated cells, grey shaded histograms: fluorescence of the cells treated with 20 μM LY294002 for 15–18 h). (C) Fluorescent microscopy imaging of DR5 in SW948 cells in untreated cells and cells exposed with 20 μM LY294002 for 17 h, stimulated or not with HGS-TR2J for 15 min. Cells were first pretreated with LY294002 and stained for cell membrane DR5. Next, cells were either kept on ice or treated with HGS-TR2J for 15 min at 37°C before fixation. One representative of at least three independent experiments is shown. (D) DR5-DISC immunoprecipitation in SW948 cells, using the HGS-TR2J antibody. Cells were pretreated or not for 17 h with 20 μM LY294002 before stimulation for 15 min using HGS-TR2J and immunoprecipitation with protein G agarose (C – antibody added after cell lysis, T – cells treated with HGS-TR2J). DR5-DISC analysis was performed at least three times. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/ACP-CLO-2010-0549.)

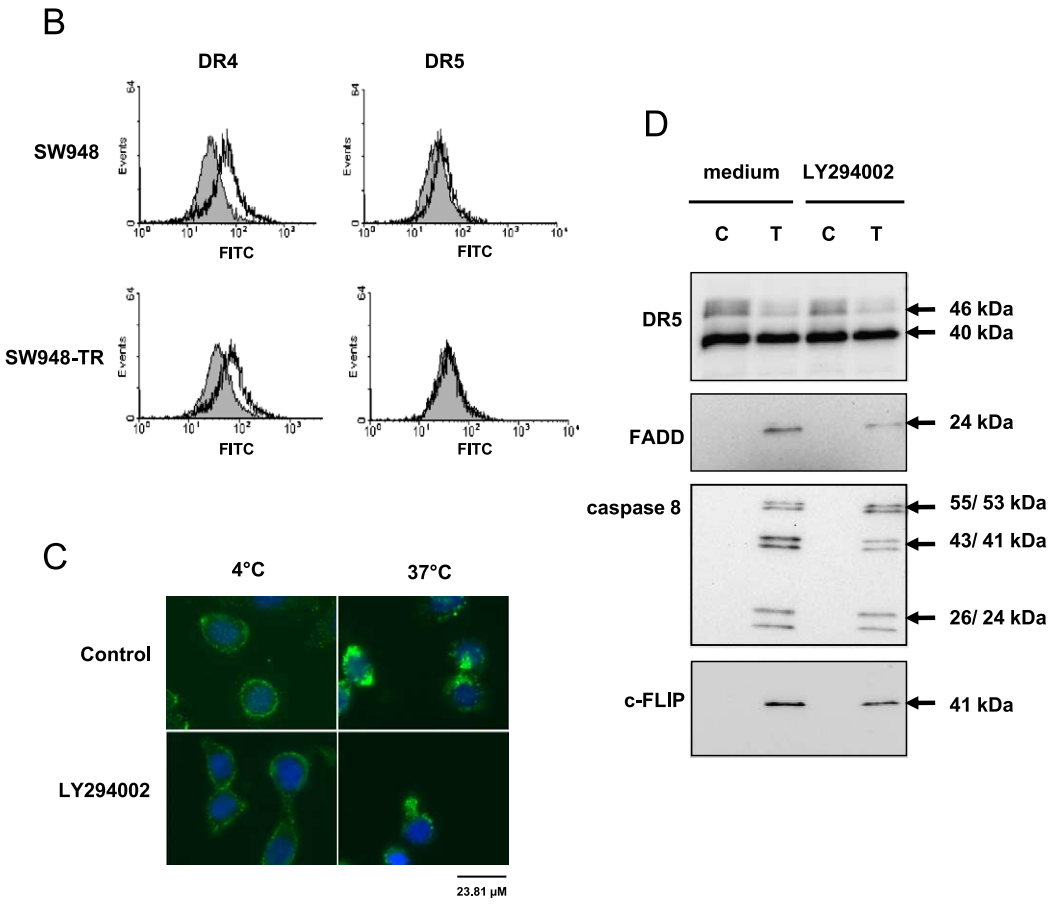

Fig. 4. (Continued.)

(Fig. 4C). Because differences in DR5 aggregation following LY294002 treatment might only occur after stimulation of the receptor, we stimulated DR5 for 30 min using HGS-TR2J. As seen in Fig. 4C, DR5 aggregated following stimulation with HGS-TR2J, but the staining pattern was similar between LY294002-treated and untreated cells.

