Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis in Cutaneous T-Cell Lymphoma Cells and Enhance Their Sensitivity for TNF-Related Apoptosis-Inducing Ligand

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Cutaneous T-cell lymphomas (CTCL) form a heterogeneous group of non-Hodgkin's lymphomas of the skin. In previous studies, we had characterized CTCL cells as resistant to the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which correlated to pronounced expression of the caspase-8/-10 inhibitor c-FLIP. For identification of proapoptotic strategies in CTCL cells and for overcoming their death ligand resistance, we investigated the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid, sodium salicylate, and diclofenac (DF). These drugs strongly enhanced apoptosis, as well as decreased CTCL cell proliferation and vitality, and DF furthermore sensitized for TRAIL-induced apoptosis. Full activation of the caspase cascade (caspase-3, -8, -9) and decreased mitochondrial membrane potential were characteristic for NSAID treatment, whereas cytochrome *c* release was seen only for DF. Downregulation of Mcl-1 and enhanced surface expression of TRAIL were seen in response to NSAIDs. Most characteristic for apoptosis induction was the downregulation of c-FLIP. In agreement with the critical role of c-FLIP for apoptosis deficiency of CTCL cells, its overexpression decreased NSAID-mediated apoptosis and its downregulation by small hairpin RNA-enhanced apoptosis. The study provides a rationale for the use of NSAIDs as a new therapeutic option for CTCL patients. Supporting this concept, *ex vivo* lymphoma cells of CTCL patients also revealed significant sensitivity for NSAID treatment.

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INTRODUCTION

Non-Hodgkin's lymphomas have shown increasing incidence in past decades, with a prevalence of 10–20 cases per 100,000 in Western countries at present (Muller *et al.*, 2005). Roughly 5% of non-Hodgkin's lymphomas are characterized by clonal proliferation of skin-homing memory T lymphocytes and primary cutaneous manifestation. The heterogeneous group of cutaneous T-cell lymphomas (CTCL) encloses as its most frequent entities mycosis fungoides, Sézary syndrome (SzS), and CD30(+) lymphoproliferative disorders. CTCL frequently reveals a rather indolent clinical course in the beginning, but may transform in its late phases to a phenotype with rapidly growing and ulcerating tumors (Willemze *et al.*, 2005).

The principal goal of cancer therapy is the elimination of tumor cells through induction of apoptosis. Thus, therapy resistance is frequently explained in terms of defects in apoptosis signaling (Reed and Pellecchia, 2005; Eberle *et al.*, 2007). Extrinsic proapoptotic pathways are initiated by death ligands as tumor necrosis factor-α, CD95L/FasL, and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), which exert particular functions in an antitumor immune response, as well as in lymphocyte autoregulation. Their binding to death receptors results in the formation of a death-inducing signaling complex, where proapoptotic initiator caspases 8 and 10 are activated (Krammer *et al.*, 2007). These caspases are negatively regulated by c-FLIP (cellular FLICE-inhibitory protein), a catalytically inactive caspase-8 homolog that binds to the death-inducing signaling complex in a competitive manner (Irmler *et al.*, 1997).

Death receptor-mediated apoptosis may be further enhanced in an amplification loop by the mitochondrial proapoptotic pathway, which is critically regulated by the Bcl-2 protein family (Reed, 2008). This is mediated by cleavage and activation of the proapoptotic Bcl-2 protein Bid through caspase-8 (Li *et al.*, 1998). Activation of the

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Abbreviations: ASA, acetylsalicylic acid; CTCL, cutaneous T-cell lymphoma; DF, diclofenac; GFP, green fluorescent protein; NaS, sodium salicylate; NSAID, nonsteroidal anti-inflammatory drug; PBS, phosphate-buffered saline; shRNA, small hairpin RNA; SzS, Sézary syndrome; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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mitochondrial apoptosis pathway results in the release of proapoptotic factors such as cytochrome *c* and subsequent activation of initiator caspase-9. Initiator caspases 8, 9, and 10 may cleave and activate the main effector caspase-3, which cleaves a large number of death substrates for complete reprogramming of the cell with the final result of DNA fragmentation (Fischer *et al.*, 2003).

Resistance to apoptosis appears as a critical feature of cancer cells, as also seen in CTCL. Thus, loss of the death receptor CD95/Fas (Contassot *et al.*, 2007, 2008), impaired activation-induced cell death due to disrupted T-cell receptor signaling (Klemke *et al.*, 2009), and constitutive activation of pro-survival transcription factors such as NF-κB (Sors *et al.*, 2006) have been reported. Particularly, c-FLIP appears as a promising target. Its dysregulated expression has been recognized in different cancer entities, including Hodgkin's lymphoma (Mathas *et al.*, 2004), and it has been described by us recently as constitutively expressed in CTCL cells (Braun *et al.*, 2007, 2010).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the clinic for treatment of pain and inflammation, and have also been established in anti-cancer therapies recently (Cha and Dubois, 2007). Cyclooxygenases (COX-1, COX-2), which catalyze prostaglandin synthesis, are important targets of NSAIDs (Castellone *et al.*, 2005; Samuelsson *et al.*, 2007). Aberrant COX-2 expression was related to tumor growth as in colorectal carcinoma (Clevers, 2006), cutaneous squamous cell carcinoma (Fecker *et al.*, 2010), and hematological malignancies (Kopp *et al.*, 2010). Selective COX-2 inhibitors such as celcoxib, valdecoxib, and rofecoxib are distinguished from nonselective inhibitors targeting both COX-1 and COX-2, such as sulindac, acetylsalicylic acid (ASA), sodium salicylate (NaS), and diclofenac (DF).

Here, we describe significant anti-neoplastic effects of NSAIDs (ASA, NaS, DF) in CTCL cell lines, as well as in *ex vivo* tumor T cells of CTCL patients, which may provide a basis for the use of NSAIDs in CTCL therapy.

RESULTS

NSAIDs decrease cell proliferation and induce apoptosis in CTCL cells

For overlooking the effects of NSAIDs in CTCL cells, HH, MyLa, HuT-78, and SeAx cell lines were treated for 40 hours with increasing concentrations of ASA and NaS (1–5 mM). Because of different sensitivities seen for DF in pilot experiments, 5–60 μg ml^{-1} was used for HH and 30–120 μg ml^{-1} was used for MyLa, HuT-78, and SeAx (Figure 1b). For time kinetic analyses, cells were treated for 16, 40, and 72 hours, respectively. Here, concentrations of 5 mM were used for ASA and NaS (5 mM), whereas DF was used at concentrations of 60 μg ml^{-1} (HH) or 120 μg ml^{-1} (MyLa, HuT-78, SeAx; Figure 1h).

According to WST-1 proliferation assays, significant dose- and time-dependent decrease of cell proliferation (between 40 and 70%, at 72 hours) was seen in all cell lines and for all treatments (Figure 1a and g). The decrease of mitochondrial enzyme activity (WST-1) was in parallel with the direct cell counts, but appeared stronger, indicating an additional effect on cell vitality (Figure 1f). Cytotoxicity appeared to have no major role, and was seen only in MyLa and HuT-78 after treatment with higher concentrations of DF and NaS (Figure 1c).

