Synergistic Effects Between Catalase Inhibitors and Modulators of Nitric Oxide Metabolism on Tumor Cell Apoptosis

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Abstract. *Inhibitors of catalase (such as ascorbate, methyldopa, salicylic acid and neutralizing antibodies) synergize with modulators of nitric oxide (NO) metabolism (such as arginine, arginase inhibitor, NO synthase-inducing interferons and NO dioxygenase inhibitors) in the singlet oxygen-mediated inactivation of tumor cell protective catalase. This is followed by reactive oxygen species (ROS)-dependent apoptosis induction. TGF-beta, NADPH oxidase-1, NO synthase, dual oxidase-1 and caspase-9 are characterized as essential catalysts in this process. The FAS receptor and caspase-8 are required for amplification of ROS signaling triggered by individual compounds, but are dispensable when the synergistic effect is established. Our findings explain the antitumor effects of catalase inhibitors and of compounds that target NO metabolism, as well as their synergy. These data may have an impact on epidemiological studies related to secondary plant compounds and open new perspectives for the establishment of novel antitumor drugs and for the improvement of established chemotherapeutics.*

Tumor cells are protected against reactive oxygen species (ROS)-mediated intercellular apoptosis induction through the expression of membrane-associated catalase (1-4). This explains the "H_2O_2-catabolizing phenotype" that is regularly acquired during experimental tumor progression and is correlated with increased tumorigenicity of malignant cells (5-9). Inactivation of tumor cell-specific protective catalase allows activation of distinct intercellular apoptosis-inducing signaling pathways whose efficiency and specificity are primarily based on NOX-dependent extracellular superoxide anion synthesis (1-4). The NO/peroxynitrite (10, 11) and the HOCl signaling pathway (10) are thereby of major importance (2-4).

NO/peroxynitrite signaling is established through formation of peroxynitrite after NO/superoxide anion interaction (10-16). Peroxynitrite is then converted to peroxynitrous acid, which readily decomposes into NO_2 and apoptosis-inducing hydroxyl radicals (17-20).

HOCl signaling requires the formation of H_2O_2 through dismutation of superoxide anions. H_2O_2 serves as substrate for DUOX-related peroxidases that synthesize HOCl. Finally, HOCl interacts with superoxide anions, resulting in the generation of hydroxyl radicals (10). Hydroxyl radicals from both signaling pathways cause lipid peroxidation that triggers ceramide synthesis and the mitochondrial pathway of apoptosis (3, 4).

Secondary plant products such as anthocyanidins and salicylic acid are known for their specific apoptosis-inducing effects on tumor cells *in vitro* (21-34) and for their antitumor effects *in vivo* (23, 28, 35, 36). Numerous publications report on the induction of the mitochondrial pathway of apoptosis by these compounds (22, 25, 27, 31, 32) and on the involvement of intracellular ROS in this process (21, 25, 31, 32). None of these studies have investigated the role of extracellular ROS of malignant cells, in relation to apoptosis induction by secondary plant compounds, though Chung *et al*. (31) demonstrated that RAC-controlled NADPH oxidase was involved in apoptosis induction. As shown by Irani *et al*. (37, 38) and confirmed by other groups (10, 11), oncogene-controlled NADPH oxidase (NOX1) generates extracellular superoxide anions. The knowledge about protection against extracellular ROS production through membrane-associated catalase (1-4) allowed to speculate that inhibition or inactivation of tumor cell catalase might be the primary target of certain plant products. Therefore the activation of the mitochondrial pathway of apoptosis might be the consequence, but not the initial step of action of apoptosis-inducing plant compounds. This assumption was proven correct in an on-going detailed study that will be presented elsewhere (Scheit and Bauer, manuscript in preparation). Thereby, salicylic acid and anthocyanidins utilized different biochemical mechanisms to inactivate

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catalase. Salicylic acid inhibited catalase in a direct way, as expected from its known potential to transform compound I of catalase into the inactive compound II *via* a one-electron transfer (39). The anthocyanidins established a complex indirect inactivation mechanism for catalase that followed the mechanism recently described for apoptosis induction by increasing arginine concentrations (3). It is based on NO metabolism, caspase-8 activity, singlet oxygen generation and catalase inactivation by singlet oxygen. As described in detail recently (3), an increase in available NO seems to cause NO-mediated transient inhibition of tumor cell protective catalase (40). This prevents decomposition of peroxynitrite and H_2O_2 that are generated by malignant cells, driven by membrane-associated NADPH oxidase and intracellular NO synthase (NOS). The reaction between peroxynitrite and H_2O_2 then leads to the formation of singlet oxygen (41) that triggers the FAS receptor in a ligand-independent mode (42). As a result, NOX1 activity is enhanced through FAS receptor-activated caspase-8 (43, 44) and NOS expression is induced (45). The increase in NOX1-dependent superoxide anion generation and in NOS-dependent NO synthesis fosters the generation of additional H_2O_2 and peroxynitrite, thus leading to the generation of an overall increased singlet oxygen concentration. This second round of singlet oxygen generation is sufficient to inactivate catalase through reaction with a histidine residue at the active center (46-48). As a result, intercellular ROS signaling is reactivated and causes apoptosis induction in the tumor cells (3). The combination of the indirect catalase inactivator cyanidin and the direct inhibitor salicylic acid caused an impressive synergistic effect, whereas the combination of two direct catalase inhibitors did not. In the present report, the significance of the synergistic principle as well as the underlying biochemical mechanisms are unravelled. Besides the intriguing chemical biology of these compounds and their modes of interactions, these data may also be relevant for the elucidation of the tumor-preventive potential of secondary plant compounds.

Materials and Methods

Materials. Arginine, artemisinin, ascorbic acid, allylisothiocyanate (AITC), diallyl sulphide (DAS), diallyl disulfide (DADS), isoxanthohumol, itraconazole, human interferon alpha A, human interferon beta 1a, human interferon gamma, methyldopa, resveratrol, salicylic acid, quercetin, xanthohumol, the NADPH oxidase inhibitor 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), the catalase inhibitor 3-aminotriazole (3-AT), the protein synthesis inhibitor cycloheximide (CHX), the singlet oxygen scavenger histidine, the NO donor DEA NONOATE, the NOS inhibitor N-omega-nitro-L-arginine methylester hydrochloride (L-NAME), Mn-containing SOD from *E. coli*, the HOCl scavenger taurine, neutralizing monoclonal antibodies against human catalase (clone CAT-505, mouse, IgG1), neutralizing monoclonal antibodies against human SOD1 (Clone SD-G6 mouse, IgG1) and control antibody directed against EGF receptor were obtained from Sigma-Aldrich (Schnelldorf, Germany). Inhibitors for caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). The catalase mimetic EUK-134 (chloro[[2,2'-[1,2-ethanediylbis[(nitrilo-κN)methylidyne]] bis[6-methoxyphenolato-κO]]]-manganese was a product of Cayman and was obtained from Biomol (Hamburg, Germany).

Peroxynitrite was obtained from Calbiochem (Merck Biosciences GmbH, Schwalbach/Ts, Germany). Photofrin (a product of Axcan, Canada) was obtained from Meduna Arzneimittel GmbH (Aschaffenburg, Germany). The arginase inhibitor N^{ω}-Hydroxy-nor-L-arginine acetate (NOR-NOHA) was obtained from Axxora (Lörrach, Germany). Detailed information on inhibitors has been previously published (1, 11, 49, 52).