Since PI3K inhibition increased caspase 8 activation, suggesting modifications in DR5-DISC formation, we performed a co-immunoprecipitation of this complex using HGS-TR2J, with or without pre-incubation with LY294002. To our surprise, DISC formation was comparable for both stimuli (Fig. 4D). HGS-TR2J could recruit DR5, FADD, caspase 8 and c-FLIP. In control cells, three caspase 8 forms were detected in the DR5-DISC: the full form, the intermediate p43/41 cleaved form and the p26/24 cleavage product. The latter indicates full cleavage of caspase 8 and therefore release of active caspase 8 from the complex. c-FLIP was mostly present in its intermediate form. In cells pretreated with LY294002, the ratio of each DISC component was similar. No DR4 was found in the DR5-DISC complex, in both LY294002-treated and -untreated cells.[3] Later time points (up to 1 h, when active caspase 8 becomes detectable in whole cell lysates from LY294002-pretreated cells) displayed similar DISC content.[4] Thus, the relative proportion of each DISC protein in the DR5-DISC is similar with and without LY294002.

3.5. The opposite effects on DR4- and DR5-sensitivity induced by PI3K signaling pathway inhibition are not specific to the SW948/SW948-TR isogenic model

To expand our results to a non-isogenic model, we also assessed the effects of PI3K-specific inhibition with LY294002 on TRAIL receptor-mediated apoptosis in Colo205. These cells were initially highly sensi-

[3]Data not shown.
[4]Data not shown.

tive to rhTRAIL and to the DR5-targeting antibody and less to the DR4-targeting antibody, indicating that here DR5 was the most potent of the two death receptors. We therefore used concentrations of 50 nM for HGS-ETR1 and 5 nM for HGS-TR2J to induce equivalent amounts of apoptosis via each death receptor. Our results show that LY294002 treatment decreased sensitivity to rhTRAIL and HGS-ETR1 (Fig. 5A). Importantly, DR5-mediated sensitivity increased following LY294002 treatment, as seen in SW948 and SW948-TR. Moreover, Colo205 cells also exhibited a reduction in surface membrane DR4 after PI3K inhibition, as seen in SW948 and SW948-TR (Fig. 5B).

4. Discussion

Prosurvival signals originating from growth factor receptors, including IGF-1R, prevent cell death induced by radiation, cytotoxic drugs and members of the tumor necrosis factor family [18,30,41,52]. In the present study, we demonstrated that inhibition of prosurvival signaling using the IGF-1R inhibitor NVP-AEW541 exclusively increased sensitivity to DR5-mediated apoptosis in SW948 and SW948-TR colon carcinoma cells. SW948 cells in particular were initially very sensitive via DR4, and therefore contained all the proteins necessary to form a DISC, but they exhibited significant resistance to apoptosis mediated via DR5. The IGF-1R inhibitor exclusively released the blockade of caspase 8 cleavage at the level of DR5. The TRAIL-resistant cell line SW948-TR became more sensitive to the anti-DR5 agonistic antibody, suggesting that the effects of IGF-1R inhibition are not restricted to rhTRAIL-sensitive cells. Surprisingly, we found that IGF-1R inhibition decreased sensitivity to rhTRAIL or an anti-DR4 agonistic antibody. The decrease in sensitivity to rhTRAIL and to the anti-DR4 antibody could be explained in part by the $\sim$50% downregulation of membrane DR4 following IGF-1R inhibition seen in all three cell lines. We correlated this DR4 downregulation with a decrease in caspase 8 cleavage following rhTRAIL or anti-DR4 treatment. This surprising decrease in sensitivity to some DR-ligand is supported by prior demonstrations that inhibition of oncogenic pathways such as HER-2 and Ras also reduces the expression of TRAIL receptors, as well as their sensitivity [10,11]. Mahalingam et al. recently found that inhibition of the c-Jun N-terminal kinase (JNK) stress pathway antagonized TRAIL-induced apoptosis but stimulated anti-DR antibody-induced apoptosis in several colon cancer cells [29]. Likewise, a proapoptotic effect of IGF-1 on rhTRAIL sensitivity in 6 out of 9 colon cancer cell lines was described [38]. We identified PI3K as the mediator of both DR4 decrease and DR5 increase in sensitivity to their respective agonist antibodies. We show that an IGF-1R inhibitor, at the concentration used, successfully prevented PI3K-dependent Akt phosphorylation induced by physiologically relevant levels of soluble IGF-1. This was accompanied by strong effects on DR-mediated apoptosis. Similar results were seen on Akt phosphorylation and DR-mediated apoptosis using the widely-accepted PI3K inhibitor LY294002, which further supports the role for PI3K in modulating DR-mediated apoptosis. Thus, as depicted in Fig. 6, our experiments provide a link between the involvement of IGF-1R in PI3K/Akt activity in colon cancer cells and the role of this pathway in death receptor-mediated apoptosis regulation.