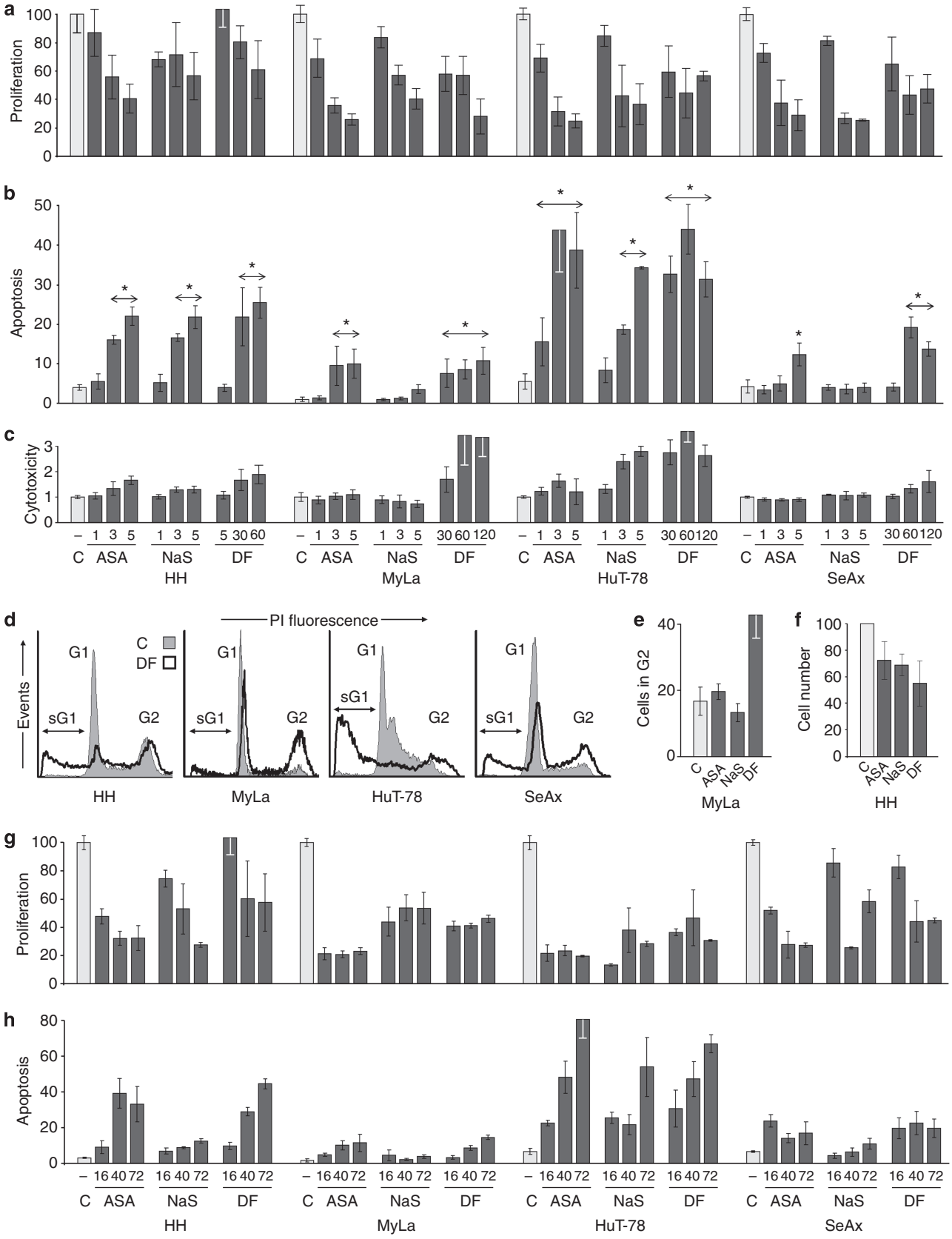
In parallel with decreased cell numbers, significant dose- and time-dependent increase of apoptosis was observed by the NSAIDs in all cell lines. Values of 80% apoptotic cells in HuT-78 and 40% in HH at 72 hours suggested a decisive role of apoptosis induction in these cells. Apoptosis was significantly less in MyLa and SeAx when treated with ASA and DF (10–20%), and did not occur after NaS treatment. Thus, in these cells, inhibited cell proliferation appeared to play the major role, also seen by strongly decreased WST-1 values at 16 hours, when apoptosis was still weak (Figure 1g and h). In agreement, a G2 cell cycle arrest was frequently seen in DF-treated MyLa and SeAx (Figure 1d and e). Thus, NSAIDs exerted substantial anti-neoplastic effects on CTCL cells related to apoptosis induction and decreased cell viability and cell proliferation.

Caspase activation by NSAIDs

For unraveling the signaling pathways, the initiator caspases-8 and -9, the main effector caspase-3, and Bid were investigated by western blot analysis. Strong and dose-dependent processing of caspase-8 and -3 was seen at 24 hours after NSAID treatment. Reduced levels of the Bid proform indicative of its activation were also seen in MyLa after DF treatment (60 μg ml^{-1}), and weak caspase-9 processing was evident in HH (Figure 2a).

Caspase involvement was further proven by the caspase inhibitor Q-VD-Oph, which significantly decreased NSAID-induced apoptosis (Figure 2b). For control, cells were also treated with an agonistic anti-CD95 antibody (CH-11, 50 ng ml^{-1}, 40 hours). This caspase-dependent induction of apoptosis was completely abrogated by Q-VD-Oph. Thus, the partially remaining proapoptotic activity in HH after

Figure 1. Nonsteroidal anti-inflammatory drugs (NSAIDs) decrease cell proliferation and induce apoptosis in cutaneous T-cell lymphoma cells. (**a**–**c**) For determination of dose response, HH, MyLa, HuT-78, and SeAx were incubated for 40 hours with increasing concentrations of acetylsalicylic acid (ASA) and sodium salicylate (NaS; 1–5 mM), as well as diclofenac (DF; 5–120 μg ml^{-1}). (**g**, **h**) For time kinetic analysis, cells were harvested at 16, 40, and 72 hours of treatment. Here, ASA and NaS were used at concentrations of 5 mM, and DF was used at 60 μg ml^{-1} for HH and 120 μg ml^{-1} for MyLa, HuT-78, and SeAx. (**a**, **g**) Cell proliferation was determined by WST-1 assay, and values are expressed as percentage of nontreated controls (C = 100%). (**b**, **h**) Apoptosis was quantified according to propidium iodide staining. Mean values ± SDs of at least six values are shown. (**c**) Cytotoxicity was determined by lactate dehydrogenase release assay, and relative values were calculated with respect to untreated controls (C = 1). (**d**) Representative histograms of DF-treated cells as determined by flow cytometry are shown. Apoptotic sub-G1 (sG1) cells, G1 and G2 populations are indicated. (**e**) Quantification of G2 population is shown for NSAID-treated (16 hours) MyLa cells. The result was highly similar for the three other cell lines (data not shown). (**f**) Cell numbers were determined using a CASY Cell Counter and presented for HH as percentage of nontreated controls (C = 100%). The result was highly similar for MyLa (data not shown). *Indicates statistical significance.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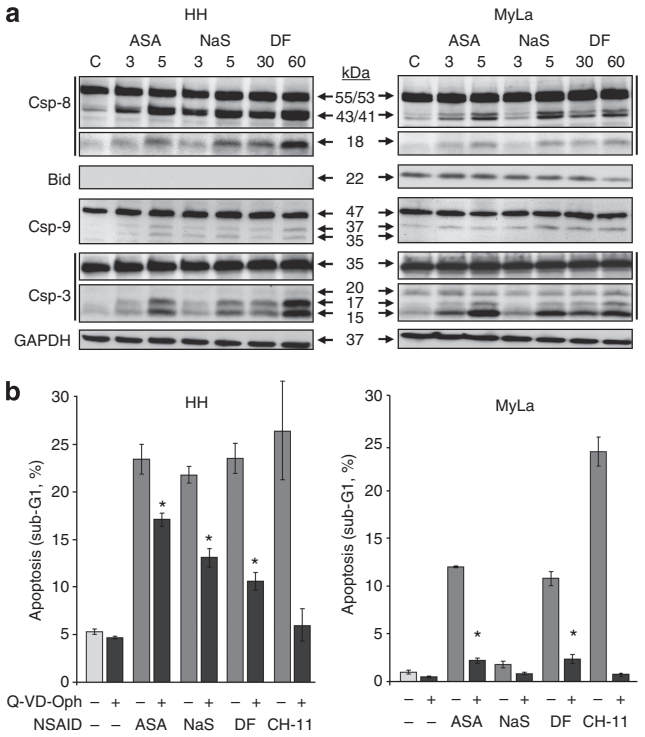

Figure 2. Activation of the caspase (Csp) signaling cascade by nonsteroidal anti-inflammatory drugs. (**a**) Processing of caspases and Bid (western blotting) is shown for HH and MyLa treated for 24 hours with acetylsalicylic acid (ASA; 3, 5 mM), sodium salicylate (NaS; 3, 5 mM), or diclofenac (DF; 30, 60 μg ml^{-1}). Nontreated cells served as controls (C). Equal loading was confirmed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. (**b**) HH and MyLa were pretreated for 1 hour with Q-VD-Oph, followed by treatment with ASA (5 mM), NaS (5 mM), or DF (60 μg ml^{-1}) for additional 24 hours. As a control, cells were treated for 40 hours with an agonistic anti-CD95 antibody (CH-11, 50 ng ml^{-1}). Apoptosis was quantified by propidium iodide staining. Means ± SDs of a representative experiment with triplicate values are shown. Another experiment revealed similar results. *Indicates statistical significance.