Cells and media for cell culture. The human gastric adenocarcinoma cell line MKN-45 (ACC 409) (established from the poorly-differentiated adenocarcinoma of the stomach (medullary type) of a 62 year-old woman) was purchased from DSMZ, Braunschweig, Germany. MKN-45 cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS). Fetal bovine serum (Biochrom, Berlin, Germany) had been heated for 30 min at 56˚C prior to use. Medium was supplemented with penicillin (40 U/ml), streptomycin (50 μg/ml), neomycin (10 μg/ml), moronal (10 U/ml) and glutamine (280 μg/ml). Care was taken to avoid cell densities below 300,000/ml and above 10^6/ml.

Methods. Autocrine apoptosis induction by intercellular ROS signaling. MKN-45 cells were seeded in 96-well tissue culture clusters at a density of 12,500 cells/100 μl of complete medium. In all experiments, assays were performed in duplicate. The concentrations of compounds that triggered apoptosis induction, as well as their combinations, are described in the respective figure legends. After the indicated time of incubation at 37˚C and 5% CO_2, the percentage of apoptotic cells was determined by inverted phase contrast microscopy based on the classical criteria for apoptosis, *i.e.*, nuclear condensation/fragmentation or membrane blebbing (2, 50, 51). The characteristic morphological features of intact and apoptotic cells, as determined by inverted phase contrast microscopy have been recently published (2, 52, 53). At least 200 neighbouring cells from randomly selected areas were scored for the percentage of apoptotic cells at each point of measurement. Control assays ensured that the morphological features 'nuclear condensation/fragmentation' as determined by inverse phase contrast microscopy were correlated to intense staining with bisbenzimide and to DNA strand breaks, detectable by the TUNEL reaction (11, 53, 54). A recent systematic comparison of methods for the quantitation of apoptotic cells has shown that there is a perfect coherence between the pattern of cells with condensed/fragmented nuclei (stained with bisbenzimide) and TUNEL-positive cells in assays with substantial apoptosis induction, whereas there was no significant nuclear condensation/fragmentation in control assays (52). Though positivity in the TUNEL reaction represents one of the clearest hallmarks for apoptosis, we found that the TUNEL reaction was not suitable for routine quantitation in our cell culture system, as the preparation of the samples for the TUNEL reaction cause a marked loss preferentially of apoptotic cells. The early apoptosis marker Annexin V positivity preceded the marker nuclear condensation and fragmentation (indicative of the completed apoptosis process). A comparison of the quantitation of Annexin V staining by fluorescence microscopy and by FACS analysis, in conjunction with

phase contrast microscopy confirmed the validity of each one of these methods (52).

siRNA-mediated knockdown of signaling components. Control siRNA and siRNAs directed against specific targets of human cells were obtained from Qiagen (Hilden, Germany). The following siRNAs were used:
A. Control siRNA ("siCo"), (catalog no. 1022076; sequences: r(UUCUCCGAACGUGUCACGU)dTdT (sense)
ACGUGACACGUUCGGAGAA)dTdT (antisense).
Control siRNA was determined by the manufacturer as not affecting the expression of any known gene).
B. High-performance validated siRNAs for the knockdown of:
TGF-beta1 ("siTGF-beta"), (Hs-TGFB1_6-HP Validated siRNA, catalog no. SI02662912; target sequence: CAG CAT ATA TAT GTT CTT CAA);
FAS receptor ("siFAS Rec.") (Hs_FAS_7_HP Validated siRNA, catalog no. SI02654463; target sequence: AAG GAG TAC ACA GAC AAA GCC);
Caspase-8 ("siCASP-8") (Hs_CASP8_11_HP Validated siRNA; catalog no. SI02661946, target Sequence: AAG AGT CTG TGC CCA AAT CAA);
Caspase-9 ("siCASP-9") (Hs_CASP9_7_HP Validated siRNA, catalog no. SI02654610, target Sequence: CAG TGA CAT CTT TGT GTC CTA);
C:HP custom siRNAs:
HP custom siRNA directed against human NOX1 ("siNOX1");
Sequences: sense: r(GACAAAUACUACUACACAA)dTdT;
antisense: r(UUGUGUAGUAGUAUUUGUC)dGdG
HP custom siRNA directed against human DUOX1 ("siDUOX1")
Sequences: sense: r(AGUCUAACACCACAACUAA)dTdT;
antisense: r(UUAGUUGUGGUGUUAGACU)dGdG
HP custom siRNA directed against human iNOS2 ("siiNOS")
Sequences: sense: r(GGGCCGUGCAAACCUUCAA)dTdT;
antisense: r(UUGAAGGUUUGCACGGCCC)dGdG

siRNAs were dissolved in suspension buffer supplied by Qiagen at a concentration of 20 μM. Suspensions were heated at 90˚C for 1 min, 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 was mixed with 12 μl HiPerFect solution (Qiagen) and 0.6 μl of specific siRNA or control siRNA. The mixture was vortexed for a few seconds and then allowed to rest for 10 min. It was then gently and slowly added to 300,000 MKN-45 cells in 1 ml RPMI 1640 medium that contained 10% FBS and antibiotics. The resulting siRNA concentration in the assay was 24 nM. The cells were incubated at 37˚C in 5% CO_2 for 24 h. The cells were centrifuged and resuspended in fresh medium at the desired concentration.

Control experiments showed that the transfection efficiency in MKN-45 cells was much greater than 90% when Hyperfect transfection reagent (Qiagen) and the protocol summarized above, were used (2).

Validation of the efficiency of knockdown of specific genes by the respective siRNAs was measured by the manufacturer through quantifying transcription levels, using RT PCR. The knockdown based on this measurement was 97% for TGF-beta1, 80% for the FAS receptor, 94% for caspase-8 and 87% for caspase-9. Consistent with these measurements, the functional evaluation of the efficiency of knockdown showed that superoxide anion production by NOX1, which depends on stimulation by TGF-beta1, had been lowered to 5% compared to the control when either TGF-beta1 or its receptor had been knocked-down by siRNA. The addition of purified TGF-beta1 restored 100% of the initial activity, indicating the specificity of the TGF-beta1 effect. Transfection with control siRNA did not affect the cells. The functional knockdown of the FAS receptor and caspases-8, 9 was more than 95% after 24 h, as apoptosis induction dependent on these proteins was nearly completely blocked. The functional knockdown of NOX1 by siNOX1 was more than 95%, as determined by direct quantification of superoxide anion generation, following the protocol recently described (55). The functional knockdown of DUOX1 and iNOS was more than 95%, as the HOCl and the NO/peroxynitrite signaling pathway were completely blocked after knockdown. The strong functional knockdown of all targets of the siNAs used indicates that the inhibition of *de novo* expression was very strong and the half-life of preexisting proteins was short.

ROS signaling relevant activities of compounds used in this study. The ROS signaling relevant activities of compounds used in this study are summarized in Table I. Direct inhibition of catalase by salicylic acid, ascorbic acid and methyldopa has been shown by other groups (39, 56, 57) and was confirmed through determination of sensitization of tumor cells for exogenous peroxynitrite as recently described (2). The dependence of catalase inhibition on the concentration of the compounds was determined. In subsequent synergy experiments (Figure 1, Figures 5-8), concentrations of the direct catalase inhibitors were used, that caused no or only marginal catalase inhibition when applied alone. Catalase inhibition by salicylic acid, ascorbic acid and methyldopa was not abrogated by the singlet oxygen scavenger histidine and therefore seemed to be direct rather than dependent on triggering singlet oxygen generation. In contrast, catalase inactivation by all compounds that affected NO metabolism was indirect as it required the generation of singlet oxygen that inactivated catalase. Singlet oxygen-dependent inactivation triggered by these compounds was tested by determining the sensitizing of tumor cells for exogenous peroxynitrite in a reaction that was abrogated by the singlet oxygen scavenger histidine, as recently described (3). The dependence of the actions on NO metabolism was determined by inhibition of compound-dependent apoptosis induction in tumor cells by 2.4 mM of the NOS inhibitor L-NAME. Inhibition of NOD was determined as increase of apoptosis induction by the exogenous NO donor DEA NONOate in the presence of 3-AT, EUK-134 and L-NAME, as recently described (3). Quercetin has been shown to be an inhibitor of NOD (58) and served as positive control in NOD inhibition assays. Induction of NOS by interferon alpha, beta and –gamma has been described (59-61) and was confirmed by inhibition of interferon action by cycloheximide and dependence of their reaction on NO synthesis.

