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Review

Tumor Cell-protective Catalase as a Novel Target for Rational Therapeutic Approaches Based on Specific Intercellular ROS Signaling

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Abstract. *Reactive oxygen species (ROS) exhibit procarcinogenic effects at multiple stages during multistep oncogenesis. As a hallmark of the transformed state, extracellular superoxide anions generated by NADPH oxidase1 (NOX1) are centrally involved in the control of the transformed state. These pro-carcinogenic effects of ROS are counterbalanced by specific ROS-dependent apoptosis induction in malignant cells, based on four interconnected signaling pathways. Tumor progression selects for a phenotype characterized by resistance to ROS-dependent apoptotic signaling. Resistance is based on membrane-associated catalase in tumor cells, which therefore represents a promising and unique target for specific tumor therapy. Novel approache, developed in vitro, utilize antibody-mediated inhibition of catalase or ROS-driven singlet oxygen generation and subsequent inactivation of tumor cell catalase as initial steps. As a consecutive step, malignant cell-generated superoxide anions then drive apoptotic signaling with high selectivity for malignant cells. We propose to translate this complex but well-established ROS-dependent signaling chemistry into novel approaches for experimental therapy in vivo.*

This article summarizes the complex and interconnected steps that are involved in multistep oncogenesis. The consequences of oncogene-dependent activation of membrane-associated NADPH oxidase1 (NOX1) are discussed in the context of proliferation stimulation and parallel induction of apoptosis, selectively in superoxide anion-generating malignant cells. The two major ROS-dependent apoptosis-inducing signaling pathways are discussed in detail. Establishment of resistance to apoptosis-inducing intercellular ROS signaling through expression of membrane-associated catalase seems to represent one of the hallmarks of tumor progression. The stringency of resistance to ROS-mediated signaling is due to the potential of catalase to decompose hydrogen peroxide as well as peroxynitrite and to oxidize NO. Therefore, several approaches to inhibit or inactivate catalase and thus to reactivate apoptotic ROS signaling are discussed. Novel experimental data on singlet oxygen generation and singlet oxygen-dependent inactivation of catalase are included and the underlying biochemistry is presented. The demonstration of multiple strategies to target protective catalase for novel approaches in tumor therapy represents the final goal of this paper.

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Multistep Oncogenesis

Multistep oncogenesis is characterized by multiple distinct steps, including abrogation of senescence control, oncogene activation, tumor suppressor gene inactivation (1-3), independence of exogenous proliferation signals through autocrine mechanisms (4-6), independence of control by neighboring cells (7-11), escape from immune surveillance (2), release of prostaglandin E2 (12-16), resistance to hypoxia-induced p53-mediated cell death (17, 18), tumor angiogenesis (19) and others (reviewed in 20-24). These basic features of oncogenesis are interconnected with three additional central mechanisms related to the action of reactive oxygen (ROS) and nitrogen species (RNS): i) oncogene-controlled extracellular superoxide anion production through NADPH oxidase (NOX1) (25-42; for review see 43-49), ii) acquisition of the 'H_2O_2-catabolizing phenotype' during tumor progression (12-16), and iii) resistance of tumor cells to intercellular ROS signaling through membrane-associated catalase expression (50-54).

ROS and Oncogenesis

Generation of extracellular superoxide anions through a membrane-associated NADPH oxidase (NOX1) is associated with oncogene activation and represents one of the hallmarks of the transformed state (25-42, 55, for review see 43-49). The rat sarcoma oncogene (RAS) and and the RAS-related small GTPase RAC1 play central roles for the activation of NOX1 (25, 56). Activated NADPH oxidase seems to be required for the control of proliferation and the maintenance of the transformed state (25-27, 29, 37, 57), changes in the cytoskeleton of transformed cells (58) and induction of cell motility (59). A causal connection between oncogene activation, superoxide anion production and transformation, as shown convincingly *in vitro*, has also been demonstrated to be relevant for the situation *in vivo* (37, 39, 40, 42, 60). Specific overexpression of NOX1 has been found in human tumors (39, 60), and is dependent on activation of RAS (39). Inhibition of NOX1 activity causes subsequent inhibition of tumor growth, as shown by the work of Mitsushita *et al*. (37). Overexpression of RAC1 in oral squamous cell carcinomas (61) and *RAC1* gene mutations in human brain tumors (62) also point to the significance of the RAS-RAC1-NOX1 network in tumor development. In addition, ROS have been shown to be relevant for tumor angiogenesis (63, 64) and metastasis (65, 66). Together with induction of genomic instability (67), the effects of ROS on angiogenesis and metastasis thus contribute to tumor progression in a highly dynamic way, beyond the establishment and the control of the transformed state. On the flip side of the coin, extracellular superoxide anions generated by transformed cells, drive both the efficiency and selectivity of intercellular induction of apoptosis specifically in malignant cells, a hitherto unrecognized potential control step during multistage oncogenesis (30, 32, 33, 35, 68-79, for review see 21-24). During intercellular induction of apoptosis, transformed cells are selectively induced to die by apoptosis after a concerted action of transformed cell-derived ROS and signaling components released by surrounding non-transformed cells (classical intercellular induction of apoptotis), or the transformed cells themselves (autocrine, ROS-mediated apoptotic self-destruction) (21-24, 30, 32, 52-54). For simplicity, the comprehensive term 'intercellular ROS signaling' is used for both aspects of ROS-mediated apoptosis induction, as they utilize the same basic signaling chemistry. Besides its potential role for the understanding of ROS-related processes in oncogenesis, intercellular ROS signaling represents a rather unique experimental system for the study of site-specific signaling effects of radical and nonradical ROS and RNS (80). The perception of ROS and RNS is often restricted to shotgun-like nonspecific damaging agents, although their specific signaling potential (in addition to their destructive potential) has been proposed many years ago (81). Specific apoptotic ROS signaling, as discussed here, depends on a fine interplay between different radical and nonradical species, with short or long free diffusion path length, whereby the membrane of malignant cells is both the origin and the target of some of the relevant molecules. Four pathways involved in intercellular ROS-mediated signaling have been elucidated so far: i) the HOCl signaling pathway (30); ii) the NO/peroxynitrite signaling pathway (30, 35, 82); iii) the nitryl chloride signaling pathway (83); and iv) the metal ion catalyzed Haber-Weiss reaction (84). The selectivity of the 'target cell function' of transformed cells is based on their specific extracellular superoxide anion production by NOX1. 'Effector function' in this system of intercellular ROS-mediated induction of apoptosis is based on the release of peroxidase and/or nitric oxide (NO). This effector function can be exerted by non-transformed as well as by transformed cells, leading either to classical intercellular induction between non-transformed and transformed cells, or autocrine ROS-mediated apoptosis induction within the population of transformed cells. Efficient apoptosis induction in this system requires a sufficient density of the target cells (to ensure optimal dismutation of superoxide anions to hydrogen peroxide) as well as a sufficient effector cell number in order to reach an optimal overall concentration of effector molecules. The combination of these two requirements represents the prerequisite for successful intercellular ROS-mediated induction of apoptosis *in vitro* and may also be relevant *in vivo*. Whereas cells transformed *in vitro* are regularly and specifically sensitive to intercellular induction of apoptosis and autocrine self-destruction, independently of the origin of tissue and the transforming principle, *bona fide* tumor cells established from tumors are resistant to intercellular induction of apoptosis (50-54). Therefore, ROS-mediated apoptotic signaling in transformed cells has been discussed as a potential early control step during oncogenesis (21). Mathematical modeling supports this assumption (85), demonstrating that elimination of malignant cells through ROS-mediated induction of apoptosis should be able to reduce the pool of transformed cells despite their ROS-driven proliferation. Malignant cells may, however, escape this control and form tumors as soon as they acquire resistance through expression of membrane-associated catalase. Tumor cells, like cells transformed *in vitro*, exhibit marked NOX1-dependent superoxide anion generation (28, 47, 52-55). The resistance of tumor cells to intercellular ROS signaling depends on the expression of membrane-associated catalase on the outer surface of the tumor cells (52-54). Catalase-mediated protection of tumor cells from intercellular ROS signaling was found in all human and rodent tumor cell lines studies so far (more than 70 human tumor cell lines have been tested; Bauer, unpublished finding). The specific location of tumor cell-protective catalase on the cell surface was proven through i) staining of live tumor cells (but not of non-