NSAID treatment was indicative of additional, proapoptotic but caspase-independent pathways.

Mitochondrial activation and NF-κB signaling

Decreased mitochondrial membrane potential ($\Delta\psi_m$), a hallmark in mitochondrial activation, was seen in HH by all three NSAIDs and in MyLa by ASA and DF (Figure 3a). A lack of $\Delta\psi_m$ decrease, seen in MyLa by NaS, correlated with a limited proapoptotic activity.

For further analysis of intrinsic apoptosis pathways, mitochondrial and cytosolic fractions of HH and MyLa were investigated for cytochrome *c* release and Bax translocation. Cells were treated for 24 hours or for 40 hours, and concentrations were at 5 mM for ASA and NaS or at 60 μg ml^{-1} for DF. In contrast to the general loss of $\Delta\psi_m$, release of cytochrome *c* and Bax translocation was seen only in DF-treated MyLa at 40 and 24 hours (Figure 3c and data not shown). In parallel, increased reactive oxygen species (ROS) levels were evident in DF-treated MyLa (Figure 3d). Thus, full activation of the mitochondrial apoptosis pathway appeared as characteristic for DF-treated MyLa, whereas decrease of $\Delta\psi_m$ in other conditions was indicative of mitochondrial dysfunction.

NF-κB signaling pathways were investigated by monitoring nuclear localization of the NF-κB subunits p65, p50, and p52 by western blot analysis in HH and MyLa cells at 16 hours of treatment. In addition, the DNA-binding capacity was monitored in nuclear extracts by an ELISA for NF-κB family members. Both assays revealed largely comparable data, namely clear downregulation of nuclear p65 in response to NSAID treatment in HH. Similarly, MyLa showed a clear tendency to downregulation of p65 and p52 nuclear levels in response to DF, as well as downregulation of p52 in response to ASA (Figure 3e and f). Thus, NF-κB pathways appeared as downregulated by NSAIDs in CTCL cells. Largely excluding any proapoptotic function of NF-κB in this setting, the I-κB kinase inhibitor BMS-345541 did not reduce apoptosis induction by DF in HH and MyLa cells (Figure 3g).

Critical role of c-FLIP in NSAID-induced apoptosis

For understanding the roles of pro- and antiapoptotic mediators in NSAID-mediated apoptosis, the expression of key regulators was investigated by western blot analysis. Whereas no significant changes were evident for Bax, Bcl-2, and XIAP, the antiapoptotic Bcl-2 protein Mcl-1 was downregulated with higher concentrations of ASA (5 mM) and DF (60 μg ml^{-1}). In addition, the antiapoptotic and pro-proliferative factor survivin was downregulated in HH and MyLa by NaS, which may contribute to its effects in these cells. On the other hand, survivin appeared as upregulated by DF in HH, which may be seen as a counter-regulatory response. The caspase-8/-10 inhibitor c-FLIP appeared to be of particular importance. Both isoforms ($FLIP_{L/S}$) were downregulated in HH and MyLa by ASA and DF. In addition, NaS resulted in a downregulation of $FLIP_S$ in HH (Figure 3h). An even stronger downregulation of c-FLIP isoforms was detected at 40 hours of DF treatment (Figure 5c).

To prove the role of c-FLIP in NSAID-induced apoptosis, viral vectors were used for its overexpression and small hairpin RNA (shRNA) for its downregulation. Apoptosis induction by ASA, NaS, or DF was significantly diminished in MyLa cells when transduced with a retroviral vector for $FLIP_L$ overexpression, whereas mock-transduced cells showed a similar response as parental cells (Figure 4a). Proving the function of c-FLIP, apoptosis by stimulation of CD95 (CH-11 agonistic antibody) was similarly reduced in FLIP-transduced cells (Figure 4a). A high transduction efficiency was monitored by green fluorescent protein (GFP) expression (70–90% GFP^+ cells, Figure 4b), and $FLIP_L$ overexpression was shown by western blot analysis (Figure 4c).

Targeting c-FLIP expression by a lentiviral $cFLIP^{RNAi}$ vector demonstrated the important role of c-FLIP for CTCL cell survival. Strongly enhanced spontaneous apoptosis was characteristic for $cFLIP^{RNAi}$-transduced HH and MyLa cells, as compared with controls. Transduction of a $Scramble^{RNAi}$ construct proved that the transduction itself had only little effect on apoptosis (Figure 4d). Additional effects of NSAIDs could not be examined in these cells, owing to the high