We investigated several hypotheses for the increase in DR5-mediated sensitivity following PI3K inhibition. No change in membrane DR5 expression was seen. It has been reported that active PI3K can directly act on the DISC and interfere with caspase 8 cleavage by up-regulating c-FLIP [33]. We found that neither basal levels of c-FLIP nor DR5-DISC c-FLIP levels were altered by the PI3K inhibitor in SW948 cells. This indicates that the effects of PI3K on the DR5-DISC are independent of c-FLIP in our model. Similar observations were made regarding the basal levels of other proteins recruited to the DISC. Furthermore, we also excluded that sensitivity to the anti-DR5 antibody following PI3K inhibition increases was caused by receptor aggregation. A number of proteins identified as downstream substrates of PI3K/Akt can modulate the mitochondrial apoptotic pathway of apoptosis, for instance caspase 9 and Bad [3,8,9]. Inhibition of caspase 9 activity using zLEHD-fmk did not protect SW948 cells from apoptosis induced by either TR2J or the combination of TR2J with LY294002, pointing toward a mitochondria-independent mechanism.[5] Following LY294002 treatment, both caspase 8 and c-FLIP cleavage was enhanced upon stimulation of DR5 with its agonistic antibody, which strongly supports the existence of a sensitizing mechanism at the DR5-DISC itself. We suggest that PI3K inhibition leads to a higher turn-over of c-FLIP and caspase 8 at the DR5-DISC, thus gradually increasing the amount of their cleaved cytoplasmic forms.

[5] Results not shown.