transformed cells) with antibody towards catalase and competition of free catalase to this staining; ii) re-establishment of intercellular ROS signaling after binding of neutralizing antibodies directed towards catalase; iii) abrogation of resistance after intensive trypsinization (54). After trypsinization, the antibody against catalase exhibited no additional effect, indicating that the same target had been attacked by trypsinization and the antibody to catalase (54). Finally, tumor cells were protected against extracellular peroxynitrite and this protection was counteracted by catalase inhibition, indicating that catalase must be active on the outside of the cell, allowing peroxynitrite to be neutralized before it reaches the cell membrane (54). In contrast, non-transformed cells were not protected against apoptosis induction by exogenous peroxynitrite. The degree of their sensitivity for peroxynitrite was not enhanced when their intracellular catalase was inhibited by a cell-permeable inhibitor, as intracellular catalase cannot prevent the reaction of peroxynitrite with the cell membrane when peroxynitrite approaches the membrane from outside the cell. Catalase-mediated protection of tumor cells from apoptotic ROS signaling is perfectly matching with the H_2O_2-catabolizing phenoptype, as defined by the classical work of Galina Deichman's group (12-16). Their experimental tumor progression studies demonstrated a remarkable increase in tumorigenicity when *in vitro* transformed hamster cell populations, that had been inoculated into syngeneic animals for the establishment of tumor formation, were compared to *bona fide* tumor cells obtained from the arizing tumors. This increase in tumorigenicity was of several magnitudes and was strictly associated with the expression of resistance to oxidative stress (H_2O_2-catabolizing phenotype). Resistance to oxidative stress was discussed as efficient protection from the attack by neutrophils and macrophages that utilize specific ROS signaling for their attack towards malignant cells (12). A direct comparison of five *in vitro* transformed cell lines (transformed by different transformation principles) with corresponding tumor cell lines that had been isolated from tumors established through inoculation of the transformed cells, confirmed that all transformed and tumor cells exhibited marked extracellular superoxide anion production, but showed that the tumor cells were protected from ROS-mediated apoptosis induction through expression of membrane-associated catalase (Deichman and Bauer, in preparation). The characterization of the H_2O_2-catabolizing phenotype of tumor cells by Deichman's group represents the central intellectual concept and experimental key for the understanding of tumor cell resistance to ROS signaling. The findings on superoxide anion production by malignant cells, the characterization of ROS-dependent intercellular induction of apoptosis specifically in malignant cells, the acquisition of the H_2O_2-catabolizing phenotype during tumor progression and the correlation between this phenotype and the occurrence of protective membrane-associated catalase, allow a rather coherent and novel picture of the role of ROS during multistep oncogenesis to be drawn (Figure 1). Activation of oncogenes, inactivation of tumor suppressor genes and acquisition of independence of senescence control seem to be the primary prerequisites for the establishment of the transformed state. Transformed cells have the potential to form tumors, but they are different from *bona fide* tumor cells (*i.e.* cells finally found in tumors), with respect to the effects of ROS signaling. Transformed cells, as well as tumor cells generate extracellular superoxide anions that establish a ROS-dependent proliferation stimulus. This proliferation stimulus has been suggested to be mediated by the superoxide anions directly (86, 87), or through their dismutation product hydrogen peroxide (55, 57, 88). The generation of superoxide anions by transformed and tumor cells occurs in a rather sustained way, whereas a limited and tightly regulated superoxide anion production also plays a role for the control of proliferation of non-transformed cells (89-91). The low level of superoxide anion production of non-transformed cells is not sufficient to establish HOCl- or NO/peroxynitrite-dependent induction of apoptosis (31, 35), in contrast to transformed cells. Whereas transformed cells are subject to ROS-dependent intercellular induction of apoptosis (either through interaction with normal neighbouring cells, or in an autocrine mode), tumor cells possess acquired resistance against intercellular ROS-mediated apoptosis induction through expression of membrane-associated catalase (52-54). The expression of membrane-associated catalase in tumor cells is under constant positive control by H_2O_2. Removal of H_2O_2, thus, has a strong modulatory effect on catalase-mediated protection of tumor cells (Bauer, unpublished findings). The difference between transformed and tumor cells with respect to the expression of membrane-associated catalase is not as clear-cut as that demonstrated in Figure 1 for reasons of simplicity, rather it represents a gradual though profound difference. Transformed cells (but not non-transformed cells) are also found to express detectable concentrations of catalase on their surface, but the concentration of the enzyme is too low to prevent ROS signaling. In contrast, tumor cells adjust their membrane-associated catalase to a level that completely blocks ROS signaling. The involvement of ROS in tumor initiation and progression, as well as further pro-carcinogenic ROS-related effects, such as the induction of genomic instability (67), effects on angiogenesis (63, 64) and modulation of the metastatic potential (65, 66), are not shown in Figure 1. Multiple pro-carcinogenic effects of ROS, thus, seem to be counterbalanced by induction of ROS-dependent apoptosis of malignant cells. The exact knowledge of the biochemical basis for intercellular ROS signaling and resistance to this feature of malignant cells allows novel therapeutic approaches to be under-taken in the future, that will be based on the

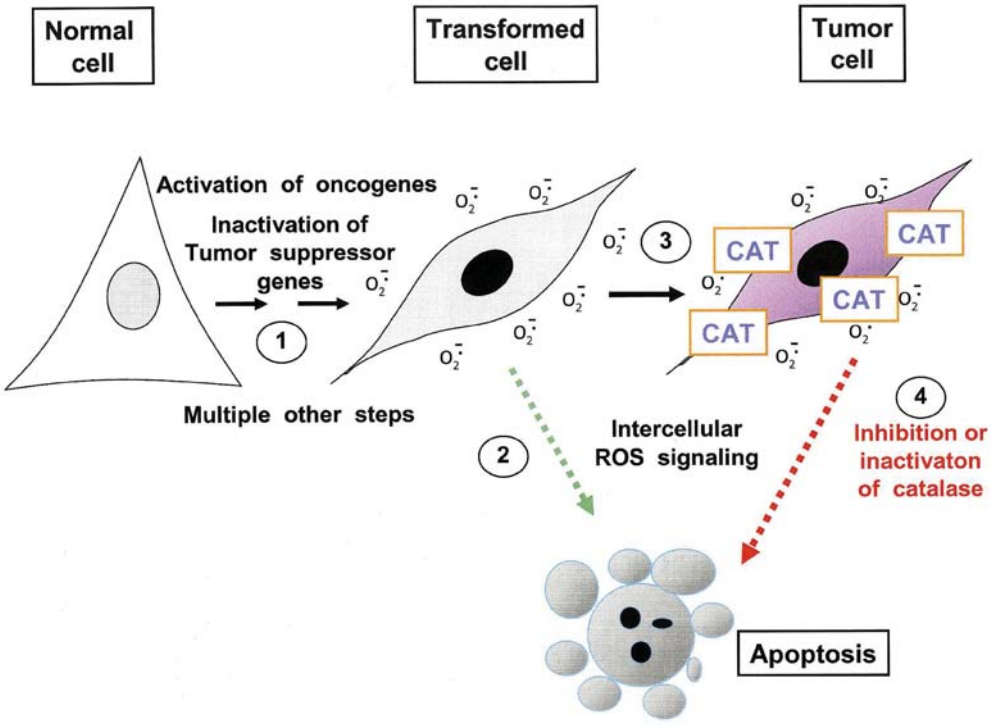

Figure 1. *Central reactive oxygen species (ROS)-related changes during transformation and tumor progression. Among many transformation-relevant cellular changes the continuous production of extracellular superoxide anions by membrane-associated NADPH oxidase (NOX1), controlled by the RAS-related small GTPase RAC1, represents one central hallmark of the transformed state (25-49, 55). Superoxide anions and their dismutation product H_2O_2 control the proliferation and the transformed state of the cells. However, they are also the basis for selective and efficient ROS-dependent intercellular induction of apoptosis that causes specific elimination of the transformed cells and that therefore seems to represent a basic tumorpreventive control system (21-24, 30, 32, 33, 35, 68-79). Tumor progression requires acquisition of resistance against intercellular ROS signaling through expression of membrane-associated catalase (CAT) (52-54), thus establishing the H_2O_2-resistant phenotype as defined by Deichman (12-16). Tumor cells maintain generation of extracellular superoxide anion through NADPH oxidase (NOX1) (28, 34, 39, 47, 52-55). Inhibition or inactivation of catalase is proposed as a novel therapeutic approach, based on the re-establishment of specific intercellular apoptotic ROS signaling of malignant cells. The figure does not show further ROS-related effects in tumor development and progression, such as induction of genomic instability, involvement in tumor inititation and tumor promotion, induction of cell motility, effects on angiogenesis and enhancement of metastasis.*

inhibition or inactivation of catalase, or the prevention of its expression. These aspects are the focus of this article.