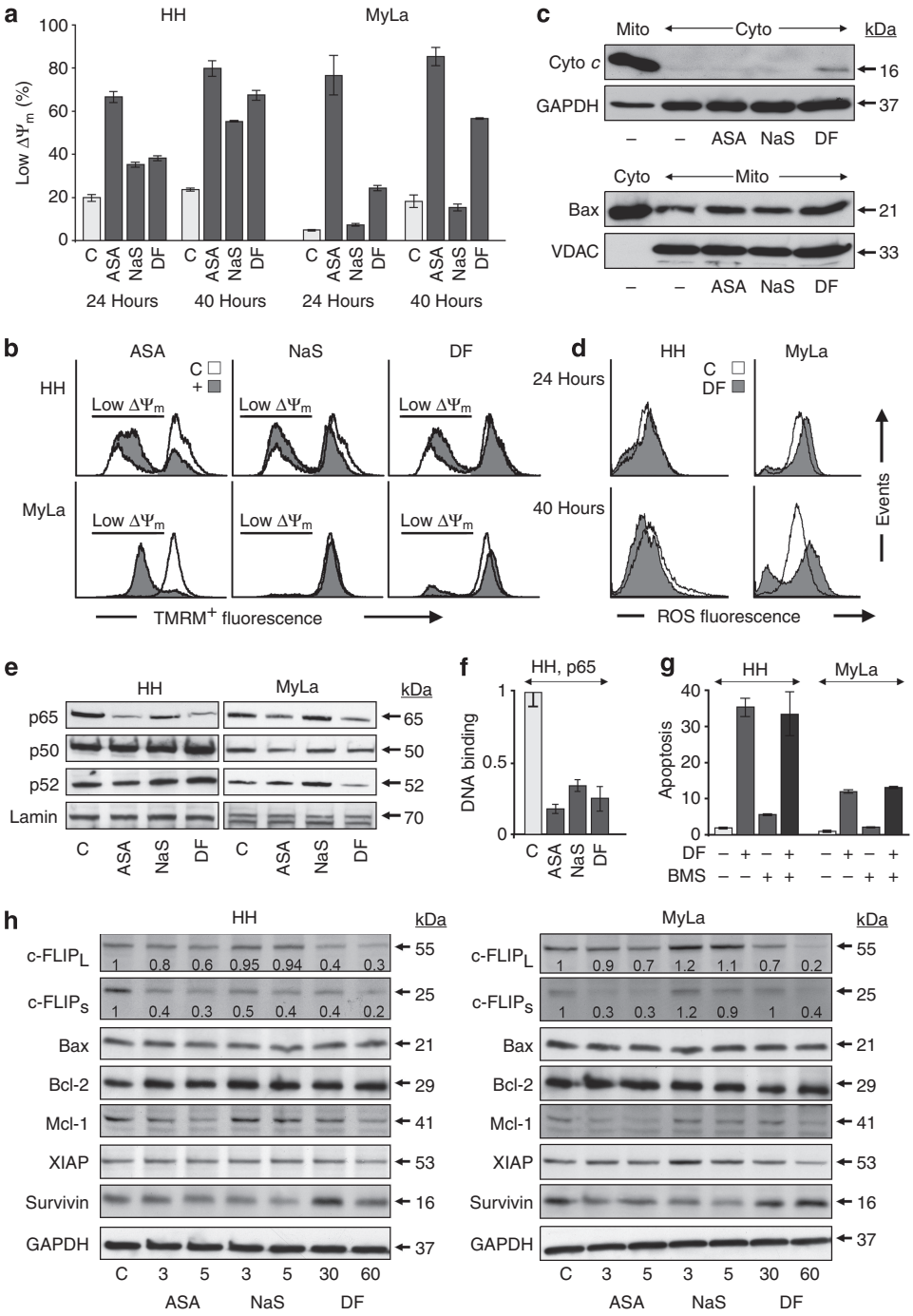

Figure 3. Loss of $\Delta\psi_m$ and downregulation of c-FLIP. (**a**) Loss of $\Delta\psi_m$ in HH and MyLa treated with acetylsalicylic acid (ASA; 5 mM), sodium salicylate (NaS; 5 mM), or diclofenac (DF; 60 μg ml^{-1}) was determined by tetramethylrhodamine methyl ester perchlorate (TMRM$^+$) staining (means ± SDs of at least six individual values). (**b**) Representative histograms of nonsteroidal anti-inflammatory drug (NSAID)-treated cells (gray) in overlays with nontreated controls (C; white). The bar indicates the populations with low $\Delta\psi_m$. (**c**) Mitochondrial (Mito) and cytosolic (Cyto) fractions of MyLa treated with NSAIDs for 40 hours (concentrations as in **a**) were investigated for cytochrome *c* (Cyto *c*) release and Bax translocation. Lane 1: cytosolic and mitochondrial controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proved equal loading of cytosolic extracts and voltage-dependent anion channel (VDAC) excluded contamination of cytosolic extracts with mitochondria. (**d**) Reactive oxygen species (ROS) levels, according to dichlorodihydrofluorescein diacetate staining, are shown in response to DF (60 μg ml^{-1}; repeated twice). (**e**) Nuclear localization of NF-κB subunits (p65, p50, and p52) is shown by western blot analysis in HH and MyLa cells treated for 16 hours with NSAIDs (ASA and NaS 5 mM, DF 60 μg ml^{-1} for HH and 120 μg ml^{-1} for MyLa). Lamin expression and ponceau staining proved equal loading. (**f**) Relative DNA-binding capacity of p65 in nuclear extracts of HH cells treated for 16 hours with NSAIDs is shown as fold change. (**g**) Apoptosis induction in HH and MyLa cells pretreated with BMS-345541 (3 μM) for 3 hours, followed by NSAID treatment for additional 40 hours is shown. Apoptosis was quantified according to propidium iodide staining. Mean values ± SDs of a representative experiment are given. (**h**) Expression of c-FLIP, Bax, Bcl-2, Mcl-1, XIAP, and survivin is shown by western blotting in cutaneous T-cell lymphoma cells treated for 24 hours with ASA (3, 5 mM), NaS (3, 5 mM), and DF (30, 60 μg ml^{-1}). Equal loading was confirmed by GAPDH and c-FLIP expression was quantified by densitometriy (fold change). The whole experiment was performed twice.

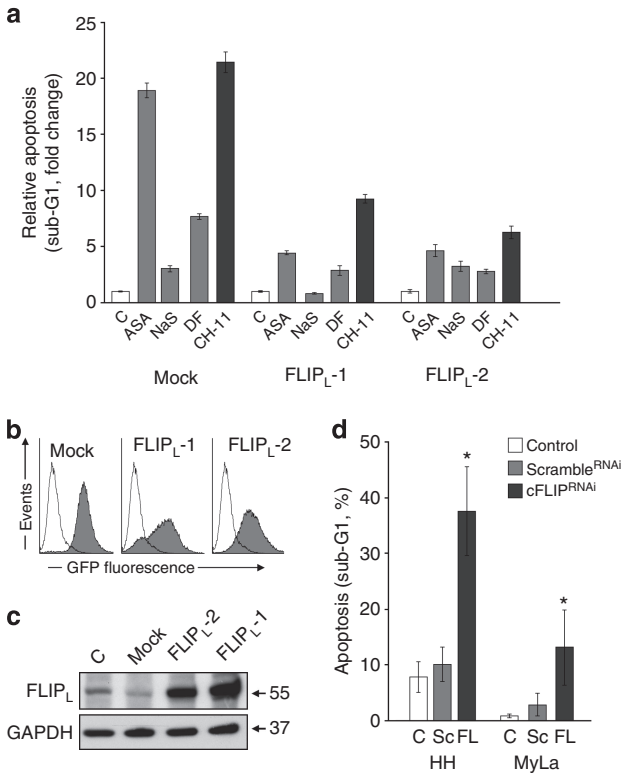

Figure 4. Decreased nonsteroidal anti-inflammatory drug-induced apoptosis after c-FLIP$_L$ overexpression. (**a**) Two independent MyLa cultures stably transduced with c-FLIP$_L$ (FLIP$_L$-1/-2) or mock retrovirus were incubated for 40 hours with acetylsalicylic acid (ASA; 5 mM), sodium salicylate (NaS; 5 mM), and diclofenac (DF; 60 μg ml^{-1}), or with CH-11 agonistic CD95 antibody (50 ng ml^{-1}, 24 hours). Apoptosis was determined by propidium iodide (PI) staining. Values were normalized with regard to nontreated control cells (C = 1). Means ± SDs of a representative experiment (one of two in triplicates) are shown. (**b**) Transduction efficiencies were monitored by green fluorescent protein (GFP; filled graphs) as compared with non-transduced controls (open graphs). (**c**) Overexpression of FLIP$_L$ in transduced MyLa cells was proven by western blot analysis as compared with mock- and non-transduced cells. (**d**) Spontaneous apoptosis (PI staining) of untreated cells in response to transduction with cFLIP^{RNAi} lentivirus (FL) as compared with Scramble^{RNAi} lentivirus (Sc) and non-transduced controls (C). Means ± SDs of two independent experiments, each performed in triplicates, are shown. *Indicates statistical significance. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

background of dying cells. After a few days, no GFP-positive cells remained. These complementary approaches were clearly indicative of the dependency of CTCL cell survival on c-FLIP expression.

Enhancement of TRAIL-induced apoptosis by DF

Death ligands are critical for autoregulation of T lymphocytes. Thus, the expression of TRAIL and TRAIL-R2/DR5 was determined after NSAID treatment. Indeed, TRAIL surface expression was increased in HH cells by all NSAIDs (Figure 5a, upper panel), whereas MyLa cells did not reveal significant TRAIL expression (data not shown). Both cell lines were characterized by strong surface expression of DR5, which, however, did not change significantly upon treatment (Figure 5a, lower panel).

With regard to the resistance of CTCL cells to TRAIL, we investigated whether NSAID treatment might sensitize for TRAIL-induced apoptosis. Indeed, enhanced TRAIL sensitivity was seen in both cell lines after treatment with DF, whereas ASA or NaS were without effect here. Pre-incubation with DF (60 μg ml^{-1}, 24 hours) followed by addition of TRAIL and combined incubation for another 16 hours resulted in 25% and 45% apoptotic cells in MyLa and HH, respectively (Figure 5b).

Enhanced apoptosis was accompanied by enhanced caspase activation, evident by strong processing of caspase-3, -8, and -9. The strongly reduced expression of c-FLIP by DF at 40 hours (Figure 5c) appeared to be of particular importance for the enhanced TRAIL sensitivity.

Correlation of *in vitro* and *ex vivo* data

For proving the impact of the *in vitro* findings for the clinical setting, the responsiveness of tumor T cells isolated from four SzS patients was investigated and compared with CD4(+) control T cells of four healthy donors (both enriched up to 90%). Clinical information of SzS patients is summarized in Table 1. Significantly enhanced apoptosis was seen in tumor T cells treated for 40 hours with ASA (5 mM), NaS (5 mM), and DF (60 μg ml^{-1}), whereas cytotoxicity remained at a low level (Figure 6). A proapoptotic response was also seen in CD4(+) normal T cells, possibly indicative of COX-2-independent effects (Figure 6). A second set of tumor T-cell preparations isolated from the same patients at 2–4 weeks later revealed largely comparable sensitivity (data not shown), thus demonstrating the principle responsiveness of patient's tumor T cells to NSAIDs.