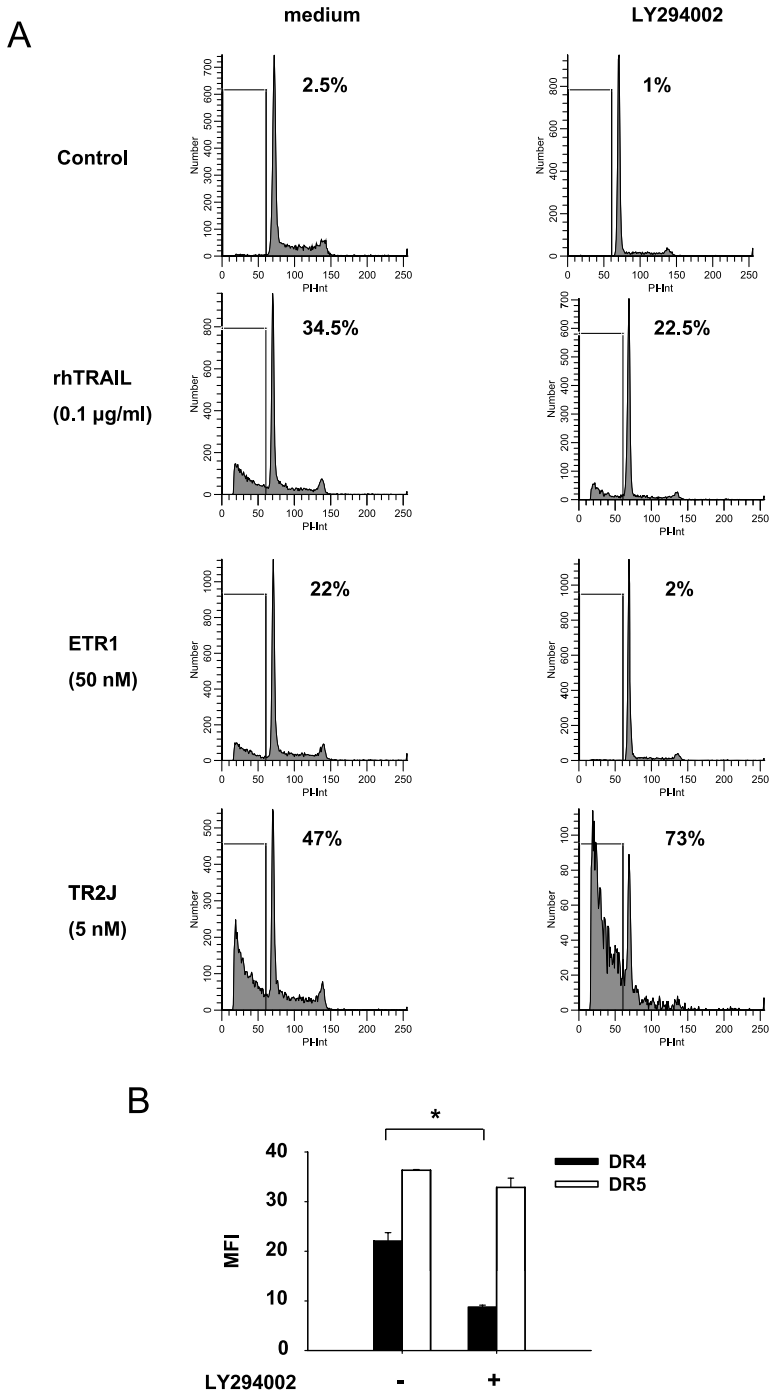

Fig. 5. The effects of PI3K inhibition on DR-mediated apoptosis can also be seen in cells that are already more sensitive to HGS-TR2J than to HGS-ETR1 initially. (A) Sub-G1 content analysis in Colo205. Cells were pre-incubated for 15–18 h with 20 µM LY294002, then either left untreated or exposed to rhTRAIL (0.1 µg/ml), HGS-ETR1 (50 nM) or HGS-TR2J (50 nM) for 3 additional hours before harvest. Nuclear DNA was then stained with propidium iodide and the cells analyzed by flow cytometry. The value in percent indicates the amount of apoptosis as measured by the count of sub-G1 events. One representative of at least 3 independent experiments is shown. (B) Surface expression of TRAIL receptors in Colo205, in untreated cells and after 15–18 h exposure to 20 µM LY294002. Receptor expression is measured as Mean Fluorescent Intensity (MFI) normalized to 100.