Statistical analysis. In all experiments, assays were performed in duplicate and empirical standard deviations were calculated. Absence of standard deviation bars indicates that the standard deviation was too small to be reported by the graphic program. Empirical standard deviations merely demonstrate reproducibility in parallel assays but do not allow statistical analysis of variance. The experiments have been repeated at least twice (with duplicate assays). The Yates continuity corrected chi-square test was used for the statistical determination of significances ($p<0.01$=significant; $p<0.001$=highly significant). As rather broad concentration ranges are used in our studies, the presentation of the data requires semilogarithmic presentation in most figures.

Table I. *ROS signaling-relevant activities of compounds used in this study*

Compound	Effect on NO metabolism	Mode of action	Direct inhibition of catalase	Singlet oxygen-dep. catalase inactivation
Salicylic acid	–	–	+	–
Ascorbic acid	–	–	+	–
Methyldopa	–	–	+	–
Arginine	+	NOS substrate	–	+
Nor-NOHA	+	Arginase inhibitor	–	+
Itraconazole	+	NOD inhibitor	–	+
Quercetin	+	NOD inhibitor	–	+
Xanthine	+	NOD inhibitor	–	+
Isoxanthine	+	NOD inhibitor	–	+
Allylisothiocyanate	+	NOD inhibitor	–	+
Artemisinin	+	NOD inhibitor	–	+
Diallyl disulfide	+	NOD inhibitor	–	+
Diallyl sulfide	–	–	–	–
Interferon	+	NOS inducer	–	+

Based on data from the literature and on own control experiments, the compounds of interest in this study were classified into direct catalase inhibitors, that do not require the generation of action of singlet oxygen for catalase inactivation, and indirect catalase inactivators that trigger the formation of singlet oxygen. All these indirect catalase inactivators were modulators of NO metabolism that either increased the substrate for NO synthesis (arginine, arginase inhibitors), prevented NO consumption by NOD or induced NOS.

Detailed statistical analysis of the data shown in Figures 1-9. Figure 1: Apoptosis induction by cyanidin (12-333 ng/ml): $p<0.001$. Synergistic effect between cyanidin and salicylic acid, methyldopa, ascorbate, anti-Catalase and anti-SOD: $p<0.001$. No significant synergistic effect between cyanidin and anti-EGFR. Figure 2: Apoptosis induction by interferon alpha (0.9-15.6 U/ml), interferon beta (1.6-31 U/ml), interferon gamma (0.9-7.5 U/ml): $p<0.001$. Inhibition of interferon effect by AEBSF, histidine, caspase-9 inhibitor: $p<0.001$. No significant inhibitory effect of caspase-8 inhibitor. Figure 3: Apoptosis induction by interferon: $p<0.001$. Inhibition of the interferon effect by CHX, histidine, L-NAME and taurine added 15 min before interferon: $p<0.001$. Inhibition of the interferon effect by histidine and L-NAME added 60 min after interferon: $p<0.01$. Inhibition of the interferon effect by taurine added 60 min after interferon: $p<0.001$. Difference between the inhibitory effects of histidine and L-NAME added 15 min before or 60 min after interferon: $p<0.001$. Figure 4: 3-AT-, interferon-, arginine and phogtofrin-dependent sensitization of MKN-45 cells for apoptosis induction by 300-500 μM H_2O_2: $p<0.001$. Prevention of sensitization by histidine: $p<0.001$. Figure 5: Apoptosis induction by arginine, NOR-NOHA, quercetin, xanthohumol, isoxanthohumol: $p<0.001$. Apoptosis induction by itraconazole: $p<0.0.1$. Synergistic effect of salicylic acid with arginine, NOR-NOHA, quercetin, xanthohumol, isoxanthohumol, itraconazole: $p<0.001$. Figure 6: Apoptosis induction by AITC, diallyl disulfide, interferon alpha, artemisinine: $p<0.001$. No significant apoptosis induction by diallylsulfide and resveratrol, when added alone. Synergistic effect between salicylic acid and AITC, DADS, interferon alpha, artemisinine, resveratrol: $p<0.001$. No synergistic effect between salicylic acid and diallylsulfide. Figure 7: Apoptosis induction by 12-333 ng/ml cyanidin: $p<0.001$. Inhibition of cyanidin-dependent apoptosis induction by histidine and caspase-8 inhibitor. $p<0.001$: Synergistic effect between cyanidin and salicylic acid: $p<0.001$. Inhibition of the synergistic effect by histidine: $p<0.001$. No significant inhibition of the synergistic effect by caspase-8 inhibitor. Figure 8: Apoptosis induction by 37-100 ng/ml cyanidin: $p<0.001$. Inhibition of cyanidin-dependent apoptosis induction by siRNA directed against NOX1, iNOS, TGF-beta1, caspase-8, caspase-9 (whole concentration range of cyanidin): $p<0.001$. Inhibition of cyanidin-dependent apoptosis induction by siRNA directed against DUOX1 (cyanidin 111-1000 ng/ml): $p<0.001$. Inhibition of cyanidin-dependent apoptosis induction by siRNA directed against the FAS receptor (cyanidin 12-333 ng/ml): $p<0.001$. Synergistic effect between salicylic acid and cyanidin: $p<0.001$. Inhibition of the synergistic effect by siRNA directed against NOX1, iNOS, TGF-beta1, caspase9: $p<0.001$. Inhibition of the synergistic effect of salicylic acid and 1.3 ng/ml cyanidin by siRNA directed against DUOX1: $p<0.001$. No significant inhibition of the synergistic effect by siRNA directed against the FAS receptor and caspase-8. Figure 9: Apoptosis induction in siCAT-transfected tumor cells and its inhibition by exogenous catalase or taurine: $p<0.001$. Apoptosis induction in a mixture of siCAT and siCo-transfected cells compared to the siCo-transfected control population: $p<0.001$. Effect of histidine on apoptosis induction in siCAT-transfected cells after 4 h: $p<0.01$.

Results

The first experiment aimed to clarify whether synergistic effects between catalase inhibitors and modulators of NO metabolism represent a general principle. Therefore, constant and low concentrations of different direct catalase inhibitors (salicylic acid, M-DOPA, ascorbic acid, antibody against catalase), as well as anti-SOD and control antibodies were combined with increasing concentrations of the NOD inhibitor cyanidin and apoptosis induction was monitored.