DISCUSSION

Defective apoptosis signaling is critical for therapy resistance and poor prognosis in neoplastic disease, and thus activation of proapoptotic pathways is a primary goal in cancer therapy (Reed and Pellecchia, 2005; Eberle *et al.*, 2007). The death ligand TRAIL is a promising antitumor agent with a preference for apoptosis induction in tumor cells (Newsom-Davis *et al.*, 2009), but induced resistance may limit its clinical applicability. Furthermore, death ligands are of particular significance for lymphomas, because of their important role in controlling lymphocyte self-regulation (Li-Weber and Krammer, 2003; Janssen *et al.*, 2005). Resistance to the CD95/CD95L-mediated control has been reported in CTCL cells and related to impaired TCR signaling and loss of the receptor CD95 (Contassot *et al.*, 2008; Klemke *et al.*, 2009). In previous studies, we have identified the pronounced resistance of CTCL cell lines to TRAIL-induced apoptosis, which was related to enhanced expression of c-FLIP (Braun *et al.*, 2007, 2010).

TRAIL resistance and c-FLIP expression may depend on NF-κB-mediated transcriptional regulation (Micheau *et al.*, 2001), and constitutive activation of NF-κB has been reported in CTCL cells, suggesting c-FLIP and possibly other NF-κB targets as important therapeutic targets (Sors *et al.*, 2006, 2008). As a further example, inhibition of NF-κB was reported to lead to reduced levels of ferritin heavy chain in

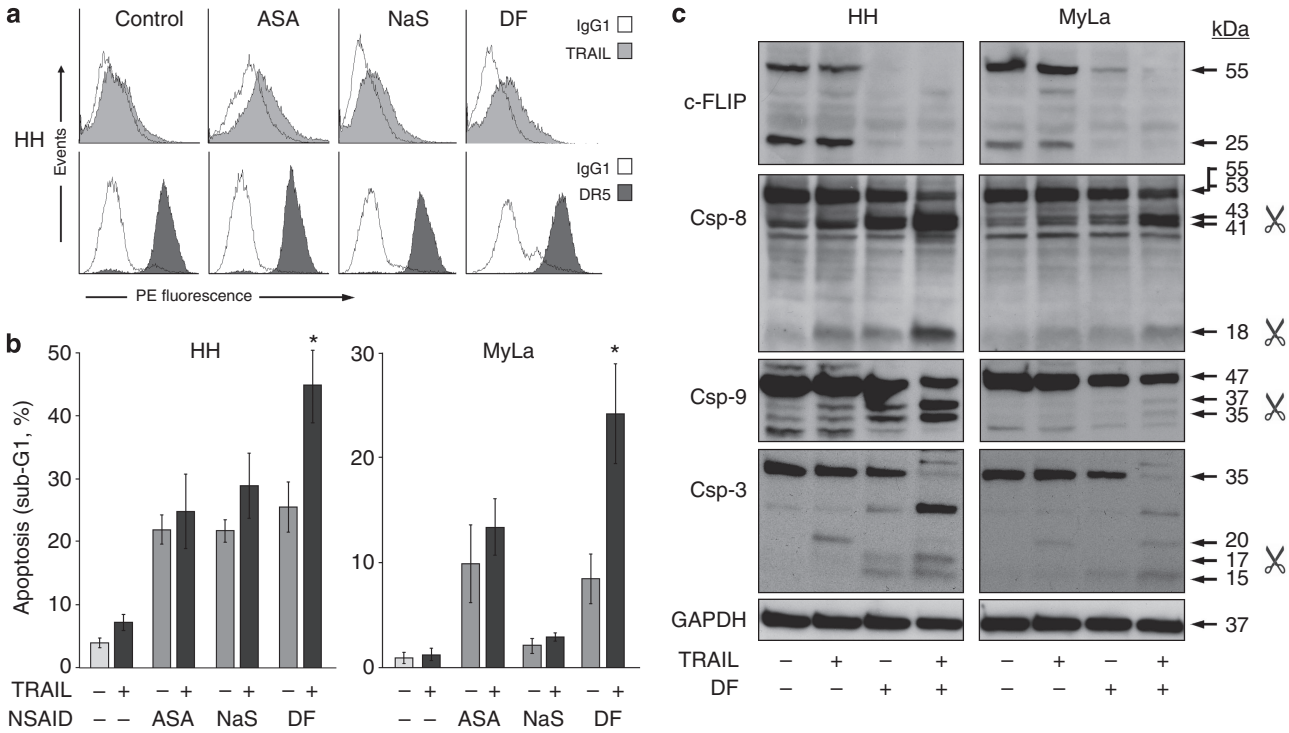

Figure 5. Enhancement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by combination with diclofenac. (**a**) Cutaneous T-cell lymphoma (CTCL) cells were treated for 24 hours with acetylsalicylic acid (ASA; 5 mM), sodium salicylate (NaS; 5 mM), and diclofenac (DF; 60 μg ml^{-1}). Surface expression of TRAIL (filled graphs, upper panel) and TRAIL-R2/DR5 (filled graphs, lower panel) in HH are shown in overlay histograms as compared with isotype controls (open graphs). Two experiments yielded similar results. (**b**) CTCL cells were treated for 40 hours with ASA (5 mM), NaS (5 mM), or DF (60 μg ml^{-1}). TRAIL was added for the last 16 hours, when indicated. Apoptosis was quantified by propidium iodide staining. Mean values ± SDs of a representative experiment are shown (two independent in triplicates). (**c**) Diclofenac and TRAIL-treated HH and MyLa cells were investigated by western blot analysis for caspase activation and c-FLIP expression. Caspase (Csp) cleavage products are indicated as (✂). Equal loading was confirmed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The experiment was repeated once, yielding highly comparable results.
*Indicates statistical significance. NSAID, nonsteroidal anti-inflammatory drugs.

Table 1. SzS patient clinical data

Patient	Age (y)	Sex	Diagnosis	WBC[1]	Tumor cell[2]	Therapy
1	64	F	SzS	30	90	INF, ECP, Ch+Pr, PUVA, MTX, Cyc, Bex+SAHA
2	73	M	SzS	30	99	UV, ECP
3	62	F	SzS	18	90	ECP
4	55	F	SzS	3.1	NA	ECP + INF

Abbreviations: Bex, bexarotene; Ch, chlorambucil; Cyc, cyclophosphamide; ECP, extracorporeal photopheresis; F, female; M, male; MTX, methotrexate; NA, not available; Pr, prednisone; PUVA, psolaren+UV; SAHA, suberoylanilide hydroxamic acid; SzS, Sézary syndrome; WBC, white blood cell; y, years.
[1]WBC count per nanoliter.
[2]Tumor cell load at the time of analysis was determined according to Vbeta staining (patient 1) or according to atypical lymphocytes (Sézary cells; patients 2, 3).

CTCL cells, thus increasing ROS levels to induce apoptosis (Kiessling *et al.*, 2009). As c-FLIP appeared to be of particular significance for TRAIL resistance, we looked for strategies to downregulate c-FLIP, thus restoring TRAIL sensitivity and decreasing lymphoma growth due to a reactivation of self-regulatory programs.

NSAIDs are in clinical use for years, and COX-dependent and independent effects have been attributed to their mode of action (Jana, 2008). Particularly, COX-2 appears as frequently upregulated in tumor cells (Greenhough *et al.*, 2009; Kopp *et al.*, 2010). The antitumor activities of NSAIDs had been recognized for colorectal cancer, and NSAIDs are used to treat patients with familial adenomatous polyposis (Cole *et al.*, 2009).