ROS-dependent Apoptotic Signaling in Malignant Cells

The basic features of intercellular ROS-dependent induction of apoptosis are presented in Figure 2: Superoxide anions that are generated specifically by transformed cells through membrane-associated NOX1 are the basis for selective induction of apoptosis through the HOCl- and the NO/peroxynitrite pathways. The HOCl signaling pathway (steps 1-7 in Figure 2) depends on the dismutation of superoxide anions to hydrogen peroxide (30). Hydrogen peroxide is then utilized by a novel peroxidase for the synthesis of HOCl. This peroxidase is released by non-transformed, as well as transformed, cells. HOCl in the micromolar concentration range does not affect the cells directly (31, 33). However, the interaction of HOCl with superoxide anions (92-94) leads to the generation of hydroxyl radicals with the ability to trigger the onset of apoptosis through lipid peroxidation. As superoxide anions and hydroxyl radicals have relatively short free diffusion path lengths (95, 96), apoptosis induction is selectively directed towards the transformed target cells. As non-transformed cells generate much fewer extracellular superoxide anions than their transformed counterparts (30-32, 35), they are not affected by HOCl in the micromolar concentration range. In the case of a vast excess of hydrogen peroxide compared to peroxidase, consumption of HOCl through the reaction with hydrogen peroxide ($H_2O_2 + HOCl \rightarrow H_2O_2 + O_2 + H^+ + Cl^-$) may lead to a significant blunting of HOCl signaling. The NO/peroxynitrite signaling pathway depends on the release of NO by non-transformed or transformed cells. NO is

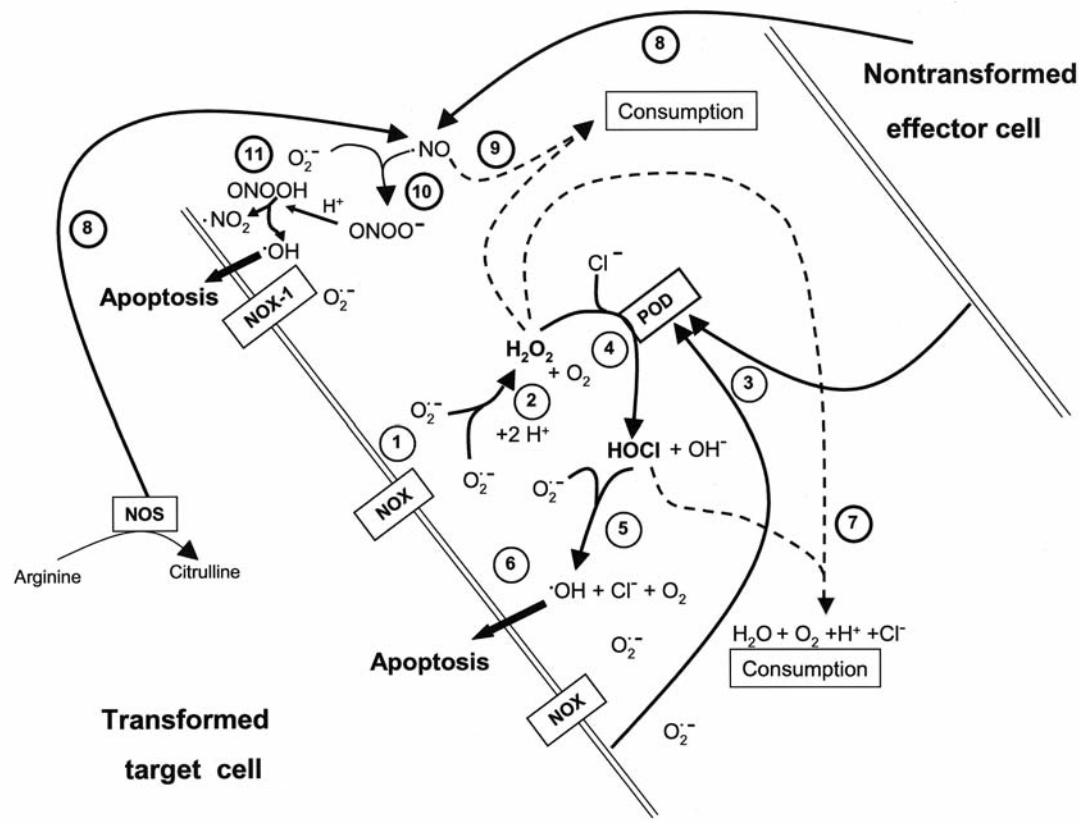

Figure 2. *Intercellular and autocrine reactive oxygen species (ROS)-dependent apoptotic signaling. Transformed target cells generate extracellular superoxide anions (step 1), whereas neighboring non-transformed cells, as well as the transformed cells themselves contribute two effector molecules: peroxidase (POD, step 3) and NO (step 8). The interplay between superoxide anions and the effector molecules establish two major signaling pathways. The HOCl pathway depends on dismutation of superoxide anions to H_2O_2 (step 2), synthesis of HOCl through peroxidase (step 4), formation of hydroxyl radicals through HOCl/superoxide anion interaction (step 5). Hydroxyl radicals induce apoptosis through lipid peroxidation, followed by sphingomyelinase activation, ceramide formation and induction of the mitochondrial pathway of apoptosis. Excess H_2O_2 blunts HOCl signaling through a consumption reaction (step 7): $H_2O_2 + HOCl \rightarrow H_2O + O_2 + H^+ + Cl^-$. NO is generated by NO synthase (NOS) (step 8) and is efficiently removed in a complex consumption reaction (step 9). Free NO readily interacts with superoxide anions and thus generates peroxynitrite (step 10). The protonated form of peroxynitrate decomposes into NO_2 and hydroxyl radicals (step 11). This figure also shows that selective apoptosis induction in transformed cells by either interaction with non-transformed cells or within the transformed cell population (autocrine self-destruction) is based on the same signaling chemistry.*

generated within the cells through the action of NO synthase (NOS), either inducible NOS (iNOS), neuronal NOS (nNOS) or endothelial NOS (eNOS), which utilize arginine as their substrate (97, 98). The available concentration of NO in the intercellular space is controlled by a complex consumption reaction between NO and hydrogen peroxide. This reaction depends on the oxidation of NO, subsequent generation of N_2O_3 and the reaction of N_2O_3 with hydroperoxide anions (Bauer, unpublished findings). Free NO and superoxide anions derived specifically from transformed cells interact and form peroxynitrite in a diffusion-controlled reaction (99-102). After protonation, peroxynitrite rapidly decomposes into NO_2 and apoptosis-inducing hydroxyl radicals (103, 104). The nitryl chloride signaling pathway (83) and the metal ion-catalyzed Haber-Weiss reaction (84) are of minor importance and are not shown in Figure 2. Both pathways depend on the availability of superoxide anions and hydrogen peroxide. The intercellular ROS-dependent signaling chemistry outlined in Figure 2 is further substantiated in Figure 3. This figure focuses on the signaling chemistry during autocrine ROS signaling for the sake of simplicity of the scheme. This reduction is justified, as the interaction between non-transformed and transformed cells follows the same signaling chemistry as the autocrine process, as can be deduced from Figure 2. This deduction has been experimentally verified. The combination of siRNA-based analysis, inhibitor studies and reconstitution experiments allowed the essential players and their reactions during ROS-mediated signaling to be defined. Figure 3 points to the role of activation of the rat sarcoma oncogene (RAS) and and the RAS-related small