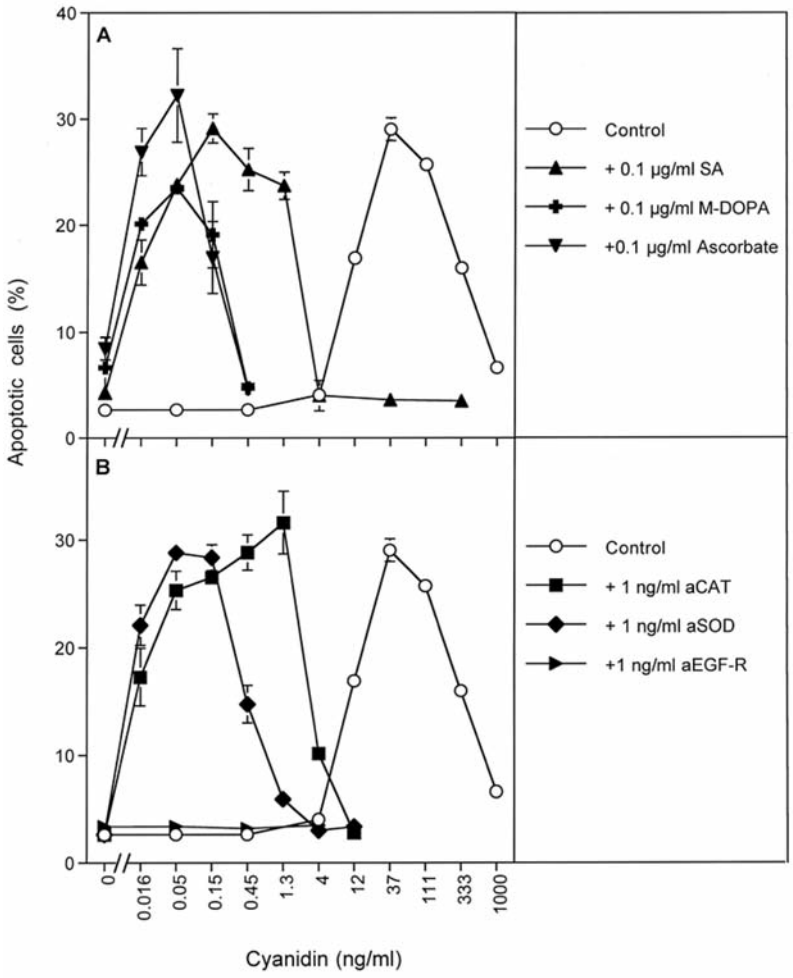

Figure 1. *Synergistic effects between catalase inhibitors and the NOD inhibitor cyanidin. MKN-45 cells under standard conditions for apoptosis induction were treated with the indicated concentrations of salicylic acid (SA), methyldopa (M-DOPA), ascorbate, neutralizing antibody directed against human catalase (aCAT), neutralizing antibody directed against human SOD (aSOD) or control antibody directed against EGF receptor (aEGF-R) for 15 min at 37˚C. Apoptosis induction was monitored after 6.5 h.*

The concentrations of the individual catalase inhibitors had been determined on the basis that they were not sufficient to cause apoptosis in tumor cells when applied alone, whereas higher concentrations caused ROS-dependent apoptosis induction (data not shown). As shown in Figure 1, all direct catalase inhibitors, as well as anti-SOD, but not control antibody, caused a strong synergistic effect with cyanidin. Therefore, the potential to induce a synergistic effect with cyanidin seems to represent a regular feature of catalase inhibitors. The effect of anti-SOD is explained as indirect inhibition of catalase by excess superoxide anions that are present in the close vicinity of tumor cells after inhibition of SOD. Superoxide anions have the potential to inhibit catalase through formation of inactive compound III ($CATFe^{III}\ O_2^-$) and through conversion of compound I ($CATFe^{IV}=O^{+\bullet}$) to the inactive compound II ($CATFe^{IV}=O$) (4, 62-66).

Recent work has demonstrated that an increase of the NO level in tumor cells may lead to complex signalling cascades that culminate in the generation of singlet oxygen, singlet oxygen-mediated inactivation of tumor cell protective catalase and subsequent reactivation of apoptosis-inducing intercellular ROS signalling (3; Scheit and Bauer, manuscript in preparation). This effect had been shown for an increase in available NO concentration through an increase in the concentration of the NOS substrate arginine (3) or alternatively through prevention of NOD-mediated consumption of NO through application of the NOD inhibitor cyanidin (Scheit and Bauer, manuscript in preparation). In order to determine whether the synergistic effect between modulators of NO metabolism and salicylic acid represented a general mechanism, several compounds that affect NO metabolism were tested for synergistic interaction with

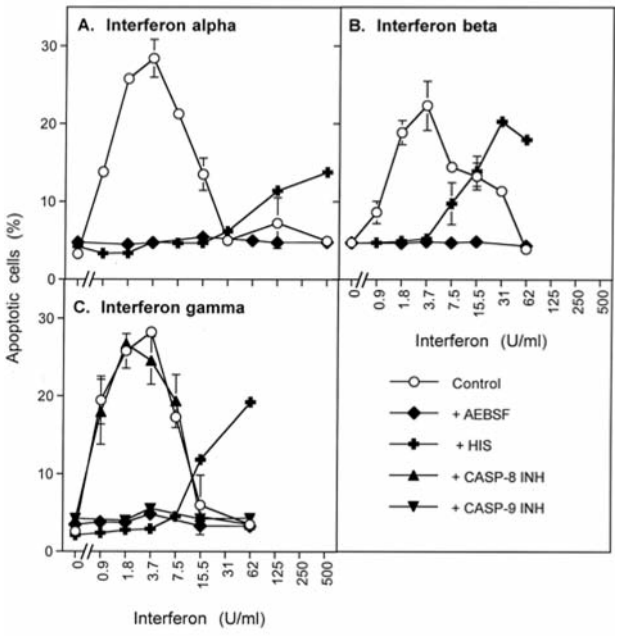

Figure 2. *Interferon induces ROS-mediated apoptosis induction in tumor cells. MKN-45 cells under standard conditions for apoptosis induction received the indicated inhibitors (100 μM of the NOX inhibitor AEBSF, 2 mM of the singlet oxygen scavenger histidine/"HIS"), 25 μM caspase-8 or caspase-9 inhibitor or remained without inhibitors. Human interferon-alpha, interferon-beta or interferon-gamma were added at the indicated concentrations and apoptosis induction was determined after 3 h.*

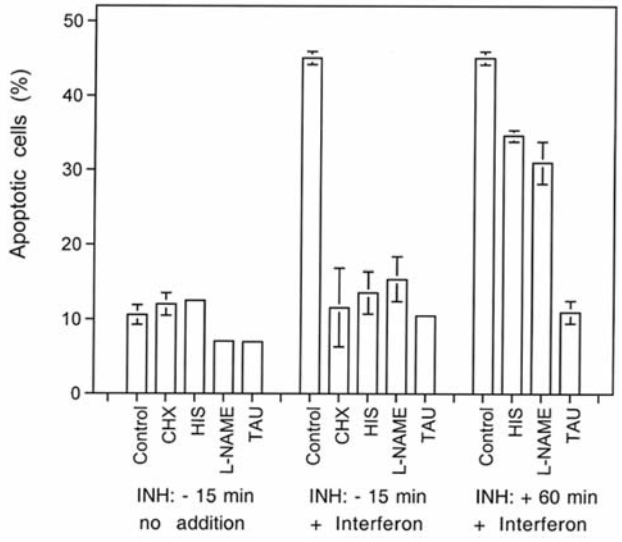

Figure 3. *Early and late signaling effects mediated by interferon. MKN-45 cells under standard conditions for apoptosis induction received 50 μg/ml of the protein synthesis inhibitor cycloheximide, 2 mM of the singlet oxygen scavenger histidine. 2.4 mM of the NOS inhibitor L-NAME or 50 mM of the HOCl scavenger taurine either 15 min prior to or 60 min after addition of 10 U/ml interferon gamma. As indicated, control assays without interferon and interferon-containing assays without inhibitor were incubated in parallel. Apoptosis induction was monitored after 3 h.*