With regard to T-cell lymphomas, however, solid data on the impact of NSAIDs are still scarce. Recently, COX-2 expression has been proven in CTCL cells, and treatment with the selective COX-2 inhibitor celecoxib resulted in decreased cell growth and viability (Kopp *et al.*, 2010). However, severe side effects such as heart failure have also been reported for

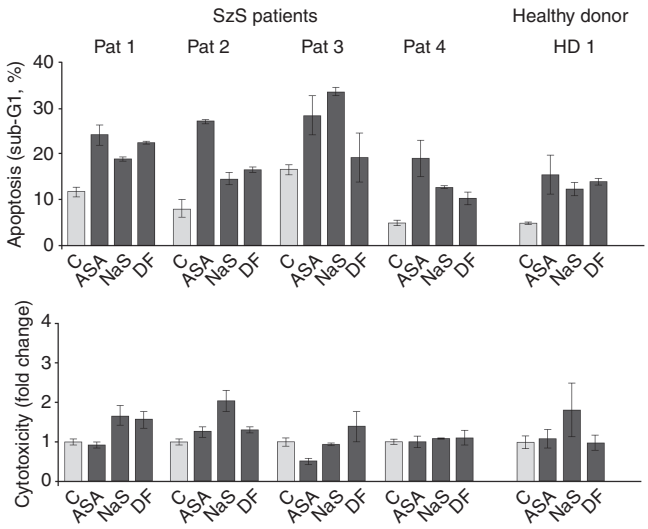

Figure 6. Nonsteroidal anti-inflammatory drugss induce apoptosis in tumor T cells of cutaneous T-cell lymphoma patients. Tumor T cells of Sézary syndrome (SzS) patients (Pat 1–4) and CD4(+) control T cells of healthy donors (HD, one is shown here) were treated for 40 hours with acetylsalicylic acid (ASA; 5 mM), sodium salicylate (NaS; 5 mM), and diclofenac (DF; 60 μg ml^{-1}). Apoptosis was quantified by propidium iodide staining (upper panel). Relative cytotoxicity was determined according to lactate dehydrogenase release. Values were normalized with respect to nontreated controls (C = 1, lower panel). Mean values of triplicates ± SDs are shown.

the selective COX-2 inhibitors, and valdecoxib and rofecoxib had to be recalled from the market (Bresalier *et al.*, 2005; Nussmeier *et al.*, 2005). Thus, nonselective COX inhibitors, namely ASA, NaS, and DF, were applied here in doses comparable to their clinical use. Thus, daily doses of up to 5 g salicylates used in high-dose treatment of type 2 diabetes (Rumore and Kim, 2010) account for a theoretical blood concentration of 1 mg ml^{-1}. On the other hand, 5 mM ASA used in our experiments correspond to almost the same concentration of 0.9 mg ml^{-1}. Of course, because of pharmacokinetics the active concentrations in the blood may be much less, but on the other hand long-term treatment and/or higher concentrations in topical applications may be applied for CTCL.

As for CTCL cell lines, NSAID treatment resulted in strong dose- and time-dependent reduction of cell proliferation, comparable to the effects of sulindac (Zhang *et al.*, 2003). Apoptosis induction, inhibition of cell proliferation, and cell vitality may be related to a common signaling cascade, but they may be self-consistent and cooperative in enhancing the anti-neoplastic efficacy. In some types of cells, apoptosis appeared as the dominant effect, whereas in others inhibition of cell proliferation appeared to have the decisive role.

With regard to the mechanisms of NSAID-induced apoptosis in CTCL cells, indications for the activation of different pathways were seen. Thus, activation of caspase-8 and -3 was evident after treatment with all three NSAIDs, suggesting an involvement of extrinsic apoptosis pathways. Besides, caspase-independent pathways may have contributory roles, as caspase inhibitors did not completely abrogate NSAID-induced apoptosis. Caspase-independent apoptosis by NSAIDs has also been discussed previously (Jana, 2008). However, typically involved mediators such as ROS or the release of mitochondrial factors have not been observed here. The downregulation of survivin detected after NaS treatment may have a contributory role, as related with anti-apoptosis and cell proliferation (Altieri, 2010).

In Jurkat systemic T-cell lymphoma cells, celecoxib-mediated apoptosis was related to mitochondrial activation via Bak, and extrinsic apoptosis pathways had been largely excluded (Jendrossek *et al.*, 2003). Also in CTCL cells, we saw mitochondrial activation as decreased mitochondrial membrane potential. Bax translocation and cytochrome *c* release was seen in DF-treated MyLa. Interestingly, HH cells showed no cytochrome *c* release and Bax translocation, which may be related to their lack of Bid expression (Braun *et al.*, 2007). Thus, high responsiveness of HH may indicate the dominance of extrinsic apoptosis pathways in CTCL cells. The mitochondrial pathway is critically controlled by pro- and antiapoptotic Bcl-2 proteins. Downregulation of Mcl-1 seen here after NSAID treatment may have a role in DF-treated MyLa; however, this was also seen under conditions in which NSAIDs did not induce cytochrome *c* release, and therefore appears not as sufficient for activating the mitochondrial pathway in CTCL cells.

The decreased mitochondrial membrane potential seen here may result from NSAID-mediated uncoupling of the electron transport chain, as had been reported for salicylates in hepatocytes (Chan *et al.*, 2005). As also caspase-9 activation was only weak in CTCL cells in response to NSAIDs, we may conclude that mitochondrial pathways were less important here.

Because of the dominant role of c-FLIP in apoptosis resistance of CTCL cells (Braun *et al.*, 2007), its general downregulation after NSAID treatment was of particular interest. In addition, in cutaneous squamous cell carcinoma (SCC) cell lines, downregulation of c-FLIP in response to DF had been seen (Fecker *et al.*, 2010). Strongly supporting an important role of c-FLIP downregulation in NSAID-mediated apoptosis in CTCL cells, its overexpression diminished apoptosis induction and in contrast its downregulation by RNA interference (RNAi) significantly induced spontaneous apoptosis. CTCL cells were also not able to survive more than a few days. These complementary approaches strengthened the particular role of c-FLIP for survival of CTCL cells.

As c-FLIP is a known target of NF-κB (Micheau *et al.*, 2001), this pathway might also be of importance for NSAID-induced apoptosis in CTCL cells. NSAIDs may indeed interfere with NF-κB signaling pathways as shown for salicylates and DF, which may bind and block I-κB kinase, thus preventing NF-κB activation (Kopp and Ghosh, 1994; Fredriksson *et al.*, 2011). NSAIDs may also interfere with proteasome functions, thus leading to a stabilization of I-κB (inhibitor of NF-κB; (Dikshit *et al.*, 2006). In agreement with these reports, our study revealed an inhibition of NF-κB signaling in CTCL cells by NSAIDs.

In line with the downregulation of c-FLIP, there was a clear sensitization for TRAIL-induced apoptosis by DF, thus showing a suitable strategy for overcoming TRAIL resistance

of CTCL. In addition, in other cell types, such as in cutaneous SCC cells, DF enhanced death ligand sensitivity (Fecker *et al.*, 2010). The fact that NaS and ASA did not enhance TRAIL sensitivity indicates principle differences in the mode of action of these drugs. Expression of TRAIL itself was upregulated in HH cells by NSAID treatment, which may further enhance extrinsic induction of apoptosis.

As a further step for proving the clinical applicability of NSAIDs in CTCL, significant responsiveness of SzS tumor T cells, treated *ex vivo*, is demonstrated here. Some sensitivity also seen in normal CD4(+) T cells might be indicative of possible side effects, which may have to be compared with the antitumor efficacy in clinical trials. Some NSAIDs are already approved for treatment of different cancer entities, such as DF for actinic keratosis (Ulrich *et al.*, 2010), whereas others are used in clinical trials, such as ASA for breast cancer (Agrawal and Fentiman, 2008) or celecoxib for colon carcinoma (Half and Arber, 2009). Overall, we demonstrate that NSAIDs exert substantial antitumor activities in CTCL cells by activation of caspases, downregulation of c-FLIP, upregulation of TRAIL, as well as by affecting cell proliferation. Taking into account the extensive experience with NSAIDs and a limited side effect profile, we may suggest the consideration of using NSAIDs alone or in combinations for therapy of CTCL.

