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Central Signaling Elements of Intercellular Reactive Oxygen/Nitrogen Species-dependent Induction of Apoptosis in Malignant Cells

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Abstract. *Intercellular reactive oxygen/reactive nitrogen species-(ROS/RNS)-dependent induction of apoptosis in malignant cells is discussed as a potential control step during oncogenesis. In previous studies, the mechanism of intercellular apoptosis-inducing signaling was mainly established through the use of specific inhibitors and scavengers. Here, a detailed analysis was carried out based on small interfering ribonucleic acid (siRNA)-mediated knockdown of central players of intercellular ROS/RNS signaling and of the mitochondrial and the FAS receptor-dependent pathway of apoptosis. The data show that transforming growth factor β1, transforming growth factor β receptor, NADPH oxidase-1 (NOX1), NOX1 organizer, and NOX1 activator control the HOCl and the NO/peroxynitrite signaling pathways. Dual oxidase-1 (DUOX1) is specifically involved in HOCl signaling, and NO synthase in NO/peroxynitrite signaling. Both pathways utilize intracellular signal transduction through protein kinase C zeta, sphingomyelinase and central elements of the mitochondrial pathway of apoptosis, whereas the FAS receptor and FAS ligand do not seem to play a role.*

Selective induction of apoptosis in transformed cells is established either through their interaction with neighboring non-transformed cells or through autocrine apoptotic self-destruction (1-3). Thereby, the HOCl (2, 5) and the nitric oxide/peroxynitrite (NO/PON) signaling pathways (2, 5, 6) are the dominant intercellular apoptosis-inducing signaling pathways [reviewed in (5)]. Both pathways are driven by superoxide anions which are generated by membrane-associated NADPH oxidase-1 (NOX1) of malignant cells (4, 7) and result in the generation of hydroxyl radicals that induce apoptosis through lipid peroxidation in the cell membrane (8). Mechanisms and the multiple modes of interaction of these pathways have been elucidated through inhibitor and reconstitution experiments [reviewed in (4, 5, 9)].

Elimination of transformed cells through selective apoptosis induction mediated by intercellular reactive oxygen species (ROS)-dependent signaling has been discussed as a potential natural tumor-preventive mechanism (4, 10, 11). This concept is in agreement with the pioneering work of Deichman *et al.* , who have shown that tumor progression requires establishment of resistance to H_2O_2 (12, 13). Resistance is achieved through the expression of membrane-associated catalase (8, 14, 15). Catalase interferes with the HOCl signaling pathway through decomposition of H_2O_2 and with NO/PON signaling through oxidation of NO and decomposition of PON (5, 8, 16, 17). Recent quantitative characterization of membrane-associated catalase has shown that localization of catalase on the outside of cells is already associated with the transformed state of the cells, however, at a concentration that is not sufficient to prevent intercellular ROS-dependent signaling (15). Catalase seems to be released by tumor cells and is covalently attached to the cells through the action of transglutaminase-2 (15).

In addition to membrane-associated catalase, tumor cells also carry superoxide dismutase (SOD) on their membrane. SOD has a co-modulatory role through preventing superoxide anion-dependent inhibition of catalase (9, 18).

The functional complex of membrane-associated NOX1 and protective catalase and SOD represents a regular characteristic of tumor cells and therefore is a focus for the establishment of novel therapeutic approaches (18-20). The complex signaling chemistry of transformed cells, or tumor cells after inhibition of their membrane-associated catalase, has, so far, been mainly elucidated through inhibitor studies and reconstitution experiments. Small interfering ribonucleic acid (siRNA)-based knockdown of defined

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potential candidates of signaling-relevant molecules as an alternative method for the stringent characterization of essential components of the ROS-driven apoptotic pathway has only been used for the dissection of the roles of NOX1 and dual oxidase-1 (DUOX1) (3). Here an endeavor was made to present a comprehensive picture of inter- and intracellular modulators of ROS-driven intercellular apoptosis induction in malignant cells based on siRNA-mediated analysis.

Materials and Methods

Materials. The NOX1 inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), the catalase inhibitor 3-aminotriazole (3-AT), arginine, catalase from bovine liver, NaOCl (for the generation of HOCl), the fast decaying NO donor diethylamine NONOate (DEA NONOate), the broad-spectrum matrix metalloproteinase (MMP) inhibitor (R)-*N*4-hydroxy-*N*1-[(*S*)-2-(1*H*-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinamide (galardin; GM6001), myeloperoxidase (MPO) from human leukocytes, the inhibitor of NO synthase (NOS) *N*-omega-nitro-L-arginine methylester hydrochloride (L-NAME), the HOCl scavenger taurine, the NO donor sodium nitroprusside (SNP) and superoxide dismutase (SOD) from bovine liver were obtained from Sigma Aldrich (Schnelldorf, Germany).

The PON decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) was obtained from Calbiochem/Merck Biosciences GmbH, Schwalbach, Germany.

The catalase mimetic EUK-8 [manganese *N,N'-bis*(salicylidene) ethylenediamine chloride] was obtained from Cayman chemicals (Ann Arbor, MI, USA) through Merck Biosciences GmbH, Schwalbach/Ts, Germany.

The mechanism-based peroxidase (POD) inhibitor 4-aminobenzoyl hydrazide (ABH) was obtained from Acros Organics, Geel, Belgium.

Transforming growth factor β1 (TGFβ1) was purified from human platelets (21) and kept as a stock solution of 1.5 μg/ml in Eagle's minimum essential medium (EMEM) plus 5% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) at −20˚C.

Media for cell culture. Cells were either kept in EMEM containing 5% FBS, or in RPMI-1640 medium containing 10% FBS, as indicated for the respective cell lines. FBS was heated for 30 minutes at 56˚C prior to use. Both media were supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). All supplements were obtained from Biochrom. Cell culture was performed in plastic tissue culture flasks. Cells were passaged once or twice weekly.

Cells. Nontransformed rat fibroblasts 208F and their derivative transformed through constitutive expression of v-src (208Fsrc3) were a generous gift from Dr C. Sers and Dr R. Schäfer, Berlin, Germany and were cultured in EMEM with 5% FBS and supplemented as indicated above.

The gastric carcinoma cell line MKN-45 was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany. Cells were grown in suspension, with some cells attaching to the plastic culture dish, in RPMI-1640, with 10% serum and supplements as described above. Care was taken to avoid cell densities below 300,000/ml and above 10^6/ml.

The human neuroblastoma cell line SHEP was obtained from Dr. J. Roessler, Department of Pediatrics and Adolescent Medicine, University Medical Centre Freiburg, Germany and was cultured in EMEM with 5% FBS and supplemented as indicated above.

Knockdown by treatment with specific siRNAs. All siRNAs were obtained from Qiagen (Hilden, Germany). The siRNAs directed towards human targets are detailed in Table I, including the respective target sequence and the order information. The sequences of custom made siRNAs are presented in Table II.

SiNOX1-a and siNOX1-b are variants of siRNAs directed to human NADPH oxidase-1. When only of variant of siNOX1 was applied, variant siNOX1-a was used and is termed "siNOX1" in the respective figures. SiDUOX1-a and siDUOX1-b are variants of siRNAs directed to human dual oxidase-1. When only one variant of siDUOX-1 was applied, variant siDUOX1-a was used and is termed "siDUOX1" in the respective figures. No sequence information was available for siRNA directed towards epidermal growth factor receptor (siEGFR) (Hs_EGFR_12 validated siRNA). In the experiment described in Figure 1, two custom-made variants of siRNA directed towards murine inducible NO synthase (siiNOS-a and siiNOS-b) were used. (siiNOS-a: target sequence: CCC GGA GCC TTT AGA CCT CAA, sense: r(CGG AGC CUU UAG ACC UCA A)dTdT; antisense: r(UUG AGG UCU AAA GGC UCC G)dGdG; siiNOS-b: target sequence:CCG ATT TAG AGT CTT GGT GAA, sense: r(GAU UUA GAG UCU UGG UGA A)dTdT; antisense: r(UUC ACC AAG ACU CUA AAU C)dGdG).

SiRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 μM. Suspensions were heated at 90˚C for 1 minute, followed by incubation at 37˚C for 60 min. Aliquots were stored at −20˚C.

Before transfection, 88 μl of medium without serum and without antibiotics were mixed with 12 μl Hyperfect solution (Qiagen) and the required volume of specific siRNA or control siRNA to reach the desired concentration of siRNA during transfection (the standard concentration of siRNA was 24 nM for MKN-45 cells and 10 nM for 208Fsrc3 cells). The mixture was treated by a vortex mixer for a few seconds and then allowed to sit for 10 min. It was then gently and slowly added to 300,000 MKN-45 cells in 1 ml RPMI-1640 medium containing 10% FBS and antibiotics (12-well plates) or to 200,000 208Fsrc3 cells/well in 2.3 ml medium supplemented with 5% FBS and antibiotics (6-well plates). The cells were incubated at 37˚C in 5% CO_2 for 24 h. Transfected cells were centrifuged and resuspended in fresh medium at the required density before use.

Determination of the efficiency of siRNA-mediated knockdown. The siRNA transfection system as described above had been optimized to allow a reproducible transfection efficiency of more than 95% of the cells and to avoid toxic effects (Bauer, unpublished data).