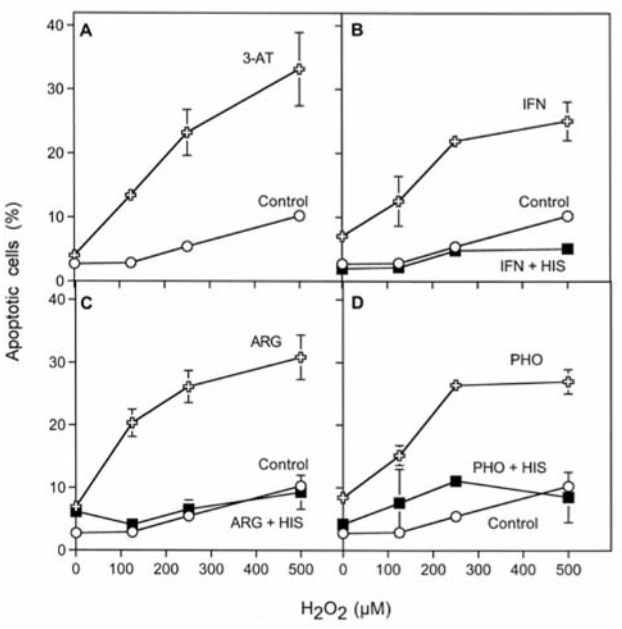

Figure 4. *Inactivation of tumor cell protective catalase. MKN-45 cells (125,000 cells/ml) remained either untreated or received 10 U/ml interferon-gamma, 5 mM arginine or 5 μg/ml of the photosensitizer photofrin in the absence or presence of 2 mM histidine, as indicated. After 20 min of incubation and illumination with visible light in the case of photofrin-containing assays, cells were washed three times to remove the additions. Control cells, previously untreated but washed received 50 mM of the catalase inhibitor 3-AT. All other cell populations were tested without further treatment. The indicated concentrations of H_2O_2 were added to the assays and apoptosis induction was monitored after 3 h.*

salicylic acid. The compounds were included in the study on the basis of their potential to affect NO metabolism at three distinc levels: i) increase of the NOS substrate arginine through either addition of arginine or arginase inhibitor; ii) induction of NOS synthesis through interferon (58-60), and iii) prevention of NO consumption by NO dioxygenase (NOD) (58, 67-69) through addition of NOD inhibitors such as itraconazole, quercetin, xanthohumol, isoxanthohumol, diallyl disulfide, allylisothiocyanide and artemisinin. In addition, resveratrol was tested, as it was found to enhance NOX1 activity in the tumor cells (Bauer, unpublished results). As a representative example for the action of modulators of NO metabolism, the biochemical pathways induced by interferons and leading to apoptosis induction in tumor a cells are shown in Figures 2-4. A detailed characterization of the biochemical features of the other compounds that follow the general scheme demonstrated for interferon will be summarized in detail elsewhere. Figure 2 shows that interferon-alpha, -beta and -gamma induce apoptosis in the human tumor cell line MKN-45 in a concentration-dependent manner, in the mode of an optimum curve. Apoptosis induction was dependent on

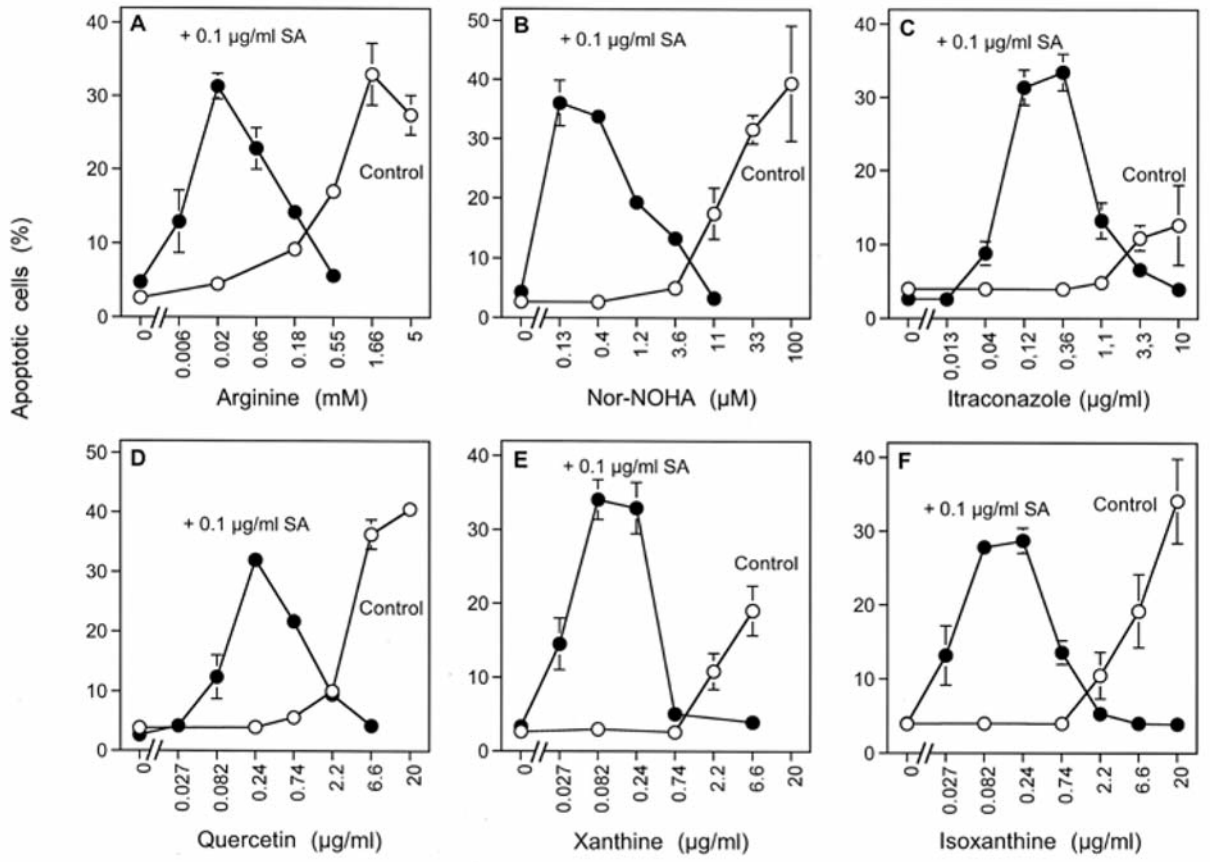

Figure 5. *Synergistic apoptosis inducing effect between salicylic acid and modulators of NO metabolism. MKN-45 cells under standard conditions for apoptosis induction were treated with 0.1 µg/m salicylic acid (SA) and then received the indicated concentrations of the NOS substrate arginine, the arginase inhibitor NOR-NOHA, the NOD inhibitors itraconazole, quercetin, xanthin and isoxanthine. Apoptosis induction was monitored after 6 h.*

superoxide anions generated by NOX1, as it was inhibited by AEBSF, and on the generation of singlet oxygen, as it was inhibited by the singlet oxygen scavenger histidine. As shown for interferon-gamma, apoptosis induction seemed to depend on the mitochondrial pathway of apoptosis, as it was inhibited by caspase-9 inhibitor. Caspase-8-dependent processes were not required, indicating that the caspase-8-dependent amplification step during singlet oxygen generation was dispensible when iNOS was induced by interferon, in contrast to the action of arginine, which required this amplification step (3). As expected from the mechanism of its action, apoptosis induction by interferon-gamma required on-going protein synthesis as it was inhibited by cycloheximide (Figure 3). Addition of inhibitors either prior to the addition of interferon or 60 min later, allowed to dissect an early step in apoptosis induction, dependent on singlet oxygen and NO synthesis from the later signaling step mediated by the HOCl pathway (Figure 3). The early step seemed to be related to inactivation of tumor cell protective catalase by singlet oxygen, as interferon-gamma (Figure 4B) and arginine (Figure 4A) treatment caused sensitization of tumor cells for exogenous H_2O_2. In line with this conclusion, singlet oxygen-mediated inactivation, mediated by interferon or arginine, caused the same biochemical result as application of the catalase inhibitor 3-AT (Figure 4A) or inactivation of catalase through singlet oxygen generated by illuminated photofrin (Figure 4D).