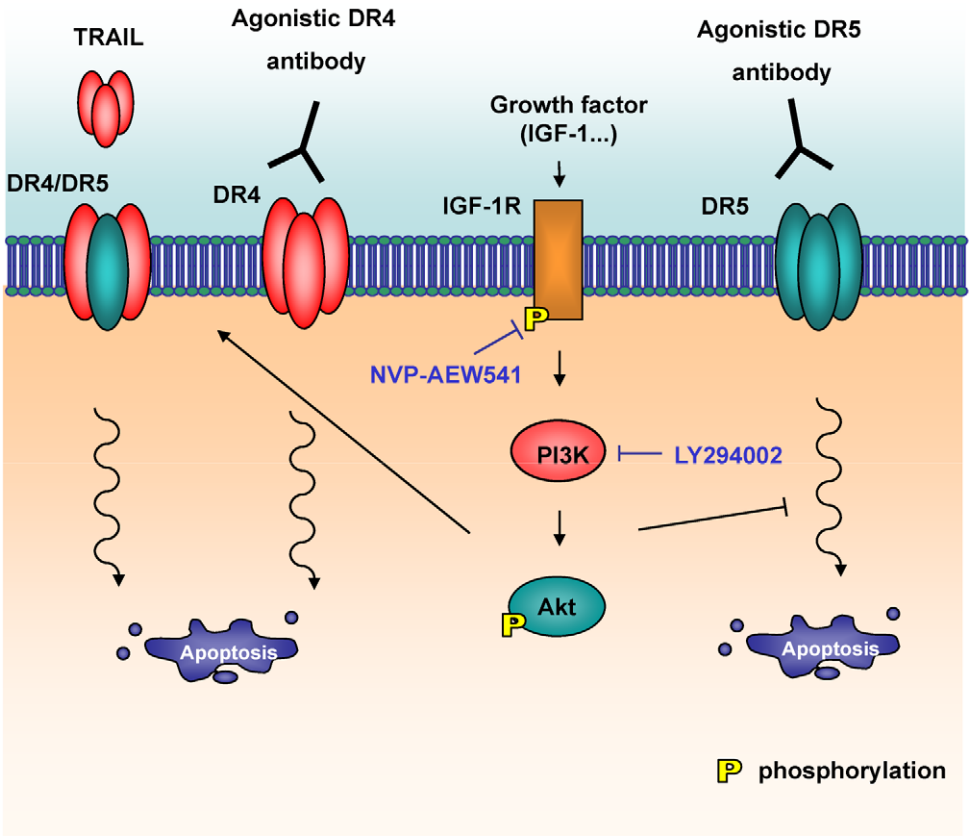

Fig. 6. Model for differential modulation of TRAIL receptor ligand-induced apoptosis by IGF-1R/I3K-dependent mechanisms. TRAIL receptor-specific agonists bind to their cognate TRAIL death receptor trimers, stimulating the TRAIL receptor-mediated apoptosic pathway. In this figure, DR4 and DR5 specifically designate homotrimers, while DR4/DR5 refers to either type of trimer, including possible heterotrimers. Upon activation of IGF-1R by growth factors such as IGF-1, the receptor becomes autophosphorylated, which activates PI3K. Active PI3K triggers Akt phosphorylation and its subsequent activation. Inhibition of PI3K using LY294002 or inhibition of IGF-1R-induced PI3K activation using NVP-AEW541 leads to a decrease in Akt phosphorylation. This is also accompanied by inhibition of TRAIL- and agonistic DR4 antibody-induced apoptosis, notably due to a decrease in membrane DR4 expression and decreased caspase 8 cleavage. Conversely, caspase 8 cleavage and apoptosis induced by agonistic DR5 antibody are increased when IGF-1R or PI3K are inhibited using NVP-AEW541 or LY294002, respectively. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/ACP-CLO-2010-0549.)

In Colo205, which is a DR5-driven cell line with regard to the relative potency of each DR to transduce apoptosis [42], sensitivity to the DR5 antibody was remarkably enhanced by LY294002. Conversely, DR4-mediated sensitivity was almost completely lost and sensitivity to rhTRAIL decreased by ∼50%. Although the downregulation of DR4 could explain part of these effects, the benefits of PI3K inhibition on DR5 signaling seemed restricted to the DR5 antibody but not to rhTRAIL. Thomas et al. established [40] that the cytoplasmic tail of DR4 and DR5 had an important role in facilitating FADD binding and DISC formation upon stimulation with DR-ligands. Depending on this cytoplasmic tail, the ability of the various DR-ligands to trigger apoptosis differed. Unlike TRAIL, the anti-DR5 agonistic antibody HGS-ETR2 could induce apoptosis independently of the last c-terminal amino-acids of DR5. In view of these findings, we suggest that PI3K inhibition might induce intracellular conformational changes at the level of DR5 that only benefits to HGS-TR2J. Recently, Hassan et al. described how inhibition of the apoptosis signal-regulating kinase 1 (ASK1)-JNK/p38 pathway prevented caspase 8 cleavage in response to the agonistic anti-Fas antibody CH11, notably via inhibition of CH11-induced FADD phosphorylation [16]. It is possible that such FADD phosphorylation mechanism differentially modulates the response to TRAIL and to the various DR agonistic antibodies. Numerous proteins can be recruited to DR4 and DR5 and modulate apoptosis signalling [35]. Our study supports the exis-

tence of pathways with opposite effects for the various TRAIL receptor ligands.

Since PI3K is an essential component in the transduction of survival signals originating from the growth factor receptors, we consider a better understanding of its influence on the TRAIL pathway necessary. Interestingly, while chemotherapy requires the p53 gene to upregulate DR5 and thereby increase death ligand sensitivity in colonic carcinoma [47], PI3K inhibition improved DR5-mediated sensitivity without requiring a functional p53 (the three cell lines used in our experiments express mutant p53). Elucidating the mechanism of action of PI3K on DR5 could therefore be decisive for future DR agonist-based therapies, since the vast majority of CRC cancers carry mutations in p53. Conversely, inhibition of PI3K combined with chemotherapy might further improve sensitivity to DR5 agonists.

DR4 and DR5 can be targeted individually (using antibodies or death-receptor specific forms of TRAIL) or simultaneously (using rhTRAIL), and these ligands will have to be combined with other therapies to overcome resistance mechanisms. We conclude that DR5-targeting agents, such as agonistic antibodies, might be optimal to elicit maximum cell death induction from DR agonist-based regimens combined with drugs inhibiting PI3K. Our results also suggest caution regarding possible antagonist effects when combining rhTRAIL or DR4 agonistic antibodies with IGF-1R or PI3K inhibitors.

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