The efficiency of specific knockdown of transforming growth factor β1 (*TGFβ1*), its receptor *TGFβR*, protein kinase C zeta (*PKC zeta*), bcl-2 homologous antagonist/killer (*BAK*), mitochondria-derived activator of caspases (*DIABLO*), epidermal growth factor receptor (*EGFR*), voltage-dependent anion channel (*VDAC*), apoptosis protease activating factor (*APAF*), caspase-3, caspase-8, caspase-9 and *FASR* mRNA had been experimentally determined by the supplier, using real-time quantitative polymerase chain reaction and was found to be more than 90% for 5 nM siRNA.

The efficiency of knockdown of *NOX1*, *DUOX1* and catalase was based on functional quantitative assays and was more than 90% (3,

Table I. *Target sequences for siRNAs directed towards human targets.*

siRNA	Target	Target sequence	Order information
siCo	Control (no target)	AAT TCT CCG AAC GTG TCA CGT	Control siRNA
siNOX1-a	NADPH oxidase-1	CCG ACA AAT ACT ACT ACA CAA	Custom made
siNOX1-b	NADPH oxidase-1	CAG GTT TGA GCA GTC ACT TTA	Custom made
siNOX3	NADPH oxidase-3	CTG GTG AAT AAT AAT TAA CTA	Hs_NOX3_1_HP siRNA
siNOX4	NADPH oxidase-4	CAA GAT GAC CGT CAC ATT ACA	Hs_NOX4_2_HP siRNA
siNOX5	NADPH oxidase-5	GAG GAG TGT GAC AAT GAG AAA	Hs_NOX5_2_HP siRNA
siNOXO1	NADPH oxidase organizer-1	CTG CAG CTG TTG GAA ACC TAT	Hs_NOXO1_2_HP siRNA
siNOXA1	NADPH oxidase activator-1	CAG GTG GAG CAA GTT GGC AAA	Hs_NOXA1_1_HP siRNA
siDUOX1-a	Dual oxidase-1	CCA GTC TAA CAC CAC AAC TAA	Custom made
siDUOX1-b	Dual oxidase-1	CCC GGG CAG ATC CGT GTG GTA	Custom made
siiNOS2	Inducible NO synthase-2	CTG GGC CGT GCA AAC CTT CAA	Custom made
sinNOS	Neuronal NO synthase	CAC AAG TGT GTC GAT CTT AGA	Custom made
siTGFβ1	Transforming growth factor β1	CAG CAT ATA TAT GTT CTT CAA	Hs_TGFB1_6_HP Validated
siTGFβR	Transforming growth factor β receptor	TCG GTT AAT AAC GAC ATG ATA	Hs_TGFBR2_7_HP Validated
siPKCzeta	Protein kinase C zeta	GAC CAA ATT TAC GCC ATG AAA	Hs_PRKCZ_6_HP Validated
siSMase	Acidic sphingomyelinase	TGG AAT TAT TAC CGA ATT GTA	Hs_SMPD1_1_HP siRNA
siBAK	BCL2 homologous antagonist/killer	AAG CGA AGT CTT TGC CTT CTC	Hs_BAK1_5_HP Validated
siDIABLO	Mitochondria-derived activator of caspases	AAT GCG TTG ATT GAA GCT ATT	Hs_DIABLO_5_HP Validated
siVDAC	Voltage-dependent anion channel-2	AAA ATA CAA GTG GTG TGA GTA	Hs_VDAC2_5_HP Validated
siCYTc	Cytochrome c	AGG CAT ATG CCT GAT GAA GTA	Hs_CYCS_6_HP siRNA
siAPAF	Apoptosis protease activating factor	AAG AGC AGC TAT GCT GAT TAA	Hs_APAF1_15_HP Validated
siCASP3	Caspase-3	CTG AGA TGG GTT TAT GTA TAA	Hs_CASP3_7_HP Validated
siCASP8	Caspase-8	AAG AGT CTG TGC CCA AAT CAA	Hs_CASP8_11_HP Validated
siCASP9	Caspase-9	CAG TGA CAT CTT TGT GTC CTA	Hs_CASP9_7_HP Validated
siFASR	FAS receptor	AAG GAG TAC ACA GAC AAA GCC	Hs_FAS_7_HP Validated
siFASL	FAS ligand	ATC GGT GAA ACT AAC AGA TAA	Hs_FASLG_1_HP siRNA
siMMP2	Matrix metalloprotease-2	CAG GCT CTT CTC CTT TCA CAA	Hs_MMP2_5_HP Validated
siSODm	Mitochondrial superoxide dismutase	ATC GTT ATG CTG AGT ATG TTA	custom made

15). The efficiency of knockdown of iNOS, neuronal NO synthase (*nNOS*), acidic sphingomyelinase (*SMAse*), NADPH oxidase organizer-1 (*NOXO1*), NADPH oxidase activator-1 (*NOXA1*), cytochrome c and mitochondrial SOD was proven through complete block of apoptosis after knockdown.

Autocrine ROS/RNS-mediated apoptosis induction. Transformed cells: 208Fsrc3 cells were seeded in 96-well plates at 12,500 cells/well with 100 µl medium and 20 ng/ml TGFβ1. All assays were performed in duplicate. The assays were cultivated at 37˚C, 5% CO_2 for 22 or 42 h and then the percentage of apoptotic cells was determined. For control, nontransformed 208F cells were cultivated under the same conditions.

Tumor cells: When seeded at appropriate density as well as cell number, tumor cells (in the presence of an inhibitor or inactivator of their protective catalase) establish apoptosis-inducing intercellular ROS/RNS signaling. Assays were performed in 96-well plates with 100 µl of complete medium per well and contained either 12,500 MKN-45 cells or 10,000 SHEP cells per well. The assays received 0-160 mM of the catalase inhibitor 3-AT for the reactivation of intercellular ROS/RNS signaling. All assays were performed in duplicate. The plates were incubated at 37˚C in an atmosphere of 5% CO_2. The percentages of apoptotic cells were then determined after 3.5-4 h for MKN-45 cells and 5-6 h for SHEP cells as described below.

Table II. *Sequences of custom-made siRNAs directed towards human targets*

siRNA		Sequence information
siNOX1-a	sense:	r(GAC AAA UAC UAC UAC ACA A)dTdT,
	antisense:	r(UUG UGU AGU AGU AUU UGU C)dGdG
siNOX1-b	sense:	r(GGU UUG AGC AGU CAC UUU A)dTdT,
	antisense:	r(UAA AGU GAC UGC UCA AAC C)dGdG
siDUOX1-a	sense:	r(AGU CUA ACA CCA CAA CUA A)dTdT
	antisense:	r(UUA GUU GUG GUG UUA GAC U)dGdG
siDUOX1-b	sense	r(CGG GCA GAU CCG UGU GGU A)dTdT,
	antisense:	r(UAC CAC ACG GAU CUG CCC G)dGdG
siiNOS2	sense	r(GGG CCG UGC AAA CCU UCA A)dTdT
	antisense:	r(UUG AAG GUU UGC ACG GCC C)dAdG
sinNOS	sense:	r(CAA GUG UGU CGA UCU UAG A)dTdT;
	antisense:	r(UCU AAG AUC GAC ACA CUU G)dTdG
siSODm	sense:	r(CGU UAU GCU GAG UAU GUU A)dTdT
	antisense:	r(UAA CAU ACU CAG CAU AAC G)dAdT

Where indicated, inhibitors were added to assays at the following final concentrations: AEBSF: 100 µM, taurine: 50 mM, FeTPPS: 20 µM, L-NAME: 2.4 mM, galardin: 10 µM.