As shown in Figures 5 and 6, all modulators of NO metabolism (arginine, arginase inhibitor, NOD inhibitors, iNOS stimulating interferon) caused a concentration-dependent induction of apoptosis in the tumor cells when applied alone. The NOX stimulator resveratrol-only showed a minor effect when applied alone. All NO metabolism-modulating compounds tested and presented in Figures 5 and 6, as well as resveratrol, established an impressive synergistic effect with 0.1 µg/ml salicylic acid. This regular and general effect of modulators of NO metabolism and NOX activity in combination with the catalase inhibitor salicylic acid seems to be specific, as replacement of dially disulfide (found to be a potent NOD inhibitor) by diallylsulphide (found not to have and inhibitory activity on

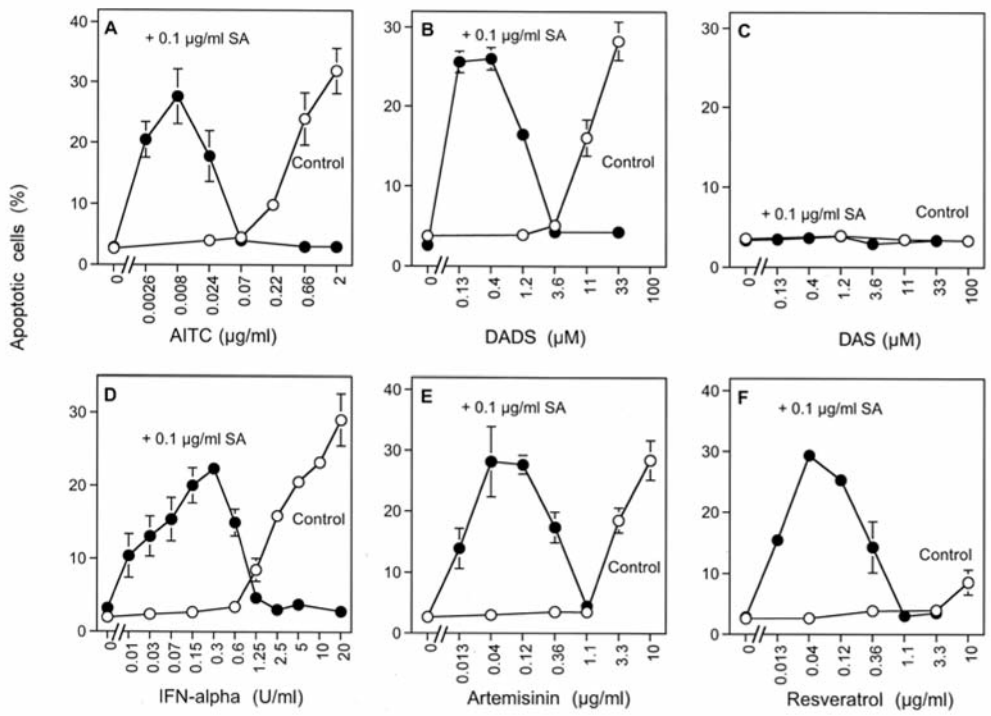

Figure 6. *Synergistic apoptosis inducing effect between salicylic acid and modulators of NO metabolism or the NOX stimulator resveratrol. MKN-45 cells under standard conditions for apoptosis induction were treated with 0.1 µg/m salicylic acid (SA) and then received the indicated concentrations of the the NOD inhibitors allyl isothiocyanate (AITC), diallyl disulfide (DADS), artemisinin, the iNOS inducer interferon alpha A and the NOX stimulator resveratrol. For control, diallyl sulphide (DAS) that lacks NOD activity was tested in parallel to diallyl disulfide. Apoptosis induction was monitored after 6 h.*

NOD) caused complete abrogation of the synergistic effect and of direct apoptosis induction by the compound itself.

An ongoing study (Scheit and Bauer, in preparation) has shown that apoptosis induction by cyanidin is dependent on an early step that is enhanced by caspase-8 and by singlet oxygen. We, therefore, asked the question whether the synergistic effect between cyanidin and salicylic acid utilizes the same biochemical mechanisms as cyanidin alone. Increasing concentrations of cyanidin were tested for their apoptosis-inducing potential, either alone or in combination with 0.1 µg/ml salicylic acid. Assays were performed in the absence of inhibitors (control) or in the presence of the singlet oxygen scavenger histidine and of caspase-8 inhibitor. As shown in Figure 7 A and B, apoptosis induction by cyanidin alone required singlet oxygen as well as caspase-8, whereas the synergistic effect between cyanidin and salicylic acid was dependent on singlet oxygen, but did not require caspase-8 activity. However, caspase-8 inhibitor caused a broadening of the optimum curve of apoptosis induction by the combination of cyanidin and salicylic acid.

For the elucidation of the molecular players involved in cyanidin-dependent apoptosis, in the absence or presence of salicylic acid, MKN-45 gastric carcinoma cells were transfected with control siRNA (siCo) or siRNA directed against NOX1, DUOX1, iNOS, TGF-beta, FAS receptor, caspase-8 and caspase-9. 24 h after beginning of siRNA treatment, the cells were challenged with increasing concentrations of cyanidin, both in the absence and presence of 0.1 µg/ml salicylic acid. As shown in Figure 8 A and B, apoptosis induction by cyanidin alone or in synergy with salicylic acid was strictly dependent on NOX1, iNOS and TGF-beta. siRNA-mediated knockdown of DUOX1 had no effect in the lower concentration range of cyanidin but blocked apoptosis at higher concentrations. For apoptosis induction by cyanidin-alone, caspase-8 and FAS-R were indispensible, whereas they were not required for apoptosis induction by synergistic cyanidin/salicylic acid interaction (Figure 8C and D). This was contrasted to the finding related to caspase-9, whose activity was necessary for apoptosis induction at all concentrations of cyanidin in the absence or the presence of salicylic acid. These data allow to differentiate between effector molecules that were necessarily involved in catalase inactivation and/or subsequent apoptosis signaling (like TGF-beta1, NOX1, iNOS, DUOX1 and caspase-9) and effectors like FAS receptor and caspase-8 that are dispensable in the presence

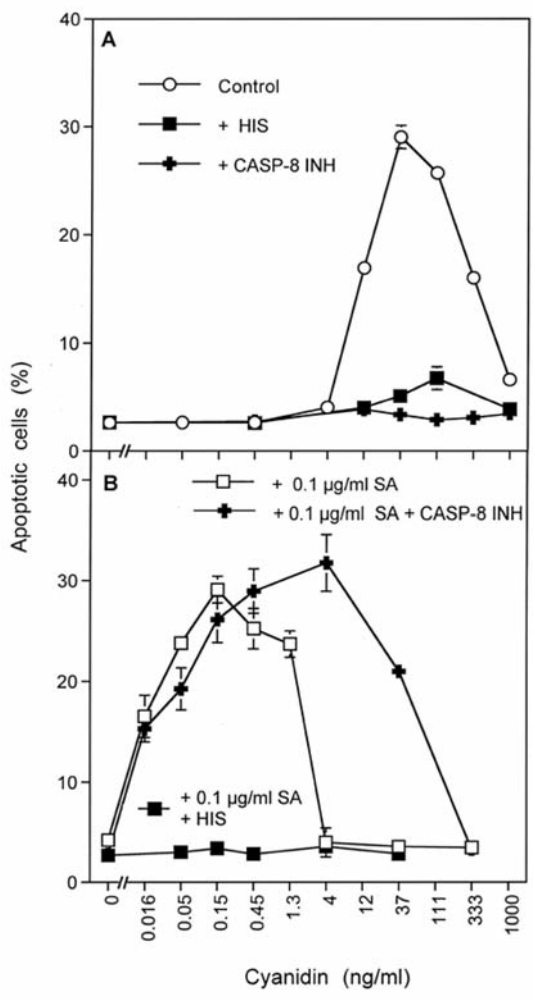

Figure 7. *Synergy between salicylic acid and cyanidin: dependence on singlet oxygen and independence of caspase-8-mediated amplification. MKN-45 cells under standard conditions for apoptosis induction remained without addition of inhibitors or received either 2 mM of the singlet oxygen scavenger histidine or the 25 µM of caspase-8 inhibitor. After 15 min, assays received either the indicated concentrations of cyanidin (A) or 0.1 µg/ml salicylic acid (SA) and the indicated concentrations of cyanidin after additional 15 min. Apoptosis induction was monitored after 6.5 h.*

of synergistically acting concentrations of the catalase inhibitor salicylic acid.