MATERIALS AND METHODS

Cell culture

The CTCL cell lines MyLa (Kaltoft *et al.*, 1992), HH (Starkebaum *et al.*, 1991; CRL-2105, ATCC, Manassas, VA), HuT-78 (Gootenberg *et al.*, 1981), and SeAx (Kaltoft *et al.*, 1987) were grown in RPMI with L-glutamine and 10% fetal calf serum at 37 °C, 5% CO_2. For apoptosis induction, KillerTRAIL (Alexis, Gruenberg, Germany, 20 ng ml^{-1}), agonistic anti-CD95 antibody (CH-11, Beckman Coulter, Krefeld, Germany; 50 ng ml^{-1}), ASA (Caelo, Hilden, Germany, 1–5 mM, corresponding to 0.18–0.9 mg ml^{-1}), NaS (Sigma-Aldrich, Taufkirchen, Germany; 1–5 mM), and DF (DF, Sigma, 5–120 μg ml^{-1}) were used. Treatments were started at 24 hours after seeding. The caspase inhibitor Q-VD-Oph (R&D Systems, Minneapolis, MN) was used at 50 μM (HH) and 10 μM (MyLa). For inhibition of NF-κB signaling, the I-κB kinase allosteric site inhibitor BMS-345541 (B9935; Sigma) was used at 3 μM with a pretreatment of 3 hours, before NSAID treatment was started.

Apoptosis, cytotoxicity, and cell proliferation

Hypodiploid cells were quantified by flow cytometry (FACSCalibur BD Biosciences, San Diego, CA) after propidium iodide staining (Riccardi and Nicoletti, 2006). Cells were harvested by centrifugation and stained for 1 hour with propidium iodide (40 μg ml^{-1} in 0.1% sodium citrate, 0.1% Triton X-100). Data were evaluated by CellQuest (BD Biosciences) or WinMDI software (Scripps Research Institute, La Jolla, CA). Cytotoxicity was determined according to lactate dehydrogenase release using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany). Cell survival and proliferation was quantified by the WST-1 assay (Roche Diagnostics) according to mitochondrial enzyme activity. Cells were seeded in 96-well plates (15,000 cells per 100 μl). After addition of WST-1 reagent, the absorbance at 450 nm was determined in an ELISA reader. Data were reported in percentage of nontreated controls. Direct cell counts were determined with a CASY Cell Counter (TTC model, Schärfe System, Reutlingen, Germany). Data were reported in percentage of nontreated controls.

Western blot analysis

Detailed protocols for protein extraction and western blot analysis were described previously (Eberle *et al.*, 2003). Primary antibodies purchased from Cell Signaling (Danvers, MA): caspase-8 (9746, 1:1,000); caspase-9 (9502, 1:1,000); active caspase-3 (9661, 1:1,000); procaspase-3 (9662, 1:1,000); Bid (2002, 1:1,000); XIAP (2042, 1:1,000); Lamin A/C (2032, 1:1,000). Antibodies obtained from Santa Cruz (Heidelberg, Germany): c-FLIP (sc-5276, 1:200); Bax (sc-493, 1:200); Bcl-2 (sc-509, 1:200); Mcl-1 (sc-12756, 1:200); Survivin (sc-17779, 1:200); GAPDH (sc-32233, 1:200); p65 (sc-109, 1:200); p50 (sc-1191, 1:200); p52 (sc-7386, 1:200). Antibodies such as cytochrome *c* (BD Biosciences, 1:1,000) and VDAC (Calbiochem, Nottingham, UK, 529536, 1:5,000) were also used. As secondary antibodies, horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse were used (Dako Cytomation, Hamburg, Germany, 1:5,000). The quantification of c-FLIP expression was obtained by densitometry.

TRAIL and TRAIL-R2/DR5 surface expression

Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), stained, and analyzed by flow cytometry. Aliquots of 1×10^6 cells in 100 μl PBS with 1% BSA were incubated for 30 minutes in the dark with PE-conjugated TRAIL antibody (RIK2, Santa Cruz, 1:5). PE-labeled isotype IgG1 antibody was used (555749, BD Pharmingen, BD Bioscience, San Diego, CA) as control. For determination of TRAIL-R2/DR5 surface expression, cells were harvested as described above. After washing with PBS, aliquots of 1×10^6 cells in 100 μl PBS, 1% BSA were incubated for 30 minutes with mAb against TRAIL-R2/DR5 (Alexis, clone HS 201, 1:100). An isotypic monoclonal mouse IgG1 antibody (Alexis, clone MOPC31C, 278-010, 1:100) was used as negative control. After washing cells twice with PBS, they were incubated for 30 minutes with a secondary phycoerythrin-labeled goat anti-mouse IgG1 antiserum (Alexis, 211-201-C050; 1:100).

Mitochondrial activation, ROS, and NF-κB

For determination of cytochrome *c* release and Bax translocation to mitochondria, cells were fractionated by a mitochondria/cytosol fractionation kit (ALX-850-276, Alexis). For determination of the mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry, harvested cells were stained with fluorescent tetramethylrhodamine methyl ester perchlorate ($TMRM^+$, Sigma; 1 μM, 15 minutes, 37 °C). For determination of intracellular ROS by flow cytometry, harvested cells were stained with fluorescent 2,7-dichlorodihydrofluorescein diacetate (Invitrogen, Eugene, OR, 50 μM, 15 minutes, 37 °C).

Nuclear translocation of NF-κB subunits (p65, p50, and p52) was determined by western blot analysis. The DNA-binding capacity of NF-κB subunits was determined by an ELISA (TransAM NF-κB family ELISA kit; Active Motif, Rixensart, Belgium). Cells (1.5×10^5 well) were seeded in a culture flask and were treated with NSAIDs for 16 hours. Nuclear extracts were prepared with a nuclear extraction kit (40010, Active Motif). Protein content was quantified using a protein assay (BCA, Pierce, Bonn, Germany). Equal amounts of nuclear proteins (6 μg) were placed in 96-well plates coated with

oligonucleotides covering the NF-κB consensus sequence. Binding of NF-κB subunits to the immobilized oligonucleotides was detected by subunit-specific antibodies, followed by immunostaining with horseradish peroxidase-labeled secondary antibodies. The chromogenic reaction was measured at 450 nm in an ELISA reader.

Viral vectors

Previously described lentiviral vectors were used for c-FLIP shRNA and control scramble shRNA expression (Rubinson *et al.*, 2003; Braun *et al.*, 2010). Cell transduction had been described previously (Al Yacoub *et al.*, 2008). A retroviral vector was used for c-FLIP$_L$ overexpression (Geserick *et al.*, 2008), which was kindly provided by Leverkus (Mannheim, Germany). Stably transduced MyLa cells were isolated for zeocin resistance (ant-zn-1, InvivoGen, San Diego, CA; 100–500 μg ml^{-1}), because of a resistance gene encoded by the retroviral vector. Transduction efficiencies were determined by flow cytometric analysis for GFP.

Isolation and enrichment of tumor T cells from SzS patients

Peripheral blood mononuclear cells from venous blood collected from SzS patients and healthy donors were isolated using density-gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden). Tumor T cells were identified by sequencing of the dominant genomic TCR-β rearrangement and were isolated using fluorochrome-conjugated Vβ chain-specific antibodies (Beckman Coulter), followed by incubation with anti-fluorochrome microbeads (130-048-801, Miltenyi Biotec, Bergisch Gladbach, Germany). Control T cells from healthy donors were enriched according to CD4 expression (130-045-101, Miltenyi Biotec). Purity of enriched cells was determined by flow cytometry using antibodies for CD3, CD4, and CD8 (BD Biosciences). Blood samples of four SzS patients were used (one male, three female; mean age: 63 years). The study was conducted according to the Declaration of Helsinki Principles. Patients had given written, informed consent on the use of the blood samples for scientific purposes. The collection and use of the probes was approved by the Ethical Committee of the Charité—Universitätsmedizin Berlin.