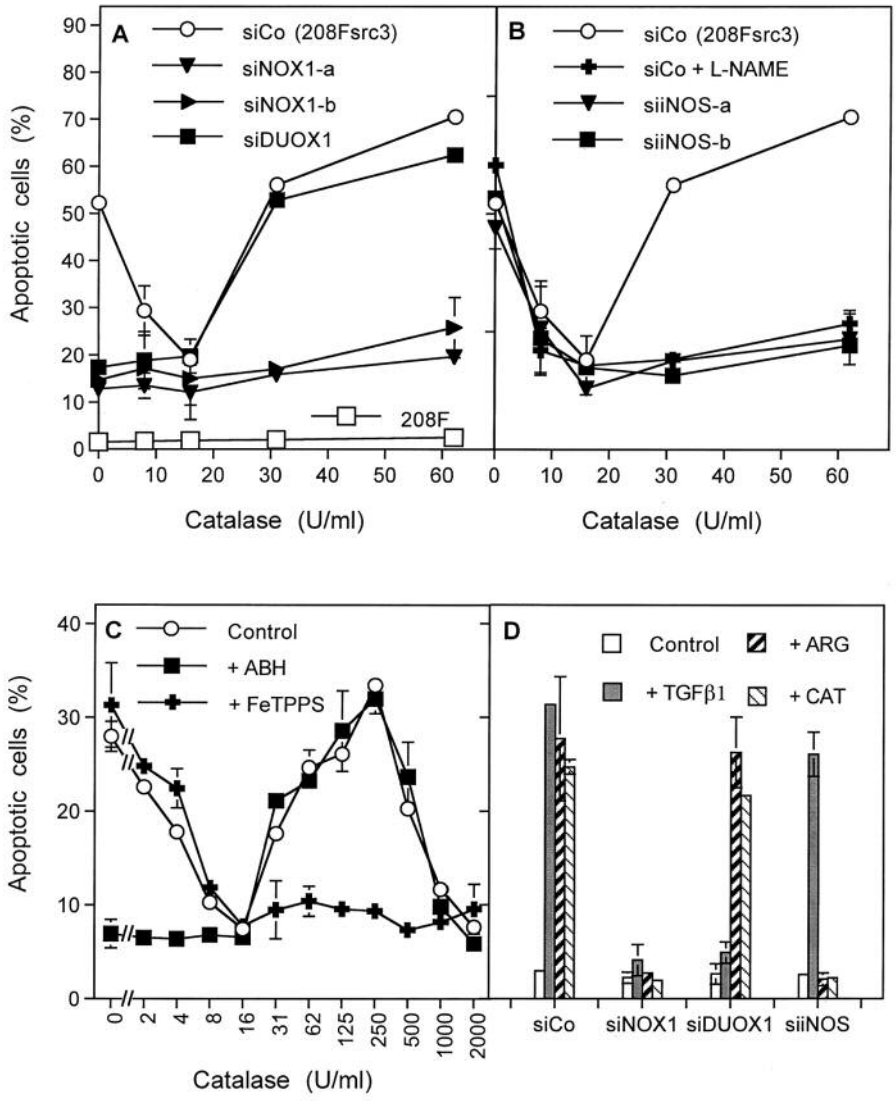

Figure 1. *Small interfering ribonucleic acid (siRNA)-based analysis of the molecular players involved in HOCl and nitric oxide/peroxynitrite (NO/PON) signaling in transformed cells. Src oncogene-transformed 208Fsrc3 cells show autocrine apoptosis induction in contrast to their non-transformed parental cells 208F. Exogenous catalase leads to a biphasic curve of apoptosis induction. SiRNA-based knockdown of NADPH oxidase-1 (NOX1) by two variants of siRNA directed towards NOX1 (siNOX1-a, siNOX1-b) abrogates apoptosis at all concentrations of catalase (A), whereas siRNA directed towards dual oxidase-1 (siDUOX1) and two variants of siRNA directed NO synthase (siiNOS-a, siiNOS-b) as well as an inhibitor of NOS [N-omega-nitro-L-arginine methylester hydrochloride (L-NAME)] act at different concentration ranges of catalase (B). This shows that HOCl signaling is inhibited by catalase, followed by resumption of NO/PON signaling. C: Apoptosis induction in 208Fsrc3 cells in the presence of a wide concentration range of catalase shows HOCl signaling [inhibited by the peroxidase inhibitor 4-aminobenzyoyl hydrazide (ABH)], followed by an optimum curve of NO/PON signaling inhibited by 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS). D: 208Fsrc3 cells in the absence of exogenous transforming growth factor β1 (TGFβ1) (control) did not show apoptosis induction, whereas exogenous TGFβ1 (20 ng/ml) caused activation of HOCl signaling, while addition of arginine (0.75 mM) or catalase (100 U/ml) caused selective activation of NO/PON signaling, as seen from the differential effect of knockdown of DUOX or iNOS.*

Apoptosis induction by exogenous HOCl. A total of 10,000 MKN-45 cells (pretreated with siRNAs for 24 h) in 100 μl complete medium in 96-well plates received 80 μM HOCl. Assays were performed in duplicate. The percentages of apoptotic cells were determined 1 hour after the addition of HOCl.

Apoptosis induction by addition of the NO donor DEA NONOate. A total of 10,000 MKN-45 cells (pretreated with siRNAs for 24 hour) in 100 μl complete medium in 96-well plates received 10 mM 3-AT and 0.5 mM DEA NONOate. Assays were performed in duplicate. The percentages of apoptotic cells were determined 1

hour after addition of the NO donor. Apoptosis induction mediated by DEA NONOate is caused by PON that is formed through the interaction of NO derived from the NO donor and NOX1-derived superoxide anions, and requires inhibition of membrane-associated catalase by 3-AT (6).

Determination of the percentage of apoptotic cells. The percentage of apoptotic cells was determined by inverted phase-contrast microscopy based on the classical criteria for apoptosis, *i.e.* nuclear condensation or fragmentation and membrane blebbing (22-25). At least 2×200 cells were scored for each point of measurement in duplicate assays.

Determination of superoxide anion production. A total of 12,500 MKN-45 cells (pretreated for 24 h with siRNAs) were cultivated in 100 μl of complete medium in 96-well tissue culture clusters in duplicate assays. SOD (Cu/Zn SOD from bovine liver) was added at 0-7 U/ml in twofold dilution steps. Apoptosis was induced through addition of 120 μM EUK-8, which establishes HOCl-dependent apoptosis induction (8). After 4 h of incubation at 37˚C in 5% CO_2, the percentage of apoptotic cells was determined by inverted phase-contrast microscopy as described above. This specific feature of the action of Cu/Zn-SOD with its characteristic curve facilitates the relative quantification of the extracellular superoxide anion concentration (26, 27), as there is a linear relationship between superoxide anion concentration and SOD required for optimal inhibition. As SOD does not penetrate the cells, only extracellular superoxide anions are determined.

Determination of peroxidase release. The assay follows the principles that were recently described (26, 28). MKN-45 cells, pretreated with siRNAs for 24 hours were washed and cultivated at a density of 300,000 cells/ml for an additional 24 h. The assays also contained 20 ng/ml TGFβ1 or 10 μM galardin. At the end of the incubation, cells were centrifuged and the supernatants were analyzed for peroxidase. The determination of peroxidase was based on destruction of HOCl generated by EUK-8 by natural peroxidase (8). A total of 10,000 208Fsrc3 cells/per 100 μl medium in the presence of 20 ng/ml TGFβ and 75 μM EUK-8 were cultivated in the presence of increasing addition of supernatants to be tested. Apoptosis induction was determined after 4 h. The resultant competition curves were compared to competition by MPO as reference. In order to ensure that peroxidase activity was measured in this competition test, some assays were cultivated in the presence of the peroxidase inhibitor ABH. Abrogation of the competition reaction by ABH ensures that indeed peroxidase had cause the competition measured.

Statistical analysis. Assays were performed in duplicate, unless otherwise stated. The empirical standard deviation was calculated and is shown in the figures. Absence of standard deviation bars for certain points indicates that the standard deviation was too small to be reported by the graphic program. Empirical standard deviations were calculated merely to determine how close the results were obtained in parallel assays within the same experiment and not with the intention of statistical analysis of variance. The Yates continuity corrected chi-square test was used for the statistical determination of significances.

For clarity, some figures use a logarithmic scale annotated with the actual concentration applied.

Results

Central elements for extracellular signaling mechanisms in transformed cells. In the presence of TGFβ1, src oncogene-transformed rat fibroblasts 208Fsrc3 (transfected with control siRNA) showed autocrine apoptosis induction ($p<0.001$), whereas their nontransformed parental cells did not (Figure 1A and B). Addition of gradually increasing concentrations of catalase up to 20 U/ml caused inhibition of apoptosis ($p<0.001$). At concentrations of more than 20 U/ml catalase, apoptosis induction resumed ($p<0.001$). Knockdown of *NOX1* by two siRNA variants directed towards *NOX1* caused complete inhibition of apoptosis at all concentrations of catalase ($p<0.001$). At concentrations of less than 20 U/ml, catalase knockdown of *DUOX* caused complete inhibition of apoptosis ($p<0.001$), whereas knockdown of *iNOS* by two variants of siiNOS or inhibition of NOS by L-NAME had no inhibitory effect. At concentrations of catalase higher than 20 U/ml, apoptosis induction was completely inhibited by knockdown or inhibition of *NOS* ($p<0.001$), whereas knockdown of *DUOX* had no effect.

In a control experiment, addition of catalase at a wider concentration range (Figure 1C) confirmed the initial inhibition of apoptosis induction by catalase ($p<0.001$), followed by resumption of apoptosis induction up to an optimum at 250 U/ml catalase ($p<0.001$). Higher concentrations of catalase then caused inhibition of apoptosis ($p<0.001$).

Apoptosis induction in 208Fsrc3 cells in the presence of TGFβ1 was confirmed to depend on NOX1 ($p<0.001$) and DUOX1 ($p<0.001$) and to be independent of iNOS (Figure 1D), whereas apoptosis induction in the presence of 100 U/ml catalase was independent of DUOX1, but dependent on NOX1 ($p<0.001$) and iNOS ($p<0.001$). The addition of the NOS substrate arginine had the same effect as the presence of catalase.