The data presented in the present manuscript show that singlet oxygen-dependent inactivation of tumor cell catalase after modulation of NO metabolism is synergistically enhanced by suboptimal concentrations of a catalase inhibitor. This allows to speculate on a seed-and-soil analogous effect, in which localized inhibited catalase enhances ROS-dependent singlet oxygen generation. To address this question, MKN-45 gastric carcinoma cells were transfected with siRNA directed against catalase and apoptosis induction in control cells, unfractionated siCAT-treated cells and a mixture of 5% siCAT-treated cells in 95% control cells were analysed for apoptosis induction. As shown in Figure 9A, siCo cells did not show significant apoptosis induction, whereas siCAT-treated cells went readily into apoptosis. The addition of the singlet oxygen scavenger histidine had a stabilizing effect on the kinetics of apoptosis induction in siCAT-treated cells. In the absence and presence of histidine, siCAT-treated cells died essentially from HOCl signaling, as taurine and exogenously added catalase caused a strong inhibitory effect, whereas the effect of the NOS inhibitor L-NAME was marginal (Figure 9A and B). When a small fraction of siCAT-treated cells were mixed with an excess of control cells, the total population showed apoptosis induction like siCAT-treated cells after a lag phase of about three hours (Figure 9C and D). Addition of histidine, L-NAME and taurine at the time point of mixing the two cell populations completely abrogated apoptosis induction. When these inhibitors were added five hs later (Figure 9D), histidine and L-NAME did not cause inhibition any more, whereas taurine caused a strong inhibition. These findings, therefore, allow to differentiate between an early singlet oxygen and NO-dependent step in the interaction between siCAT and siCo cells, and a later signaling step, exclusively through HOCl signaling.

Discussion

These data demonstrate that direct catalase inhibitors (such as salicylic acid, ascorbate, methyldopa and neutralizing antibodies directed against catalase), as well as modulators of NO metabolism that target different levels of regulation of available cellular NO, cause ROS-dependent apoptosis induction in tumor cells. In contrast to the action of direct catalase inhibitors, the effect of the modulators of NO metabolism depends on the generation of singlet oxygen and subsequent singlet oxygen-dependent catalase inactivation, as recently shown for arginine (3) and verified exemplarly for NOS-inducing interferons in this manuscript. The combination of direct catalase inhibitors with modulators of NO metabolism results in an impressive synergistic effect that utilizes singlet oxygen. This synergistic effect represents a rather general principle to induce apoptosis in tumor cells. Salicylic acid is a prototype of catalase inhibitors that transforms the active catalase intermediate compound I into the inactive compound II through a one-electron transfer (39): ($CATFe^{IV}=O^{+\bullet}$ + e^{-} → $CATFe^{IV}=O$).

The potential to execute a one-electron transfer is related to the antioxidant nature of salicylic acid. It is noteworthy that in the context of the biological system described here, the reaction of the antioxidant causes inactivation of catalase and therefore a subsequent increase in free ROS such as H_2O_2 and peroxynitrite. This represents an interesting biochemical

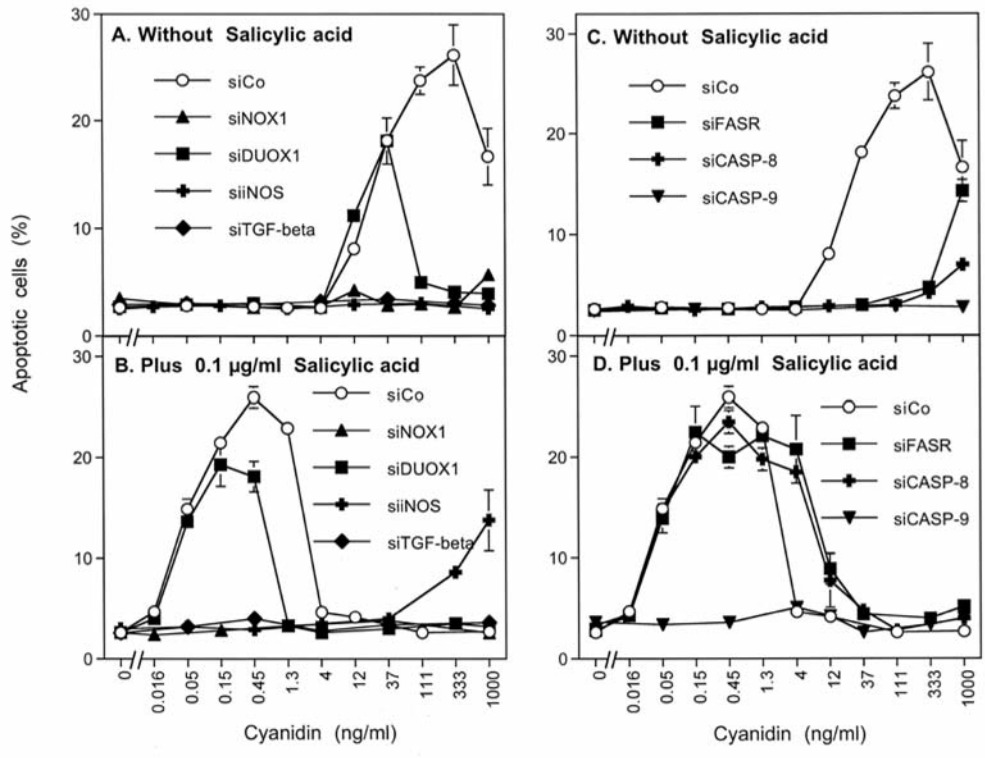

Figure 8. *siRNA-based analysis of the mechanism of cyaniding and salicylic acid action on tumor cells. MKN-45 cells were transfected with 24 nM of control siRNA (siCo), siRNA directed against human NOX1 (siNOX1), human DUOX1 (siDUOX1), human iNOS (siiNOS), TGF-beta1 (siTGF-beta), FAS receptor (siFASR), caspase-8 (siCASP-8) or caspase-9 (siCASP-9). 24 h after transfection, the cells were seeded under standard conditions and challenged with the indicated concentrations of cyanidin either directly (A, C) or after 15 min of incubation in the presence of 0.1 µg/ml salicylic acid. Apoptosis induction was monitored 4 h after addition of cyanidin.*

example for the potential of certain antioxidants to provoke a prooxidant action. Ascorbic acid and methyldopa seem to use the same strategy for catalase inhibition as salicylic acid, *i.e.* formation of inactive compound II through a one-electron transfer (56, 57). In contrast, antibodies directed against catalase need to bind to catalase in a way that the enzymatic function is inhibited. This may be achieved by binding at the the entry site for the active center or at a position that causes allosteric inhibition after binding of the antibody. The inhibitory effect of anti-SOD on catalase is only seen in the presence of active NOX, *i.e.* in the situation of a tumor cell where NOX1, catalase and SOD are expressed in the cell membrane. As a result of SOD inhibition, local availability of superoxide anions increases as their enzymatic dismutation is inhibited. This allows for superoxide anion-dependent inhibition of catalase, through a one-electron transfer on compound I and in addition through compound III formation (4, 62-66). As all of these different modes of catalase inhibition cause synergy with modulators of NO metabolism, the general concept with respect to the role of catalase inhibition as an essential partner in the synergistic effect described here, is established.