Statistics

Experiments usually consisted of triplicate values, and were performed 2–3 times. Statistical significance was determined by Student's *t*-test and is indicated in the figures by asterisks ($P<0.05$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Agrawal A, Fentiman IS (2008) NSAIDs and breast cancer: a possible prevention and treatment strategy. *Int J Clin Pract* 62:444–9

Altieri DC (2010) Survivin and IAP proteins in cell-death mechanisms. *Biochem J* 430:199–205

Al Yacoub N, Romanowska M, Krauss S *et al.* (2008) PPARdelta is a type 1 IFN target gene and inhibits apoptosis in T cells. *J Invest Dermatol* 128:1940–9

Braun FK, Fecker LF, Schwarz C *et al.* (2007) Blockade of death receptor-mediated pathways early in the signaling cascade coincides with distinct apoptosis resistance in cutaneous T-cell lymphoma cells. *J Invest Dermatol* 127:2425–37

Braun FK, Hirsch B, Al Yacoub N *et al.* (2010) Resistance of cutaneous anaplastic large-cell lymphoma cells to apoptosis by death ligands is enhanced by CD30-mediated overexpression of c-FLIP. *J Invest Dermatol* 130:826–40

Bresalier RS, Sandler RS, Quan H *et al.* (2005) Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 352:1092–102

Castellone MD, Teramoto H, Williams BO *et al.* (2005) Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. *Science* 310:1504–10

Cha YI, Dubois RN (2007) NSAIDs and cancer prevention: targets downstream of COX-2. *Annu Rev Med* 58:239–52

Chan K, Truong D, Shangari N *et al.* (2005) Drug-induced mitochondrial toxicity. *Expert Opin Drug Metab Toxicol* 1:655–69

Clevers H (2006) Colon cancer—understanding how NSAIDs work. *N Engl J Med* 354:761–3

Cole BF, Logan RF, Halabi S *et al.* (2009) Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. *J Natl Cancer Inst* 101:256–66

Contassot E, Gaide O, French LE (2007) Death receptors and apoptosis. *Dermatol Clin* 25:487–501

Contassot E, Kerl K, Roques S *et al.* (2008) Resistance to FasL and tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in Sezary syndrome T-cells associated with impaired death receptor and FLICE-inhibitory protein expression. *Blood* 111:4780–7

Dikshit P, Chatterjee M, Goswami A *et al.* (2006) Aspirin induces apoptosis through the inhibition of proteasome function. *J Biol Chem* 281: 29228–35

Eberle J, Fecker LF, Forschner T *et al.* (2007) Apoptosis pathways as promising targets for skin cancer therapy. *Br J Dermatol* 156 (Suppl 3):18–24

Eberle J, Fecker LF, Hossini AM *et al.* (2003) CD95/Fas signaling in human melanoma cells: conditional expression of CD95L/FasL overcomes the intrinsic apoptosis resistance of malignant melanoma and inhibits growth and progression of human melanoma xenotransplants. *Oncogene* 22:9131–41

Fecker LF, Stockfleth E, Braun FK *et al.* (2010) Enhanced death ligand-induced apoptosis in cutaneous SCC cells by treatment with diclofenac/hyaluronic acid correlates with downregulation of c-FLIP. *J Invest Dermatol* 130:2098–109

Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 10:76–100

Fredriksson L, Herpers B, Benedetti G *et al.* (2011) Diclofenac inhibits tumor necrosis factor-alpha-induced nuclear factor-kappaB activation causing synergistic hepatocyte apoptosis. *Hepatology* 53:2027–41

Geserick P, Drewniok C, Hupe M *et al.* (2008) Suppression of cFLIP is sufficient to sensitize human melanoma cells to TRAIL and CD95L-mediated apoptosis. *Oncogene* 27:3211–20

Gootenberg JE, Ruscetti FW, Mier JW *et al.* (1981) Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. *J Exp Med* 154:1403–18

Greenhough A, Smartt HJ, Moore AE *et al.* (2009) The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 30:377–86

Half E, Arber N (2009) Colon cancer: preventive agents and the present status of chemoprevention. *Expert Opin Pharmacother* 10:211–9

Irmler M, Thome M, Hahne M *et al.* (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* 388:190–5

Jana NR (2008) NSAIDs and apoptosis. *Cell Mol Life Sci* 65:1295–301

Janssen EM, Droin NM, Lemmens EE *et al.* (2005) CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434:88–93

Jendrossek V, Handrick R, Belka C (2003) Celecoxib activates a novel mitochondrial apoptosis signaling pathway. *FASEB J* 17:1547–9

Kaltoft K, Bisballe S, Dyrberg T *et al.* (1992) Establishment of 2 continuous T-cell strains from a single plaque of a patient with mycosis-fungoides. *In Vitro Cell Dev Biol* 28:161–7

Kaltoft K, Bisballe S, Rasmussen HF *et al.* (1987) A continuous T-cell line from a patient with Sezary syndrome. *Arch Dermatol Res* 279:293–8

Kiessling MK, Klemke CD, Kaminski MM *et al.* (2009) Inhibition of constitutively activated nuclear factor-kappaB induces reactive oxygen species- and iron-dependent cell death in cutaneous T-cell lymphoma. *Cancer Res* 69:2365–74

Klemke CD, Brenner D, Weiss EM *et al.* (2009) Lack of T-cell receptor-induced signaling is crucial for CD95 ligand up-regulation and protects cutaneous T-cell lymphoma cells from activation-induced cell death. *Cancer Res* 69:4175–83

Kopp E, Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 265:956–9

Kopp KL, Kauczok CS, Lauenborg B *et al.* (2010) COX-2-dependent PGE(2) acts as a growth factor in mycosis fungoides (MF). *Leukemia* 24:1179–85

Krammer PH, Arnold R, Lavrik IN (2007) Life and death in peripheral T cells. *Nat Rev Immunol* 7:532–42

Li HL, Zhu H, Xu CJ *et al.* (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94:491–501

Li-Weber M, Krammer PH (2003) Function and regulation of the CD95 (APO-1/Fas) ligand in the immune system. *Semin Immunol* 15:145–57

Mathas S, Lietz A, Anagnostopoulos I *et al.* (2004) c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med* 199:1041–52

Micheau O, Lens S, Gaide O *et al.* (2001) NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* 21:5299–305

Muller AM, Ihorst G, Mertelsmann R *et al.* (2005) Epidemiology of non-Hodgkin's lymphoma (NHL): trends, geographic distribution, and etiology. *Ann Hematol* 84:1–12

Newsom-Davis T, Prieske S, Walczak H (2009) Is TRAIL the holy grail of cancer therapy? *Apoptosis* 14:607–23

Nussmeier NA, Whelton AA, Brown MT *et al.* (2005) Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery. *N Engl J Med* 352:1081–91

Reed JC (2008) Bcl-2-family proteins and hematologic malignancies: history and future prospects. *Blood* 111:3322–30

Reed JC, Pellecchia M (2005) Apoptosis-based therapies for hematologic malignancies. *Blood* 106:408–18

Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 1:1458–61

Rubinson DA, Dillon CP, Kwiatkowski AV *et al.* (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 33:401–6

Rumore MM, Kim KS (2010) Potential role of salicylates in type 2 diabetes. *Ann Pharmacother* 44:1207–21

Samuelsson B, Morgenstern R, Jakobsson PJ (2007) Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacol Rev* 59:207–24

Sors A, Jean-Louis F, Begue E *et al.* (2008) Inhibition of IkappaB kinase subunit 2 in cutaneous T-cell lymphoma down-regulates nuclear factor-kappaB constitutive activation, induces cell death, and potentiates the apoptotic response to antineoplastic chemotherapeutic agents. *Clin Cancer Res* 14:901–11

Sors A, Jean-Louis F, Pellet C *et al.* (2006) Down-regulating constitutive activation of the NF-kappaB canonical pathway overcomes the resistance of cutaneous T-cell lymphoma to apoptosis. *Blood* 107:2354–63

Starkebaum G, Loughran TP, Waters CA *et al.* (1991) Establishment of An Il-2 independent, human T-cell line possessing only the P70 Il-2 receptor. *Int J Cancer* 49:246–53

Ulrich M, Drecoll U, Stockfleth E (2010) Emerging drugs for actinic keratosis. *Expert Opin Emerg Drugs* 15:545–55

Willemze R, Jaffe ES, Burg G *et al.* (2005) WHO-EORTC classification for cutaneous lymphomas. *Blood* 105:3768–85

Zhang CL, Kamarashev J, Qin JZ *et al.* (2003) Expression of apoptosis regulators in cutaneous T-cell lymphoma (CTCL) cells. *J Pathol* 200: 249–54