Central elements for extracellular signaling mechanisms in tumor cells. In order to study the signaling chemistry of reactivated intercellular ROS/RNS signaling after inhibition of catalase of tumor cells, human MKN-45 gastric carcinoma cells were transfected with irrelevant control siRNA (siCo) or with siRNAs directed towards *NOX1, DUOX1* and *iNOS*. One day after transfection, siCo-transfected cells were treated with increasing concentrations of the catalase inhibitor 3-AT, in the absence of inhibitors or in the presence of the HOCl scavenger taurine, the PON decomposition catalyst FeTPPS or the NOX1 inhibitor AEBSF (Figure 2A). In parallel, apoptosis induction by increasing concentrations of 3-AT in siCo-transfected MKN-45 cells was compared to that in siDUOX1-, siiNOS- and siNOX1-transfected cells (Figure 2B). SiCo-transfected cells showed an optimum curve for apoptosis induction by 3-AT ($p<0.001$). At all concentrations of 3-AT, apoptosis induction was inhibited by

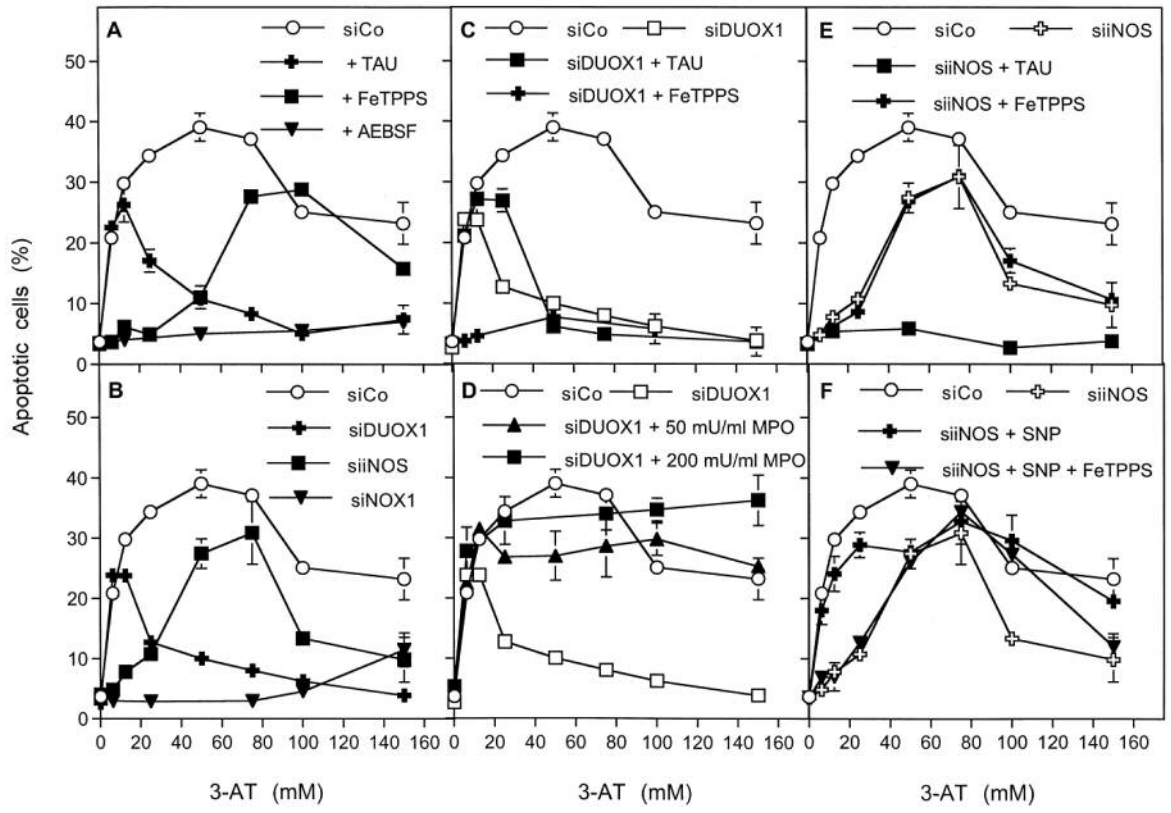

Figure 2. *Small interfering ribonucleic acid (siRNA)-based analysis of reactive oxygen species/reactive nitrogen species (ROS/RNS) signaling in tumor cells after gradual inhibition of catalase by 3-aminotriazole (3-AT). A: The HOCl scavenger taurine (TAU) and the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) allow dissection of autocrine apoptosis induction in MKN-45 gastric carcinoma cells in the presence of increasing concentrations of the catalase inhibitor 3-AT into NO/PON and HOCl signaling, whereas the NOX1-inhibitory effect of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) indicates that both processes are dependent on superoxide anions. B-F. siRNA based knockdown of dual oxidase 1 (siDUOX1), inducible NO synthase (siiNOS) and NADPH oxidase-1 (siNOX1) allow their roles in both pathways to be defined. This is counter-controlled by the additional presence of taurine, FeTPPS and by supplementation with myeloperoxidase (MPO) or the NO donor sodium nitroprusside (SNP) at 15 μM.*

AEBSF ($p<0.001$), whereas inhibition by FeTPPS was restricted to the lower concentrations of 3-AT ($p<0.001$) and inhibition by taurine to the higher concentrations of 3-AT ($p<0.001$). Knockdown of *NOX1* caused nearly complete inhibition of apoptosis induction at all concentrations of 3-AT ($p<0.001$) (Figure 2B). siRNA directed towards *iNOS* caused inhibition of apoptosis induced by low concentrations of 3-AT ($p<0.001$), in analogy to the PON decomposition catalyst FeTPPS, whereas siRNA towards *DUOX1* caused a complete inhibition of apoptosis at high concentrations of 3-AT ($p<0.001$) and thus paralleled the inhibition pattern of the HOCl scavenger taurine.

3-AT-mediated apoptosis induction in siDUOX1-transfected cells was completely inhibited by FeTPPS ($p<0.001$), but was not affected by taurine (Figure 2C), indicating that siDUOX1 abrogated HOCl signaling but did not interfere with NO/PON signaling. Addition of MPO to siDUOX1-treated cells abrogated the inhibitory effect of siDUOX ($p<0.001$) (Figure 2D). A complementary result was seen for siiNOS-transfected cells, as i) their residual apoptosis induction was inhibited by taurine ($p<0.001$); ii) residual apoptosis induction was not affected by FeTPPS, iii) the NO donor SNP abrogated the inhibitory effect of siiNOS ($p<0.001$), and iv) the abrogating effect of SNP was inhibited by FeTPPS ($p<0.001$) (Figure 2E and F).

3-AT-mediated apoptosis induction in MKN-45 cells was completely inhibited through pretreatment with siRNA directed towards TGFβ1 ($p<0.001$), TGFβR ($p<0.001$), PKC zeta ($p<0.001$) and sphingomyelinase (SMAse) ($p<0.001$), whereas treatment with siRNA directed towards the EGF receptor only showed a modulatory effect on apoptosis induction (Figure 3A-C). The effect of treatment with siTGFβ1, but not that with siTGFβR was completely abrogated through addition of exogenous TGFβ1 ($p<0.001$) (Figure 3A and B).

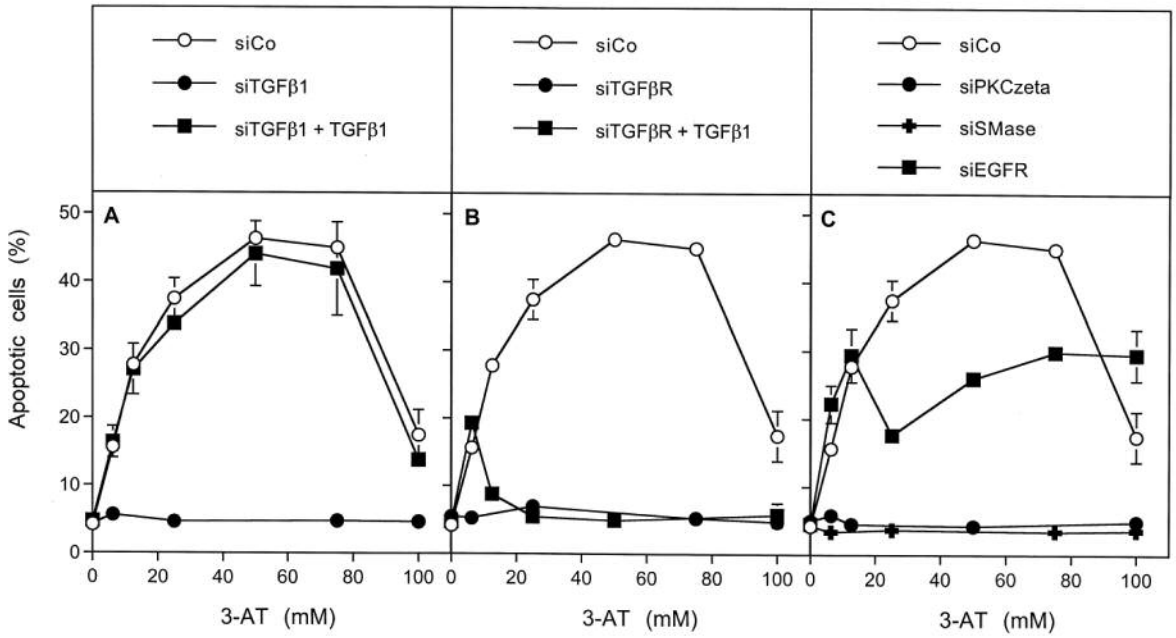

Figure 3. *Modulators of autocrine apoptosis induction in MKN-45 tumor cells. Compared to cells transfected with control small interfering ribonucleic acid (siRNA) (siCo), transfection with siRNA directed towards transforming growth factor beta-1 (siTGFβ1) (A), TGFβ receptor (siTGFβR) (B), protein kinase C zeta (siPKCzeta) or acidic sphingomyelinase (siSMASE) (C) completely prevented apoptosis induction, whereas knockdown of the epidermal growth factor receptor (siEGFR) only caused a slight decrease in apoptosis (C). The effect of knockdown of TGFβ1 was completely abrogated through supplementation with exogenous TGFβ1, whereas supplementation with TGFβ1 had no effect on cells with knockdown of the TGFβR.*

Quantitative and functional aspects of NOX1-dependent superoxide anion generation. A recently described functional assay allowed quantification of the relative concentration of extracellular superoxide anions (27). It is based on the bell-shaped inhibition curve of superoxide anion-dependent signaling by Cu/Zn SOD. Figure 4A exemplarily demonstrates the application of this assay and shows that exogenous TGFβ1 enhanced superoxide anion production about fourfold ($p<0.001$), whereas siRNA-mediated knockdown of either *NOX1* or *TGFβ1* caused a strong reduction in superoxide anion production ($p<0.001$).