The synergy partner reacting with a catalase inhibitor needs to cause an increase in available NO. This can be achieved by increasing the concentration of arginine (the substrate of NOS) either by adding arginine or preventing its arginase-mediated decomposition. Alternatively, the concentration of NOS and thus that of NO can be enhanced by NOS-inducing compounds such as interferons (59-61). Prevention of NO consumption through NO dioxygenase represents another alternative to increase the steady-state levels of NO (58, 67-69). Thus, a multitude of different chemicals can synergize with catalase inhibitors in the induction of singlet oxygen-mediated apoptosis in tumor cells. Their common feature is enhancement of the available NO concentration, independent of the level of control that is targeted.

The ROS-dependent apoptosis-inducing effects of interferons, either applied alone or in synergy with catalase inhibitors may contribute to the established antitumor potential of interferons. According to our data, the effect of interferon directed against tumor cells is based specifically on the potential of interferons to induce NOS and thus to increase the available NO concentration.

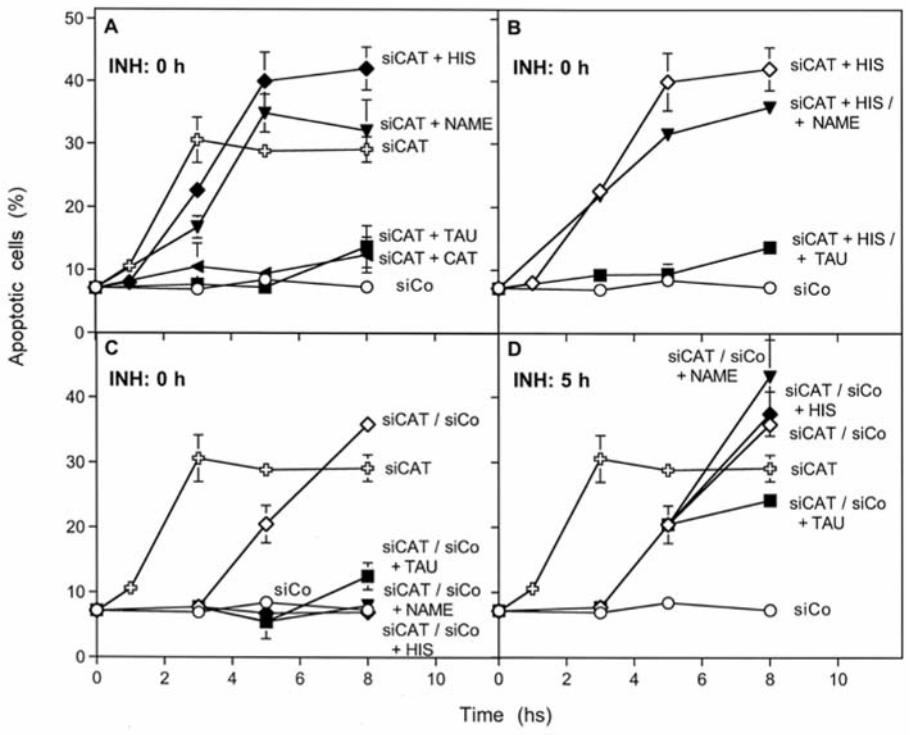

Figure 9. *Seed and soil effect between catalase-depleted and control tumor cells. MKN-45 cells were transfected either with 12 nM control siRNA (siCo) or siRNA directed against catalase (siCAT). 24 h after transfection the cells were either seeded as pure cultures ("siCo" or "siCAT") or as mixture consisting of 5% siCAT-pretreated and 95% siCo-pretreated cells ("siCAT/siCo"). Where indicated, the assays received 2 mM histidine, 2.4 mM L-NAME, 50 mM taurine or 30 U/ml bovine liver catalase ("+CAT"). The inhibitors were either added at the beginning of culture or 5 h later. In the case of siCAT/siCo, the inhibitors were added to siCo cells before the siCAT cells were added. Apoptosis induction was monitored kinetically, as indicated in the Figure.*

The common effective and central mechanism of these modulators of NO metabolism is their contribution to catalase inactivation through singlet oxygen generation, as shown recently for arginine (3) and exemplarily for interferon gamma in this paper (Figure 4). The same effect can be achieved by direct application of singlet oxygen through illumination of the photosensitizer photofrin, thus demonstrating singlet oxygen to be the central principle for inactivation of catalase. NO is confirmed as one central initial element in this complex reaction through the analogous effects of various modulators of its concentration and through inhibition of the reaction by L-NAME. The synergistic effect between catalase inhibitors and modulators of NO concentration is defined as singlet oxygen-dependent inactivation of catalase. Thereby, singlet oxygen seems to be generated through the interaction between cell-derived peroxynitrite and H_2O_2 (3).

The central molecular players required to allow for the synergistic effect are the same as for induction of apoptosis by individual reactants, *i.e.* TGF-beta1, NOX1, DUOX, NOS and caspase-9. Their effect is controlled by singlet oxygen. However, whereas apoptosis induction by compounds like cyanidin requires amplification through the FAS receptor and caspase-8 (3), the synergistic effect is independent of these two partners. This confirms that the FAS receptor and caspase-8 have a ROS-mediated function during an early step, which leads to catalase inactivation, and have no direct death receptor-related apoptosis inducing effect in this context. It can be speculated that successful inactivation in a few catalase molecules in the first round of ROS signaling causes enhancement of subsequent signaling events that lead to more catalase inactivation independent of the primary amplification step. Based on a previous report (3), this can be achieved by the interaction between H_2O_2 and peroxynitrite, resulting in the formation of singlet oxygen specifically at those sites of the membrane where catalase is inactivated. Thus, it was predicted and experimentally confirmed here (Figure 9) that the addition of few catalase-deficient tumor cells to intact cells should mimick the situation of an initial suboptimal catalase inactivation that causes a seed and soil-like induction of a self-amplificatory mechanism that finally leads to catalase inactivation at a sufficient level to allow efficient intercellular ROS-dependent apoptosis induction.

The biological consequences of these findings are intriguing. The outlined synergistic effects allow inactivation of tumor cell protective catalase and subsequent ROS-dependent selective apoptosis induction in tumor cells at concentrations of the individual compounds that are too low to be active against tumor cells when present alone. It can be speculated that this synergism may have a positive impact on the control of spontaneous or induced microtumors through dietary secondary plant products. It is obvious that the chance to establish synergistic effects depends on the variety of plant-derived food intake. The efficiency of low concentrations of various compounds during synergistic interaction adds another advantage in favour of elimination of malignant cells. As many of the active compounds also have additional antioxidant function (which reflects their potential for one electron transfers), higher concentrations of the compounds can interfere with the ROS signaling that they helped initiate. This negative effect on ROS signaling may be avoided when low concentrations of the compounds are sufficient to establish the synergistic effect on catalase inactivation, but are not sufficiently high to interfere with subsequent apoptosis-inducing intercellular ROS signaling.

It also has to be pointed-out, that the synergistic effects described here may raise significant problems for the interpretation of epidemiological studies. When the effect on tumor prevention by regular intake of individual plant compounds such as salicylates, anthocyanidins or other flavonoids is monitored, the unknown intake of one of the potential synergy partners may enhance the effect and the dose-dependency of the compound studied, according to the synergy effects described here. The impact of this synergy on the outcome of the study can neither be foreseen nor easily analyzed by the investigator. These aspects deserve further conceptional and experimental work in the future.

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