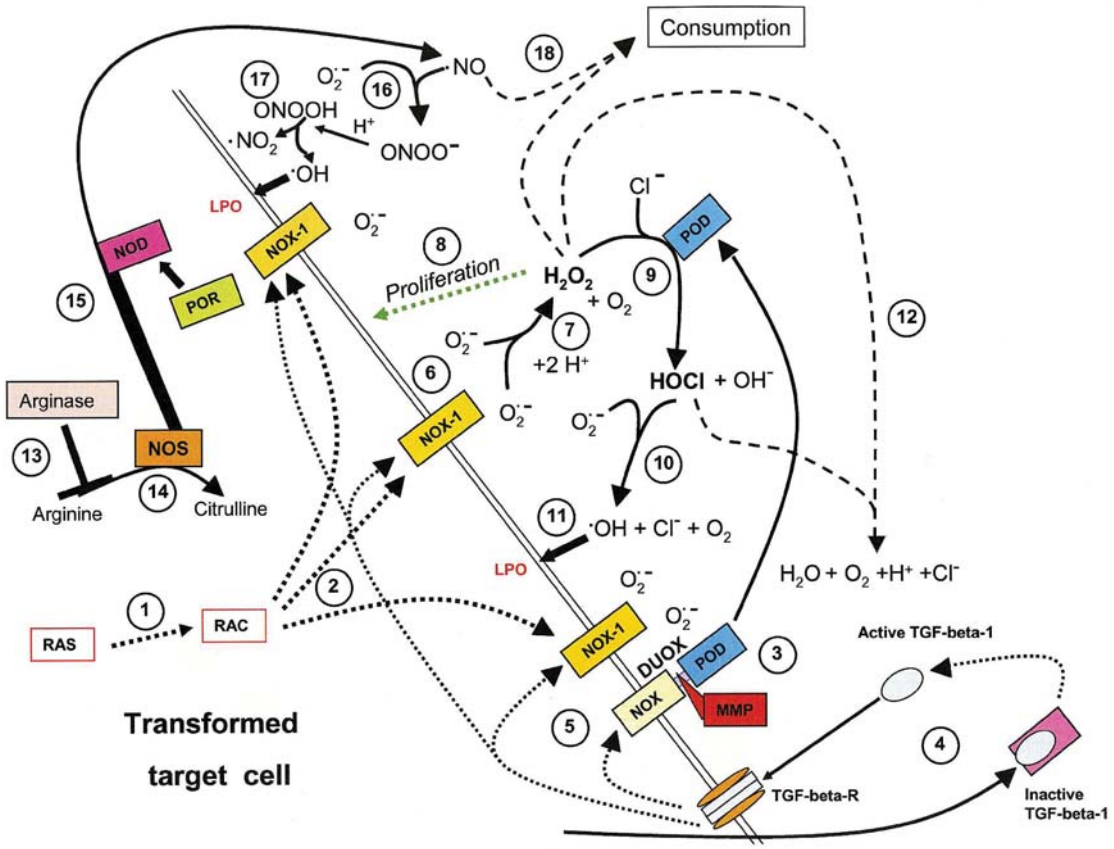

Figure 3. *Detailed picture of central players of intercellular reactive oxygen species (ROS)-mediated apoptotic signaling. siRNA-based analysis, reconstitution experiments and inhibitor studies have allowed to define the essential players in intercellular ROS signaling. NOX1 represents the NADPH oxidase that is activated by rat sarcoma oncogene (RAS) through the RAS-related small GTPase RAC1 (25). The peroxidase domain of dual oxidase (DUOX) is removed from DUOX through matrix metalloproteases (MMP). Inactive transforming growth factor type-β (TGF-β) is released by the cells and, upon activation through conformational change activates its receptor. As a result, synthesis of NOX1 and DUOX is increased, resulting in enhanced superoxide anion production and peroxidase release. Arginase controls the levels of arginine, the substrate for NO synthase (NOS). A substantial part of NO generated by NOS is dioxygenated by NO dioxygenase (NOD) that is controlled by cytochrome P450 oxido-reductase. Please find details in the text.*

GTPase RAC for the activity of the NADPH oxidase NOX1 (steps 1 and 2). The HOCl-synthesizing peroxidase is coded by the peroxidase domain of dual oxidase (DUOX) (105-107). The peroxidase domain is freed through the action of matrix metalloproteases that are inhibited by galardin (step 3) (108). Release of the peroxidase is not a prerequisite for its activity. The cells release inactive transforming growth factor type-beta1 (TGF-β1), a complex between TGF-β1 and the large latency-associated protein, which is subsequently activated through a change of the conformation of the inhibitory latency-associated protein (step 4). The interaction of active TGF-β1 with its receptor leads to an induction of NOX1 as well as to DUOX expression. *In vitro*, intercellular ROS signaling is significantly stimulated by the addition of exogenous TGF-β1 and severely inhibited by neutralizing antibodies directed towards TGF-β1, or through siRNA-based knockdown of either TGF-β1 or its receptor. The level of free NO is controlled at several central points that co-operate in the modulation of the efficiency of the NO/peroxynitrite signaling pathway. First of all, the level of arginine is controlled by the activity of cellular arginase (step 13). The concentration of active NO synthase then defines the rate of NO synthesis (step 14). A substantial amount of NO is converted to nitritate through the action of NO dioxygenase (NOD) (step 15) (109-112), which is interconnected to the activity of cytochrome P450-dependent oxido-reductase POR (111). NO passes the cell membrane readily and is then channelled either into the complex consumption reaction with hydrogen peroxide (step 18) or to the interaction with superoxide anions, resulting in the formation of peroxynitrite (step 16). The complex scenario outlined in Figure 3 also shows the positive effect of ROS signaling for the cells,