Based on siRNA-mediated analysis and application of this assay system, it was shown that siRNA-mediated knockdown of *TGFβR* had a similar strong inhibitory effect on extracellular superoxide anion production ($p<0.001$) as knockdown of *NOX1* or *TGFβ1*, whereas knockdown of *DUOX1* had no inhibitory effect (Figure 4B). Supplementation with exogenous TGFβ1 abrogated the inhibitory effect of siTGFβ1 ($p<0.001$), but not that of siTGFβR. The inhibition of extracellular superoxide anion generation through treatment with siNOX1 was dependent on the concentration of the siRNA and was paralleled by an inhibition of autocrine apoptosis induction (data not shown), demonstrating the functional relevance of NOX1-dependent superoxide anion generation as well as the potential to modulate the strength of a specific signaling effect through siRNA-based methodology.

Quantitative and functional aspects of DUOX1-dependent peroxidase release. A recently described functional assay for the quantiation of peroxidase (28) is characterized by the concentration-dependent inhibition of EUK-8-mediated apoptosis induction ($p<0.001$), as demonstrated for the reference enzyme MPO in Figure 5A. Inhibition by MPO was abrogated in the presence of the peroxidase inhibitor ABH ($p<0.001$), confirming that the enzymatic activity of the peroxidase was responsible for the observed effect. The application of this assay system in combination with specific siRNA-treatment showed that transfection with two different siRNAs directed towards *DUOX1* prevented the TGFβ1-triggered release of peroxidase into the supernatant ($p<0.001$), whereas control siRNA and siRNA directed towards NOX1 had no inhibitory effect (Figure 5B and C). Addition of the MMP inhibitor galardin to cells treated with siCo prevented the release of peroxidase ($p<0.001$) and thus pointed to the role of MMP in this system. Treatment with siTGFβR caused complete ($p<0.001$) and siPKC zeta a substantial inhibition ($p<0.001$) of peroxidase release (Figure 5C).

The role of NOS isoforms for intercellular apoptosis signaling. Whereas knockdown of iNOS was shown to prevent NO/PON-mediated signaling effects in gastric

carcinoma cells (Figure 2) and in other cell systems of epithelial or fibroblast lineage (Bauer, unpublished results), apoptosis induction in 3-AT-treated SHEP neuroblastoma cells was not inhibited by siiNOS, but inhibition required siRNA directed towards nNOS ($p<0.001$) (Figure 6). In line with previous findings that these cells only establish NO/PON signaling without contribution of the HOCl pathway (8), siNOX1 caused an inhibitory effect ($p<0.001$), but siDUOX1 did not (Figure 6).

The interplay between extracellular and intracellular signaling effects. As reactivation of intercellular ROS/RNS-mediated apoptosis signaling in MKN-45 gastric carcinoma cells through inhibition of protective catalase has been shown to be dependent on NO/PON signaling at low concentrations of the catalase inhibitor (such as 6 mM 3-AT) and dominating HOCl signaling at higher concentrations of the inhibitor (such as 75 mM 3-AT), whereas no apoptosis induction occurred in the absence of inhibitor (8), siRNA-based analysis should allow for elucidation of the distinctive as well as the common biochemical features of the two signaling pathways (Figure 7). Whereas knockdown of *DUOX1* seemed to play no role in NO/PON signaling at 6 mM 3-AT (Figure 7B), its knockdown completely inhibited HOCl signaling at 75 mM 3-AT ($p<0.001$) (Figure 7C). In contrast, knockdown of *iNOS* strongly interfered with NO/PON signaling ($p<0.001$) (Figure 7B), but only had a minor effect on HOCl-dependent apoptosis induction in the presence of 75 mM 3-AT (Figure 7C). All other components of both signaling pathways and their intracellular effects seemed to be identical, as both pathways required active NOX1, TGFβ1, TGFβR, functional PKC zeta and SMAse ($p<0.001$). Both pathways seemed to depend on functional VDAC, BAK, DIABLO, mitochondrial SOD, and caspase-9 ($p<0.001$). Both pathways seemed to be independent of the activity of the FAS receptor and caspase-8, but required the activity of caspase-9 and caspase-3 ($p<0.001$).

The data established by siRNA-based analysis, as shown in Figure 7, comprise the molecular players that establish intercellular ROS signaling and those that contribute to the intracellular execution of apoptosis induction. In order to selectively focus on the players involved in intracellular apoptosis signaling after application of HOCl or NO, MKN-45 cells transfected with control siRNA or siRNA directed towards molecular players of interest were either confronted with exogenous HOCl or the NO donor DEA NONOate, and their apoptotic response was measured. As determined before, the apoptotic response of the tumor cells to HOCl did not require parallel inhibition of catalase, whereas NO-mediated, PON-dependent apoptosis induction did. siRNA-based analysis of apoptosis induction in tumor cells through addition of exogenous NO or HOCl showed identical patterns for both pathways (Figures 8 and 9): Both pathways

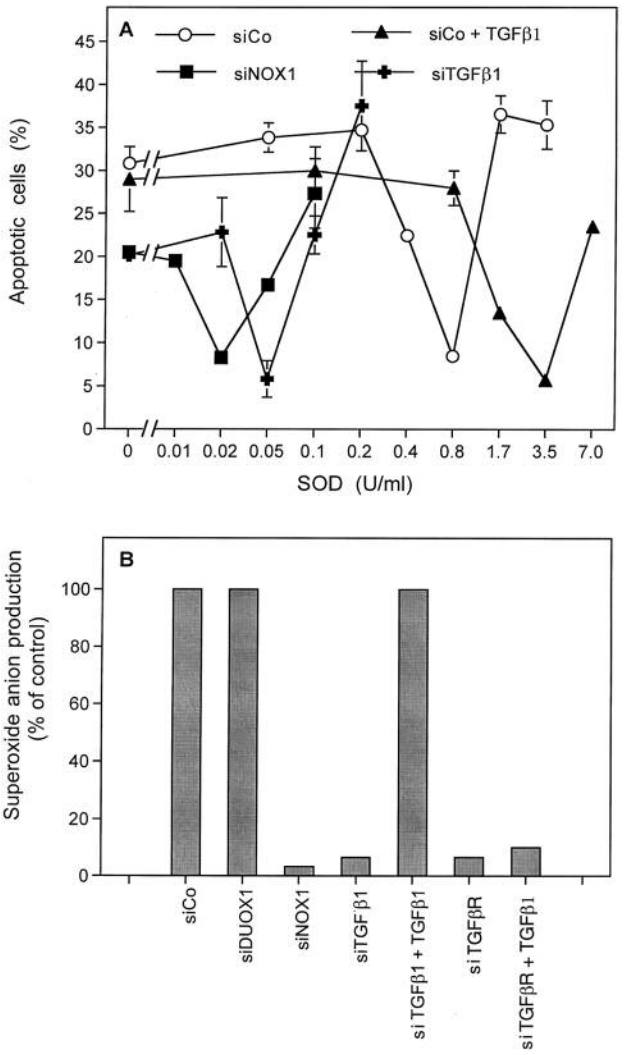

Figure 4. *Quantitation of extracellular superoxide anions generated by NADPH oxidase 1 (NOX1). A: An example of quantification of extracellular superoxide anions, based on SOD-mediated inhibition of apoptosis induction (27). Exogenous transforming growth factor beta-1 (TGFβ1) increases superoxide anion production (resulting in a rightward shift of the inhibiton curve), whereas siRNA-based knockdown of NADPH oxidase 1 (NOX1) or of TGFβ1 causes a decrease in superoxide anion production (resulting in a leftward shift of the inhibition curve). B: Quantification of extracellular superoxide anion production by MKN-45 cells transfected with control siRNA (siCo, representing 100% superoxide anion production) compared to cells with knockdown of dual oxidase-1 (siDUOX1), NOX1, TGFβ1 and its receptor (TGFβR), in the absence or presence of exogenous TGFβ1.*

were driven by NOX1, as seen by the effect of knockdown of *NOX1, NOXO1* and *NOXA1* ($p<0.001$), whereas knockdown of *NOX3, NOX4* and *NOX5* had no effect. Importantly, the inhibitory effect of knockdown of NOX1 and its regulators NOXO1 and NOXA1 confirmed that PON

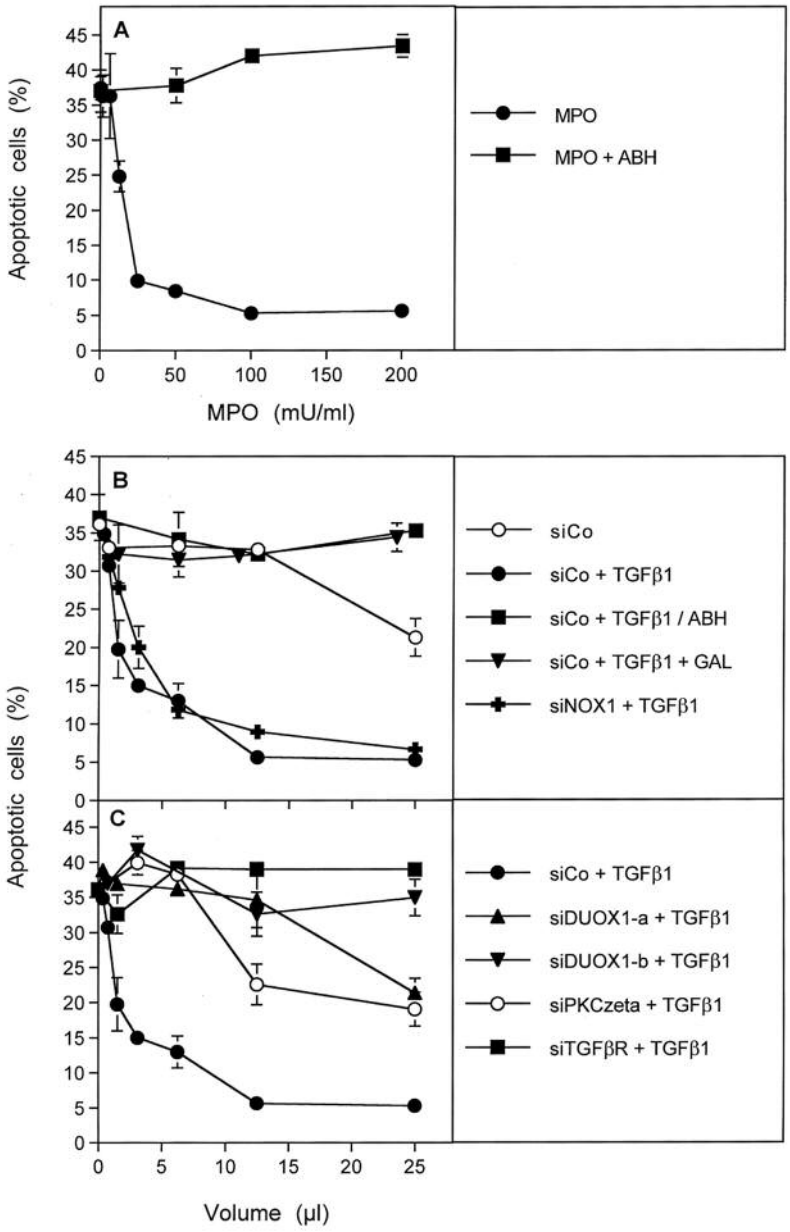

Figure 5. *Quantification of peroxidase release. The assay is based on competition of peroxidase in the test sample with the peroxidase activity of EUK-8, which results in a decrease in EUK-8-mediated apoptosis induction (28). A: Reference competition curve for the quantification of myeloperoxidase (MPO) in the competition assay was applied. Competition by MPO was abrogated by the peroxidase inhibitor 4-aminobenzoyl hydrazide (ABH). B: Release of competing peroxidase from MKN-45 cells transfected with control siRNA (siCo) required the action of transforming growth factor beta-1 (TGFβ1) and was prevented by the matrix metalloprotease inhibitor galardin (GAL). Inhibition of competition by ABH indicates that the competing activity is based on peroxidase activity. Knockdown of NADPH oxidase-1 (NOX1) did not influence the release of peroxidase. C: Knockdown of dual oxidase-1 (DUOX1) by two variants of siDUOX1, of the TGFβ receptor (TGFβR) and of protein kinase C zeta (PKC zeta) inhibited the release of peroxidase.*

formation through the interaction between NO generated by DEA NONOate and NOX1-derived superoxide anions was essential for apoptosis induction. The dependence of HOCl-mediated apoptosis induction on NOX1 ($p<0.001$)and its modulators confirmed that HOCl/superoxide anion interaction, leading to the generation of hydroxyl radicals is essential for HOCl-mediated apoptosis induction. The role of TGFβ1, TGFβR and PKCzeta for both pathways was also confirmed ($p<0.001$). Likewise, confirmation of function for SMase and components of the mitochondrial pathway of

apoptosis (BAK, DIABLO, VDAC, cytochrome c, APAF1, caspase-9) were achieved ($p<0.001$). As knockdown of the FASR and caspase-8 had no inhibitory effect on apoptosis induction, the death receptor-mediated FAS pathway does not seem to be required for apoptosis induction by both pathways, whereas caspase-3 seems to act as final caspase ($p<0.001$). Knockdown of *MMP2* caused inhibition of NO/PON ($p<0.001$) and of HOCl signaling ($p<0.001$), mediated through a peroxidase reaction, as seen through the counter effect of the mechanism based peroxidase inhibitor ABH ($p<0.001$).

Discussion

These data confirm that oncogene-transformed cells that are cultivated at sufficient cell density and cell number show autocrine apoptosis induction, whereas their non-transformed parental cells do not. As transformed cells, in contrast to their nontransformed parental cells, are known to express NOX1 (4, 7, 29), and as knockdown of *NOX1* prevented apoptosis induction, this difference can be attributed to the selective expression of NOX1 by transformed cells. Autocrine apoptosis induction by transformed cells is enhanced by the addition of exogenous TGFβ1. Without addition of TGFβ1, the kinetics is delayed (9). This finding is explained by the stimulatory role of TGFβ1 on NOX1 activity (27) and on peroxidase release (28). As apoptosis induction in transformed cells was abrogated through siRNA-based knockdown of *NOX1* and *DUOX1*, whereas knockdown of *iNOS* had no inhibitory effect, it seemed to be exclusively due to HOCl signaling. This is in line with a previous report (3). As gradual addition of exogenous soluble catalase initially inhibited HOCl signaling and then allowed for resumption of apoptosis induction that was specifically inhibited by knockdown of *iNOS* and *NOX1* (indicative of the NO/PON pathway), it becomes overt that H_2O_2 not only fosters HOCl signaling, but also interferes with NO/PON signaling. This counterbalance is further substantiated through the effect of the NOS substrate arginine, which leads to NO/PON signaling and inhibition of DUOX-dependent HOCl signaling and thus causes an result analogous to that of exogenous catalase, although through a different mechanism. Whereas catalase removes H_2O_2 that competes with NO, increased arginase concentration fosters NO synthesis and in this way competes with H_2O_2. Competition between NO and H_2O_2 was recently shown (8) and the complex underlying mechanisms have been discussed (5). Very high concentrations of soluble catalase finally inhibited NO/PON signaling. The high concentration of soluble catalase was required to mimic the inhibitory effect of high local catalase concentration on the surface of tumor cells on NO/PON signaling. This high local concentration is required for kinetic reasons, as catalase has to compete with the generation of PON/peroxynitrous acid in close vicinity to the membrane (8).

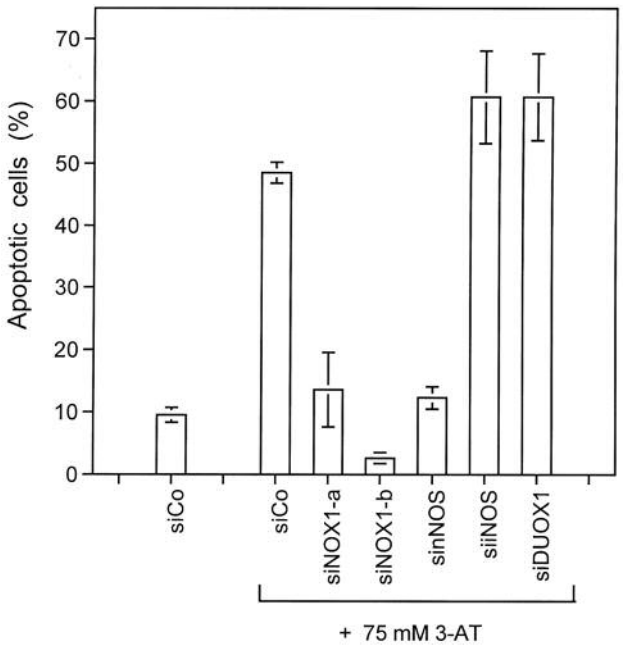

Figure 6. *Autocrine apoptosis induction in neuroblastoma cells depends on neuronal NO synthase (nNOS) and not on inducible NO synthase (iNOS). Apoptosis induction in SHEP cells transfected with control small interfering ribonucleic acid (siRNA) (siCo) was shown to depend on catalase inhibition by 3-aminotriazole (3-AT) and was prevented by knockdown of NADPH oxidase-1 (NOX1) (by two variants of siNOX1) and knockdown of neuronal NO synthase (nNOS), but not by knockdown of inducible NO synthase (iNOS) or dual oxidase-1 (DUOX1).*

When the protective catalase of tumor cells is inhibited and apoptosis induction is reactivated, the same essential players as seen before for ROS/RNS signaling of transformed cells seem to be relevant. These are NOX1, DUOX1, iNOS or nNOS in tumor cells or neural origin. Gradual inhibition of catalase initially allows NO/PON signaling, as seen by the effects of knockdown of NOX1 and NOS, whereas at higher concentrations of catalase inhibitor, HOCl signaling prevails, as seen by the knockdown of *DUOX1* and *NOX1*. The results of the knockdown experiments are shown to be conclusive, according to the additional control through inhibitor experiments. Furthermore, as supplementation with MPO abrogated the effect of knockdown of *DUOX*, and supplementation of the NO donor SNP abrogated the effect of knockdown of iNOS, the specificity of the respective siRNA treatment was confirmed. There is no experimental way to control the effect of knockdown of *NOX1* by supplementation with a superoxide anion-generating system, as this would require a superoxide anion generating system that acted exclusively in close vicinity of the cell membrane as overall superoxide anion synthesis has an inhibitor effect on intercellular ROS/RNS signaling (30). This negative effect