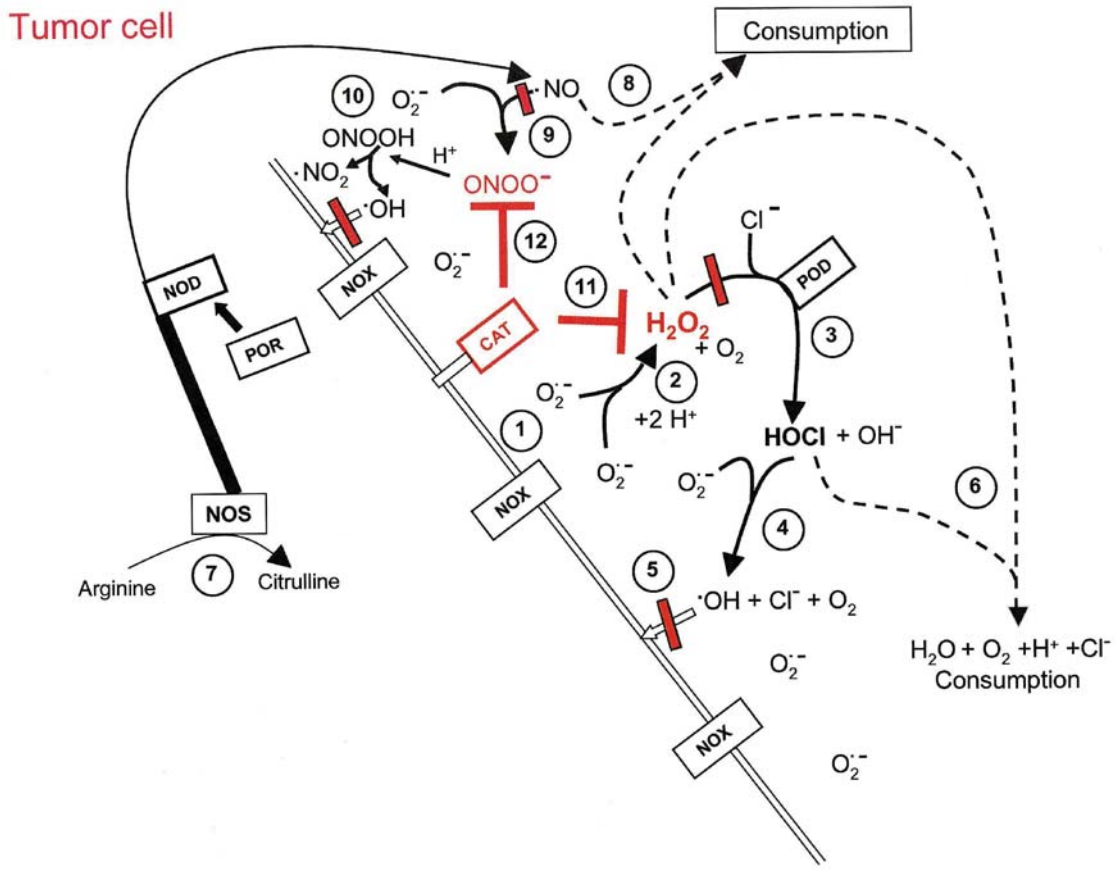

Figure 4. *Catalase protects tumor cells against intercellular reactive oxygen species (ROS) signaling. Efficient tumor progression resulting in tumor formation requires the establishment of resistance to ROS signaling (52-54). This is achieved uniformly in all tumor cell systems studied so far, through expression of membrane-associated catalase on the outer surface of the tumor cells. Catalase interferes with HOCl signaling (steps 1-5) through decomposition of H_2O_2 (step 11) and with the NO/peroxynitrite pathway (steps 9 and 10) through decomposition of peroxynitrite (step 12) and oxidation of NO through compound I (not shown in Figure 4, for details see Figure 5). Decomposition of H_2O_2 also inhibits the nitryl chloride pathway and the metal ion-catalyzed Haber-Weiss reaction (data not included in this figure).*

simplified by the proliferation-stimulating activity of hydrogen peroxide (step 8) (55, 57). Other groups suggest a direct role of superoxide anions for proliferation stimulus (86, 87). These positive ROS-dependent effects on the cells are counterbalanced by severe negative effects directed against transformed cell survival. These effects are mediated by hydroxyl radicals derived either from the interaction between superoxide anions and HOCl (92-94), or the decomposition of protonated peroxynitrite (103, 104). Hydroxyl radicals cause lipid peroxidation that transmits the apoptosis-inducing signal through sphingomyelinase activation, ceramide formation, activation of the mitochondrial pathway of apoptosis, caspase-9 and caspase-3 activity (data not shown). During tumor progression, tumor cells acquire resistance to intercellular ROS signaling through expression of membrane-associated catalase (52-54). The tumor cell phenotype characterized by the combination of activated NOX1 and membrane-associated catalase correlates with the hydrogen peroxide-catabolizing phenotype of tumor cells as characterized by Deichman *et al*. (12-16). Figure 4 demonstrates that membrane-associated catalase of tumor cells interferes with HOCl signaling through removal of hydrogen peroxide (and thus prevention of HOCl production) and with NO/peroxynitrite signaling, through decomposition of peroxynitrite. Catalase also efficiently blocks the nitryl chloride signaling pathway, as the inhibition of HOCl production also prevents formation of nitryl chloride through HOCl/nitrite interaction. Finally, catalase prevents the metal ion-dependent Haber-Weiss reaction, as this signaling pathway depends on decomposition of hydrogen peroxide through Fenton chemistry. Thus, the expression of only one phenotypic marker, *i.e.* membrane-associated catalase, prevents induction of apoptosis by all four ROS-dependent intercellular signaling pathways in tumor cells. This feature

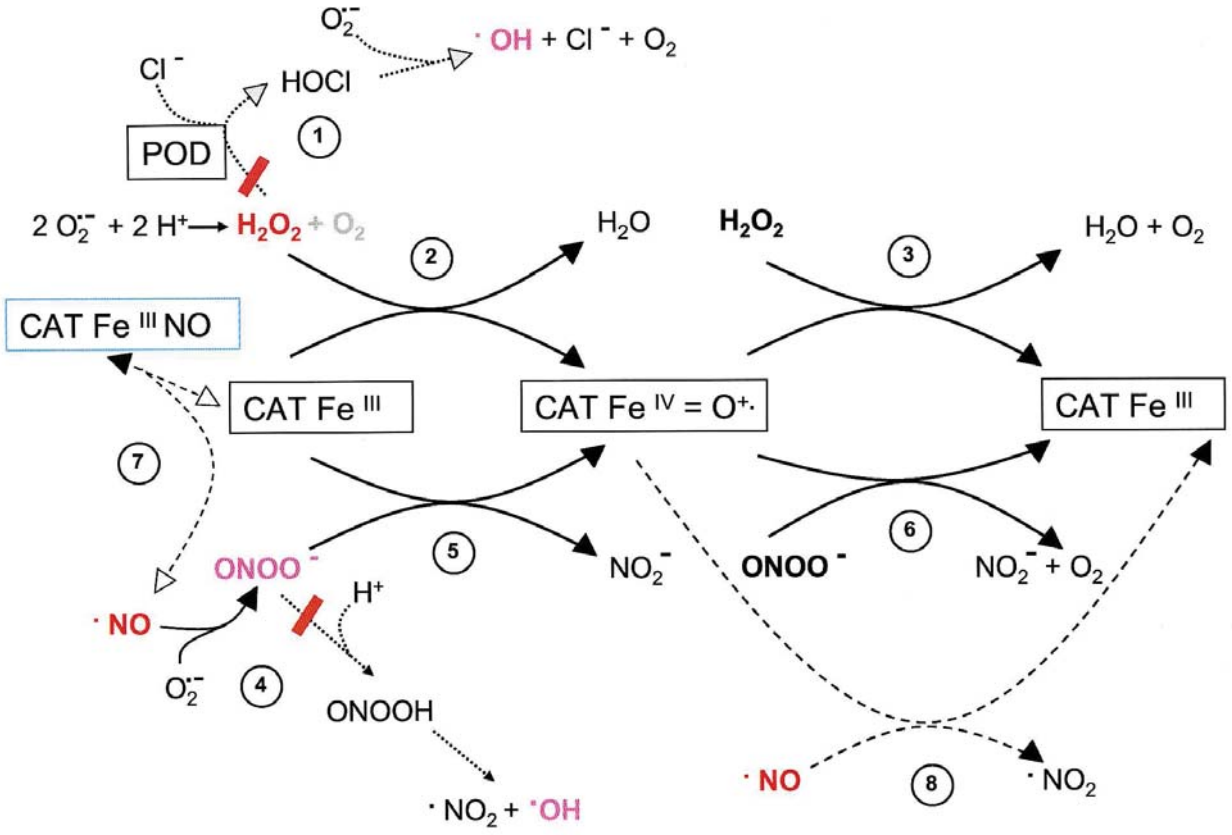

Figure 5. *Multiple protective functions of catalase against reactive oxygen species (ROS) signaling. HOCl signaling (step 1) is prevented through removal of H_2O_2 in a two-step reaction (steps 2 and 3), in which native catalase (CAT Fe^{III}) reacts with the first molecule of H_2O_2, resulting in formation of water and compound I (CAT Fe^{IV}=$O^{+\cdot}$), which, in a reaction with H_2O_2, generates water and molecular oxygen and is thereby converted back to CAT Fe^{III}. Peroxynitrite is generated through the reaction between superoxide anions and NO (step 4). Peroxynitrite is decomposed by catalase in a two-step reaction (steps 5 and 6) in analogy to H_2O_2, involving intermediate formation of compound I. Free NO can inhibit catalase in a reversible mode (step 7, K_i=0.18 μM) or is oxidized by compound I (step 8) and thus removed from signaling.*