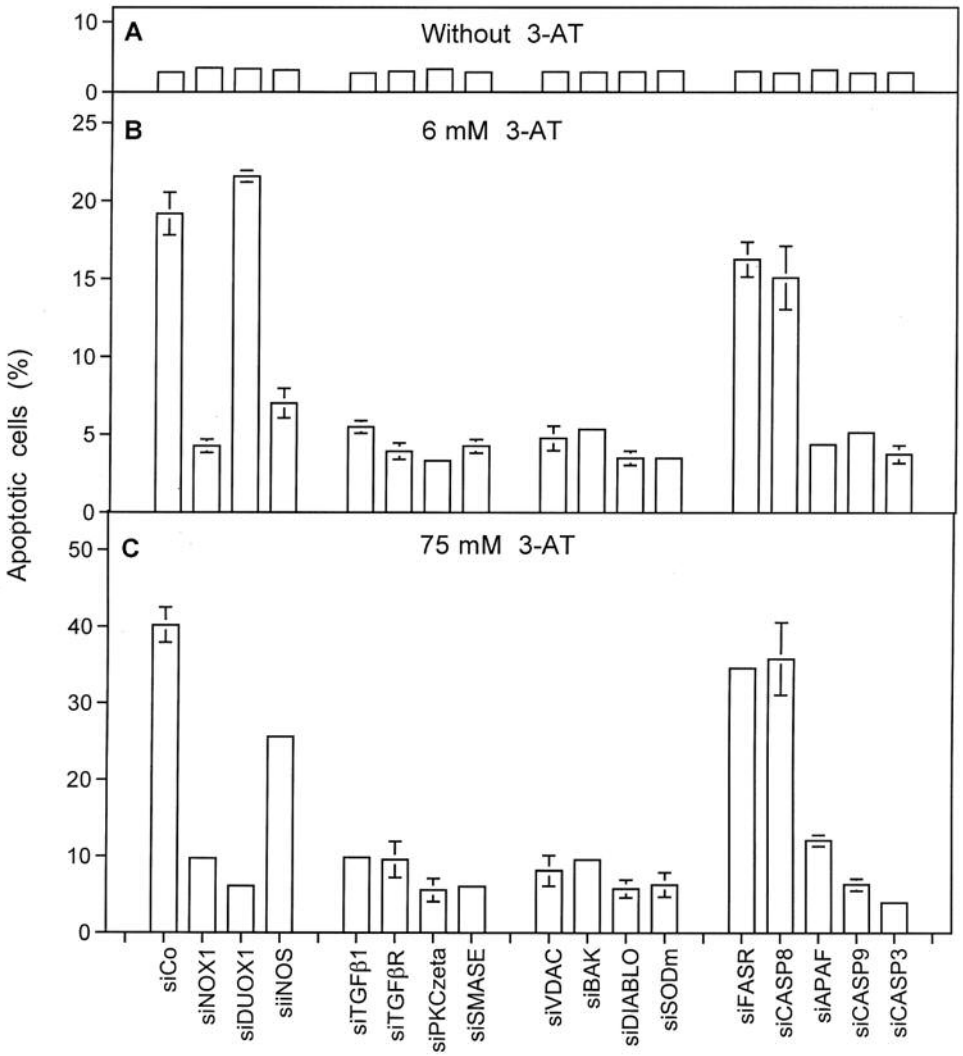

Figure 7. *Small interfering ribonucleic acid (siRNA)-based analysis of autocrine apoptosis induction by the nitric oxide/peroxynitrite (NO/PON) and the HOCl signaling pathway in MKN-45 cells after differential inhibition of catalase by 3-aminotriazole (3-AT). As determined by preceding inhibition studies, 6 mM 3-AT reactivated NO/PON signaling, whereas 75 mM 3-AT caused reactivation of HOCl signaling. The figure shows the effect of knockdown of essential molecular players on these signaling events. Compared to control siRNA (siCo), siRNA directed towards NADPH oxidase-1 (siNOX1), transforming growth factor β1 (siTGFβ1), TGFβ receptor (siTGFβR), protein kinase C zeta (siPKC zeta), acidic sphingomyelinase (siSMase), voltage-dependent anion channel (siVDAC), bcl-2 homologous antagonist/killer (siBAK), mitochondria-derived activator of caspases (siDiablo), mitochondrial superoxide dismutase (siSODm), apoptosis protease activating factor (siAPAF), caspase-9 (siCASP9) and caspase-3 (siCASP-3) caused strong inhibition of apoptosis at 6 mM and 75 mM 3-AT, whereas siRNA directed towards the FAS receptor (FASR) and caspase-8 (siCASP8) had no effect at both concentrations of 3-AT. SiRNA directed towards dual oxidase-1 (siDUOX1) had no effect at 6 mM 3-AT, but caused strong inhibition at 75 mM 3-AT. In contrast, siRNA directed towards inducible NO synthase (siiNOS) caused a strong inhibitory effect at 6 mM 3-AT and a marginal inhibitory effect at 75 mM 3-AT.*

is due to direction of generation of central elements such as hydroxyl radicals or PON away from the target cell membrane (30). However, control experiments ensured that the effect of knockdown of *NOX1* resulted in a reduction of extracellular superoxide generation to less than 10% that of the control. Based on the cell impermeability of SOD used for this determination, the extracellular localization of NOX1-derived superoxide anions was assured. Likewise, the effect of knockdown of *DUOX1* was also directly determined by a strong decrease in peroxidase release from siRNA-treated cells, following the analysis recently described (28).

Knockdown of *TGFβ1* or the *TGFβR* in tumor cells had a very strong inhibitory effect on intercellular ROS/RNS-dependent apoptosis-inducing signaling at all concentrations

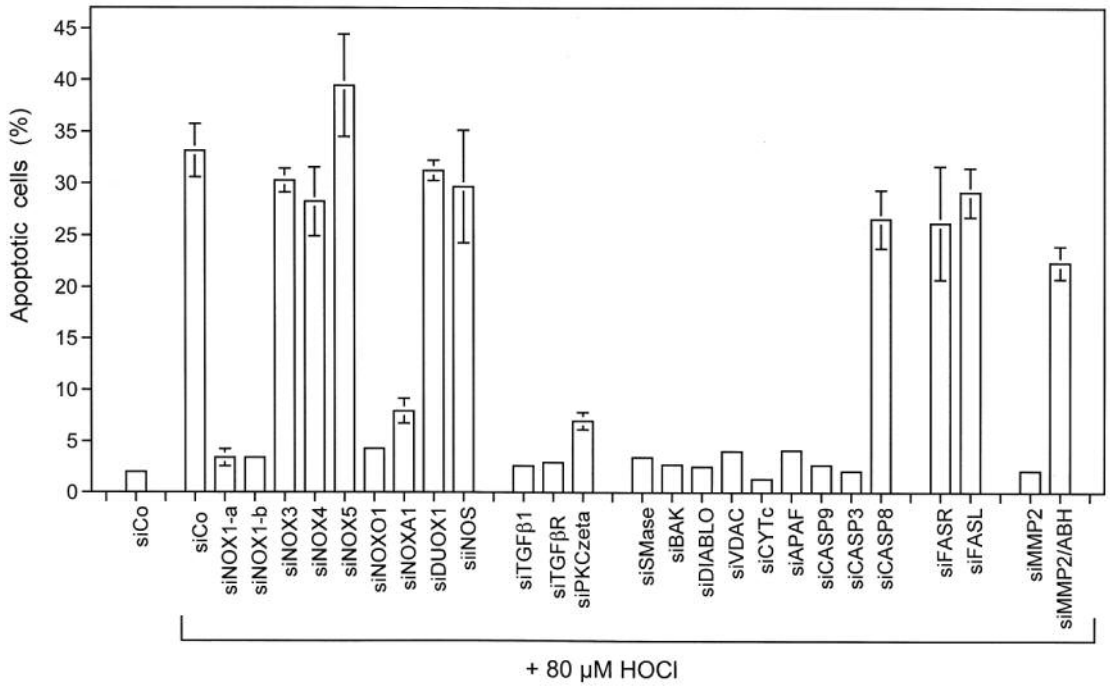

Figure 8. *Effect of knockdown of extra- and intracellular signaling molecules on apoptosis induction in MKN-45 cells by exogenous HOCl. Control small interfering ribonucleic acid (siRNA) (siCo), siRNA directed towards NADPH oxidase 1, 3, 4 and 5 (siNOX1, -3, -4, -5), NOX organizer 1 (siNOXO1), NOX1 activator (siNOXA1), dual oxidase-1 (siDUOX1), inducible NO synthase (siiNOS), transforming growth factor β-1 (siTGFβ1), TGFβ receptor (siTGFβR), protein kinase C zeta (siPKCzeta), acidic sphingomyelinase (siSMASE), bcl-2 homologous antagonist/killer (siBAK), mitochondria-derived activator of caspases (siDiablo) (siDiablo), voltage-dependent anion chanel (siVDAC), cytochrome c (siCYTc), apoptosis protease activating factor (siAPAF), caspase-9, -3 and -8 (siCASP9, -3, -8), FAS receptor (siFASR), FAS ligand (siFASL), matrix metalloproteinase-2 (siMMP2) and siMMP2 plus 150 μM 4-amino benzoyl hydrazide (ABH).*

of the catalase inhibitor. The specificity of this approach was assured as the effect of siTGFβ1 was compensated for by the addition of exogenous TGFβ1, whereas the effect of siTGFβR was not, as expected. This finding shows that tumor cells seem to produce sufficient concentration of TGFβ1 required for the control of their intercellular signalling and also have a functional receptor to react to their own TGFβ1. In contrast, transformed cells such as 208FSrc3 cells seem to produce less TGFβ1 and therefore require supplementation with exogenous TGFβ1 under experimental conditions. Based on our analysis, TGFβ1 seems to have two central targets in this context: it seems to control the activity of NOX1 (27) and to drive the release of the peroxidase domain of DUOX that is split from DUOX by the action of MMP (28). The strong inhibitory effect of knockdown of *PKCzeta* is in line with these findings, as PKC zeta is involved in the control of NOX1 (31) and a partial dependence of DUOX-coded POD release on PKC zeta was directly demonstrated here.