defines membrane-associated catalase of tumor cells as an attractive target for selective antitumor therapy based on ROS-mediated induction of apoptosis. The protective function of catalase depends on its multiple reaction pathways, as summarized in Figure 5: HOCl signaling (step 1) is prevented through the classical catalase reaction (steps 2 and 3), in which the native catalase ($CATFe^{III}$) interacts with hydrogen peroxide in a first step, resulting in the formation of compound I (CAT FeIV=$O^{+\cdot}$) and water. Compound I then interacts with a second molecule of hydrogen peroxide, yielding native catalase, water and molecular oxygen. The inhibitory effect of catalase on the NO/peroxynitrite pathway (step 4) depends on the potential of catalase to use peroxynitrite as a substrate for a two-step reaction (steps 5 and 6), that involves compound I formation in an analogous way as during the interaction of the enzyme with hydrogen peroxide. The differentiation between true peroxynitrite decomposition by catalase from a theoretically conceivable action of catalase on an hydrogen peroxide-mediated step induced by peroxynitrite was recently shown (54). The differentiation utilized the ability of catalase compound I to subtract two hydrogen atoms from methanol, resulting in formation of formaldehyde that can be easily detected by purpald staining. It was shown that in the presence of peroxynitrite (and the absence of hydrogen peroxide), catalase indeed caused formaldehyde formation from methanol. This confirms the direct interaction of peroxynitrite with catalase, leading to enzymatic peroxynitrite decomposition. This conclusion is in perfect agreement with the findings of Gebicka and Didil (113) and Kono *et al.* (114). The work of other groups has shown that NO can inhibit catalase reversibly (step 7) (115). This inhibition is characterized by an enzyme inhibitor constant (Ki) of 0.18 μM NO. Finally, compound I has the potential to oxidize NO and thus to remove NO from the system (step 8) (116). This potential adds to the protection of tumor cells as it counterbalances the inhibitory effect of NO on catalase and also interferes with NO/peroxynitrite signaling.

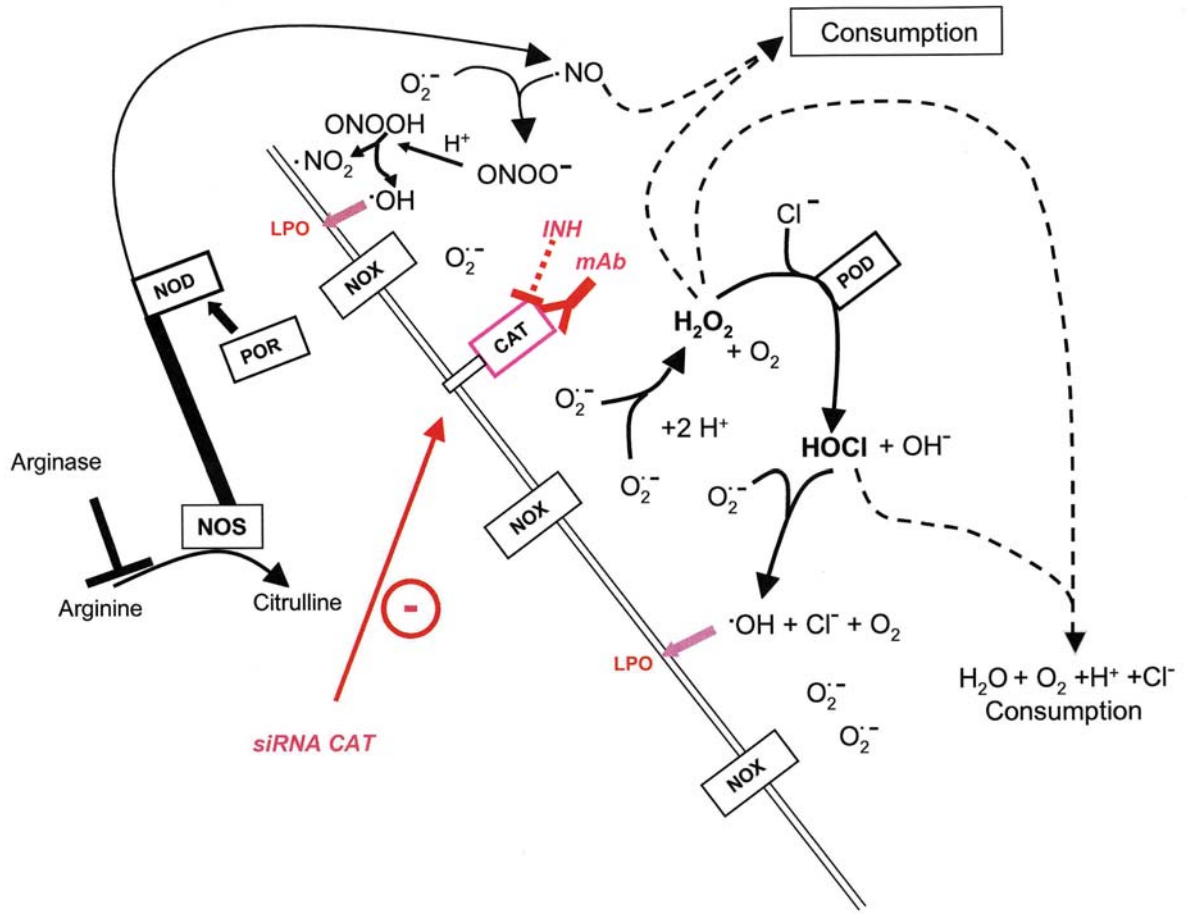

Figure 6. *Restoration of intercellular reactive oxygen species (ROS)-mediated apoptotic signaling of tumor cells through inhibition of catalase activity or its expression. Inhibitors of catalase like 3-aminotriazole (3-AT), monoclonal antibodies towards catalase, and siRNA-mediated knockdown of catalase have the potential to restore intercellular ROS signaling and subsequent selective apoptosis induction in tumor cells.*

Sensitization of Tumor Cells to ROS-mediated Intercellular Apoptotic Signaling through Catalase Inhibition or siRNA-mediated Knockdown of its Expression

Figure 6 demonstrates that tumor cells can be sensitized to their own specific intercellular ROS signaling when membrane-associated catalase is inhibited by small inhibitors such as 3-aminotriazole (3-AT), or monoclonal antibodies directed towards catalase, or when the expression of catalase is prevented through siRNA-based knockdown (52-54). These three approaches have been utilized for the analysis of catalase-mediated protection of tumor cells (52-54). The specificity of these approaches was assured as i) intercellular ROS signaling-controlled induction of apoptosis was activated after catalase inhibition or knockdown of its expression, as ii) the apoptosis-inducing ROS-signaling dependent effects of specific antibody towards catalase or siRNA-based knockdown were specifically competed with by the addition of exogenous catalase, and iii) the apoptosis-inducing effect of the catalase inhibitor 3-AT was abrogated through addition of the catalase mimetic chloro[[2,2'-[1,2-ethanediylbis [(nitrilo-κN)methylidyne]]bis[6-methoxyphenolato-κO]]]-manganese (EUK-134). The salen-manganese complex EUK-134 exhibits an activity analogous to that of catalase but is not inhibited by 3-AT (117). Whereas 3-AT and siRNA-based knockdown affect both membrane-bound catalase (specific for tumor cells) and intracellular peroxisomal catalase (which is not specific for tumor cells), the monoclonal antibody acts exclusively on the membrane-associated catalase due to its lack of cell permeability. The inactivation of intracellular catalase may contribute to the efficiency of ROS-mediated intercellular induction of apoptosis, as this process is linked to intracellular ROS-dependent effects. Normal cells which lack the potential for intercellular signaling and have no extracellular catalase, were not affected by the inhibition of their intracellular

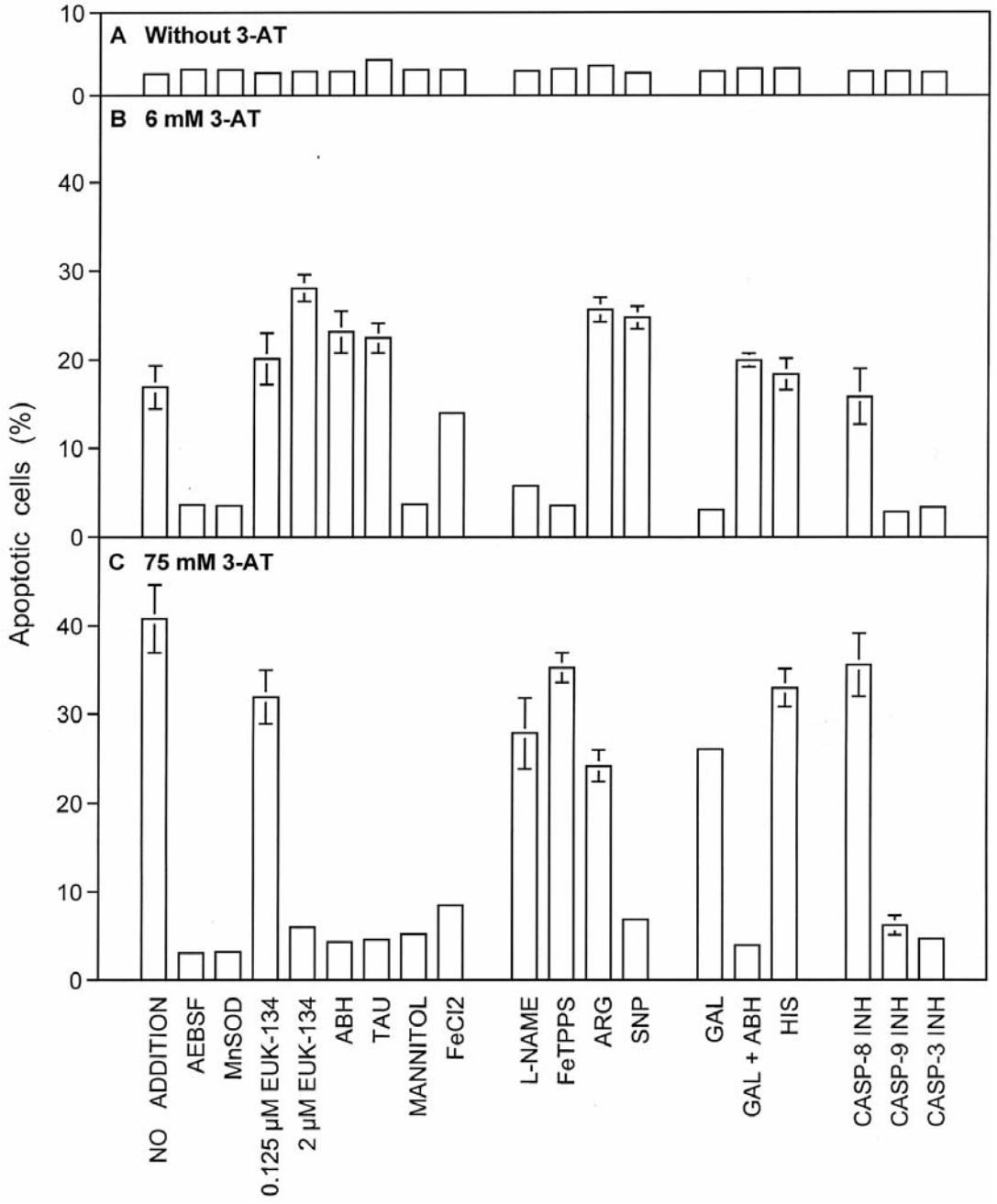

Figure 7. *NO/peroxynitrite and HOCl signaling after differential inhibition of catalase in MKN-45 gastric carcinoma cells. A total of 12,500 MKN-45 human gastric carcinoma cells in 100 µl RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), were incubated in the absence or the presence of 6 mM or 75 mM or the catalase inhibitor 3-aminotriazole (3-AT). Assays contained no further additions or the indicated inhibitors: 100 µM of the NADPH oxidase inhibitor 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF), 60 U/ml Mn superoxide dismutase (MnSOD), 0125 µM or 2 µM of the catalase mimetic chloro[[2,2'-[1,2-ethanediylbis[(nitrilo-κN)methylidyne]]bis[6-methoxyphenolato-κO]]]-manganese (EUK-134), 150 µM of the peroxidase inhibitor 4-amino benzoyl hydrazide (ABH), 50 mM of the HOCl-scavenger taurine, 10 mM of the hydroxyl radical-scavenger mannitol, 20 µM $FeCl_2$, 2.4 mM of the NOS inhibitor N-ω-nitro-L-arginine methylester hydrochloride (L-NAME), 20 µM of the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS), 0.75 mM arginine, 0.05 mM of the NO donor sodium nitroprusside (SNP), 10 µM of the matrix metalloprotease inhibitor galardin, 2 mM of the singlet oxygen-scavenger histidine, 25 µM of the caspase-8 inhibitor, 25 µM of the caspase-9 inhibitor or 50 µM of the caspase-3 inhibitor. After 4 h at 37°C, the percentage of apoptotic cells was determined by the classical morphological criteria for apoptosis (nuclear condensation, nuclear fragmentation and membrane blebbing). Please find technical details for cell culture, reagents and quantitation of apoptotic cells in reference 54 and the discussion of the signaling chemistry in the text.*