The combination of the siRNA-based data obtained here with results obtained in recently published inhibitor and reconstitution experiments now allows a rather complete picture to be drawn of the extracellular ROS/RNS-dependent signaling pathways of tumor cells with inhibited or inactivated catalase: i) TGFβ1 and its receptor control the activity of NOX1 and the release of the POD domain from DUOX1; ii) NOX1 generates extracellular superoxide anions that either dismutate to H_2O_2, or react with HOCl to generate hydroxyl radicals or react with NO, resulting in the formation of PON; iii) the POD domain of DUOX generates HOCl, utilizing H_2O_2 as substrate; iv) the interaction between HOCl and NOX1-derived superoxide anions yields hydroxyl radicals in close vicinity to the cell membrane, resulting in lipid peroxidation; v) PON generated through the interaction between NOX1-derived superoxide anions and NOS-derived NO is protonated by proton pumps in the membrane and yields peroxynitrous acid. Peroxynitrous acid then decomposes into NO_2 and hydroxyl radicals. PON that diffuses away from the membrane preferentially reacts with CO_2 and does not contribute to apoptosis induction (5).

The siRNA-based analysis of tumor cells in which the NO/PON or HOCl signaling pathway are differentially expressed by use of adequate concentrations of the catalase inhibitor 3-AT or in which these two pathways are separately and selectively induced through the addition of HOCl or NO

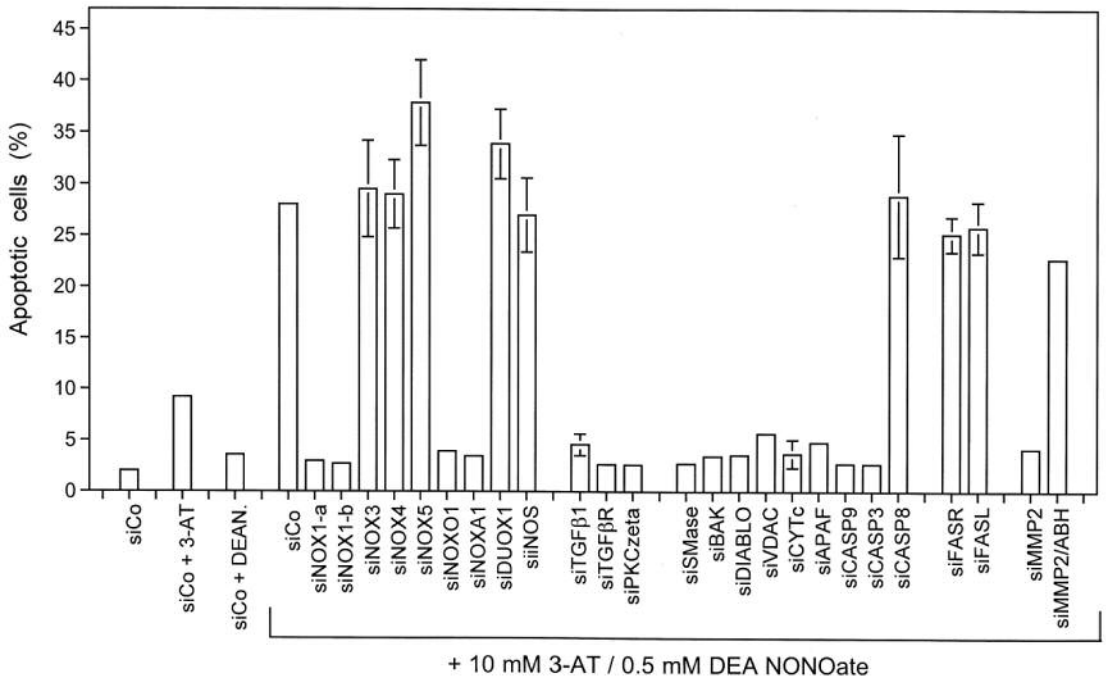

Figure 9. *Effect of knockdown of extra- and intracellular signaling molecules on apoptosis induction in MKN-45 cells by the exogenous NO donor diethylamine NONOate (DEA NONOate). MKN-45 tumor cells transfected with control small interfering ribonucleic acid (siRNA) (siCo) did not show substantial apoptosis induction when cultured alone or in the presence of 10 mM 3-aminotriazole (3-AT) or 0.5 mM DEA NONOate (siCo + DEAN). Apoptosis was induced in siCo cells when 10 mM 3-AT and 0.5 mM DEA NONOate were combined. The figure shows the differential effect of knockdown of extra- and intracellular players on apoptosis induction in the presence of 10 mM 3-AT and 0.5 mM DEA NONOate. Control small interfering ribonucleic acid (siRNA) (siCo), siRNA directed towards NADPH oxidase 1, 3, 4 and 5 (siNOX1, -3, -4, -5), NOX organizer 1 (siNOXO1), NOX1 activator (siNOXA1), dual oxidase-1 (siDUOX1), inducible NO synthase (siiNOS), transforming growth factor β-1 (siTGFβ1), TGFβ receptor (siTGFβR), protein kinase C zeta (siPKCzeta), acidic sphingomyelinase (siSMASE), bcl-2 homologous antagonist/killer (siBAK), mitochondria-derived activator of caspases (siDiablo) (siDiablo), voltage-dependent anion chanel (siVDAC), cytochrome c (siCYTc), apoptosis protease activating factor (siAPAF), caspase-9, -3 and -8 (siCASP9, -3, -8), FAS receptor (siFASR), FAS ligand (siFASL), matrix metalloproteinase-2 (siMMP2) and siMMP2 plus 150 μM 4-amino benzoyl hydrazide (ABH).*

(in the presence of 10 mM 3-AT) allowed the description of HOCl/superoxide anion interaction, NO/superoxide anion interaction and of the intracellular events following lipid peroxidation through hydroxyl radicals derived from HOCl or NO/PON signaling. Thereby, the role of NOX1 and its associated activities NOX1 organizer and NOX1 activator (32) were confirmed. Most likely, lipid peroxides generated through the action of hydroxyl radicals activate SMAse (33, 34). The strong effect of the knockdown of SMAse indicates that ceramides might indeed play a crucial role in intracellular signalling pathways after ROS/RNS-dependent extracellular effects. Ceramides have been shown to target mitochondria to trigger the mitochondrial pathway of apoptosis (35-37). In line with this assumption, knockdown of central elements of the mitochondrial pathway of apoptosis (38-40), such as BAK, DIABLO, mitochondrial SOD, APAF, and caspase-9 had a strong inhibitory effect on apoptosis induction in 3-AT-treated tumor cells. VDAC seems to play a central role for apoptosis induction as well. The exact function of VDAC is, however, a matter of scientific dispute (41). The effect of the knockdown of caspase 3 may be taken as an indication that this is the final executing caspase, triggered by caspase-9, whereas the death receptor-dependent pathway through FASR, FASL and caspase-8 do not seem to play a role here.

As the release of cytochrome *c* during the execution of the mitochondrial pathway of apoptosis causes a breakdown of the respiratory chain, free superoxide anions are generated and dismutated to H_2O_2 by mitochondrial SOD. This increase in intracellular ROS is a consequence of apoptosis induction and has to be clearly differentiated from the initial extracellular ROS/RNS effects that trigger the onset of apoptosis induction.

The literature on apoptosis usually differentiates between an extrinsic pathway, mediated by death receptors, and the intrinsic mitochondrial pathway. However, the findings here show that the mitochondrial pathway can also be triggered by extrinsic ROS/RNS signaling that targets the membrane without affecting death receptors. Therefore, this suggests the

need to differentiate between extrinsic death receptor-dependent and extrinsic death receptor-independent pathways mediated by the intracellular mitochondrial pathway. It also suggests the term "intrinsic apoptosis induction" should be used only when the initial signal is truly acting inside the cells.

Interestingly, the siRNA-based analysis given in Figures 8 and 9 also shows that knockdown of *MMP2* caused inhibition of apoptosis induction by exogenous HOCl or NO. As its inhibition was abrogated by the peroxidase inhibitor ABH, it seemed to depend on an enzymatic reaction of peroxidase. This finding is explained by the destruction of HOCl and oxidation of NO/decomposition of PON by membrane-associated peroxidase domain of DUOX1 (30). This finding, therefore, explains the need for the release of the POD for functioning signalling pathways and also points out the dependency of intercellular ROS/RNS signalling on specific steric constellations.

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