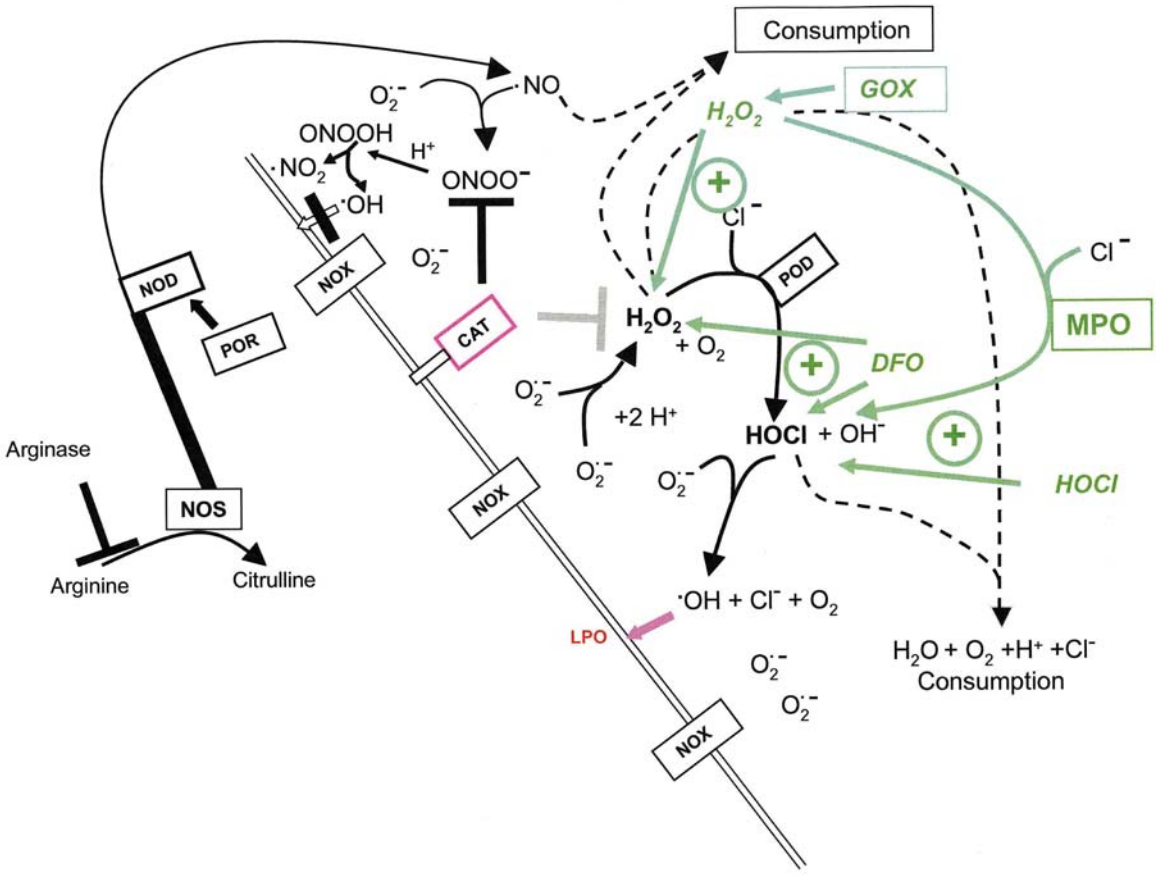

Figure 8. *Establishment of intercellular reactive oxygen species (ROS) signaling through addition of exogenous signaling components. Addition of exogenous HOCl causes apoptosis induction in tumor cells through HOCl/superoxide anion interaction and subsequent hydroxl radical generation. Whereas tumor cell catalase efficiently prevents HOCl synthesis of tumor cells, it ensures optimal action of exogenously added HOCl through removal of H_2O_2. In the presence of the ferric ion chelator deferoxamine (DFO), consumption of HOCl and H_2O_2 through Fenton chemistry is inhibited. Fenton chemistry distant from the cell membrane does not contribute to apoptosis induction in the tumor cells (as HOCl and H_2O_2 are relatively far-ranging molecular species and hydroxyl radicals generated through the action of ferrous ions cannot reach the cell membrane) but rather inhibits the signaling process. Abrogation of HOCl and H_2O_2 consumption through removal of ferric ions (which are centrally involved in Fenton chemistry after being reduced to ferrous ions by superoxide anions) causes apoptosis induction due to enhancement of the specific signaling effects at the cell membrane that are no longer controlled by catalase. Addition of H_2O_2 –generating glucose oxidase (GOX) causes apoptosis induction through re-establishment of the HOCl signaling pathway. This effect is synergized by addition of exogenous peroxidase such as myeloperoxidase (MPO). Re-establishment of HOCl signaling has been initially interpreted as mere overrunning of the protective potential of catalase (51, 52). Recent experiments show that a more complicated process is involved in this process, as further discussed in Figure 9.*

catalase. This is explained by the lack of extracellular ROS-derived lipid peroxidation and the redundancy of intracellular antioxidant defence, where catalase, glutathione, glutathione peroxidase and other enzymes work together to cope with intracellular oxidative stress. Nevertheless, the selective inhibition of tumor cell membrane-associated catalase by monoclonal antibodies seems to represent a superior approach for future therapeutic approaches based on catalase inhibition and induction of ROS-mediated induction of apoptosis due to its specific action on tumor cell membrane-associated catalase. Experimental data demonstrating the sensitization of tumor cells to ROS-mediated induction of apoptosis after catalase inhibition are shown in Figure 7. The data summarize our findings for the action of the catalase inhibitor 3-aminotriazole (3-AT) on the gastric carcinoma cell line MKN-45. Analogous results have been obtained for the application of antibodies towards catalase and for knockdown of catalase by increasing concentrations of specific siRNA (54). Figure 7 demonstrates that untreated MKN-45 tumor cells only have a minor background apoptotic activity in the absence of the catalase inhibitor. At low concentrations of 3-AT (6 mM), apoptosis was induced and depended exclusively on the NO/peroxynitrite pathway, as it was blocked by inhibition of NADPH oxidase through 4-(2-aminoethyl)benzene-sulfonyl

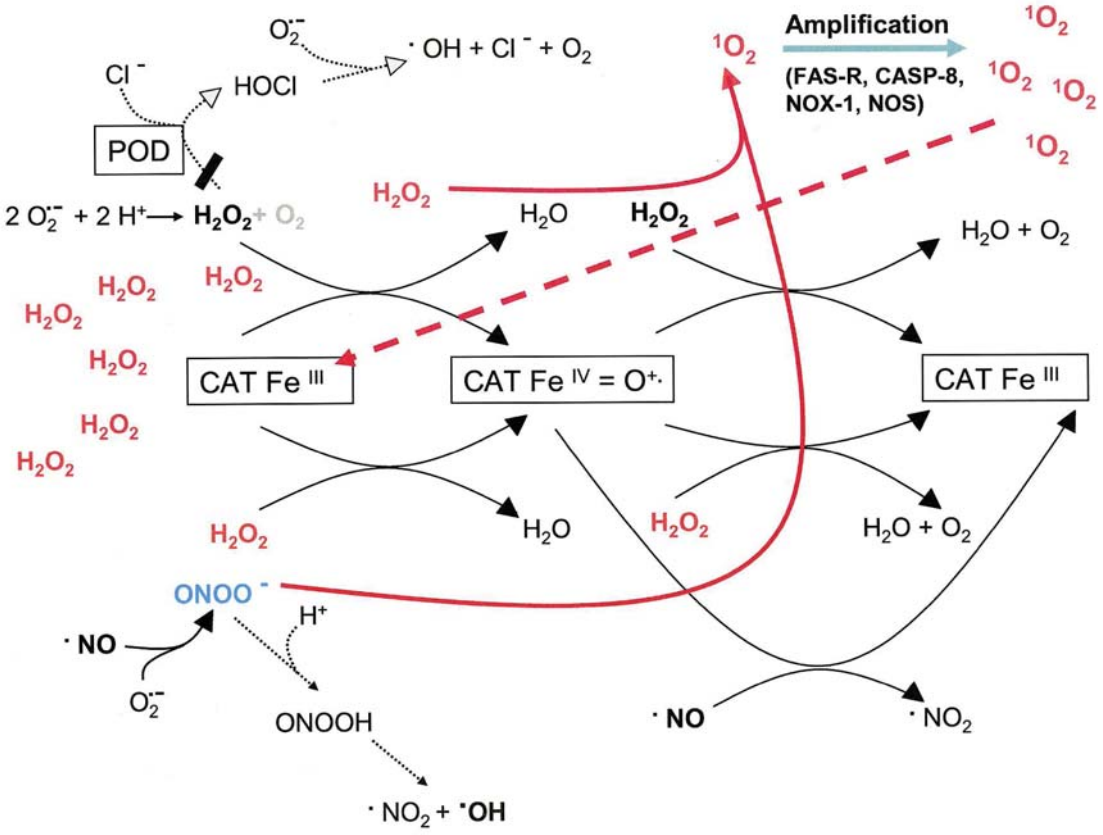

Figure 9. *Excess H_2O_2 causes singlet oxygen generation through displacement of peroxynitrite from catalase. Excess H_2O_2 generated by exogenously added glucose oxidase (GOX) competes with peroxynitrite for catalase and leads to the displacement of peroxynitrite. As a result, H_2O_2 and peroxynitrite interact and form singlet oxygen. Singlet oxygen causes a APO/FAS receptor and caspase-8-mediated amplification step through induction of NADPH oxidase (NOX1) and NO synthase (NOS). As a result, increased concentrations of H_2O_2 and peroxynitrite generate more singlet oxygen that inactivates catalase and allows subsequent intercellular ROS signaling. Please find details of the reactions in the text.*

fluoride (AEBSF), scavenging of superoxide anions through superoxide dismutase (SOD), inhibition of NO synthesis through the NOS inhibitor N-ω-nitro-L-arginine methylester hydrochloride (L-NAME), decomposition of peroxynitrite through the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) and scavenging of hydroxyl radicals through mannitol. The effect of 6 mM 3-AT was slightly increased when either an exogenous NO donor was added, or endogenous NO generation was enhanced by the addition of arginine. Inhibitors specific for the HOCl pathway [such as the HOCl scavenger taurine and the peroxidase inhibitor 4-aminobenzoyl hydrochloride (ABH)] did not inhibit apoptosis induced by 6 mM 3-AT. Prevention of peroxidase release by galardin-mediated inhibition of matrix metalloproteinase activity caused abrogation of NO/peroxynitrite signaling, as the peroxidase localized at the cell membrane decomposes peroxynitrite in a specific enzyme reaction, as seen by the interference with the peroxidase inhibitor ABH. This site-specific effect is not seen when the peroxidase is released from the cells and thus its local concentration at the membrane becomes much lower. When 75 mM 3-AT were added, overall induction of apoptosis increased and were mainly dependent on HOCl signaling, as seen from the inhibition profile. Decomposition of hydrogen peroxide by 2 μM of the catalase mimetic EUK-134, peroxidase inhibition by ABH as well as scavenging HOCl by taurine, caused nearly complete inhibition of apoptosis induction. The involvement of superoxide anions and hydroxyl radicals in intercellular signaling under these conditions was assured by the inhibitory effects of AEBSF, SOD and mannitol. The addition of $FeCl_2$ caused a nearly complete block of the reaction, as this leads to Fenton chemistry-dependent decomposition of hydrogen peroxide and HOCl, distant from the membrane. The resulting hydroxyl radicals are too far away to reach and attack the cell membrane, due to their very low free diffusion path length. At the same time, essential components of HOCl signaling, *i.e.* H_2O_2 and HOCl, then no longer contribute to specific

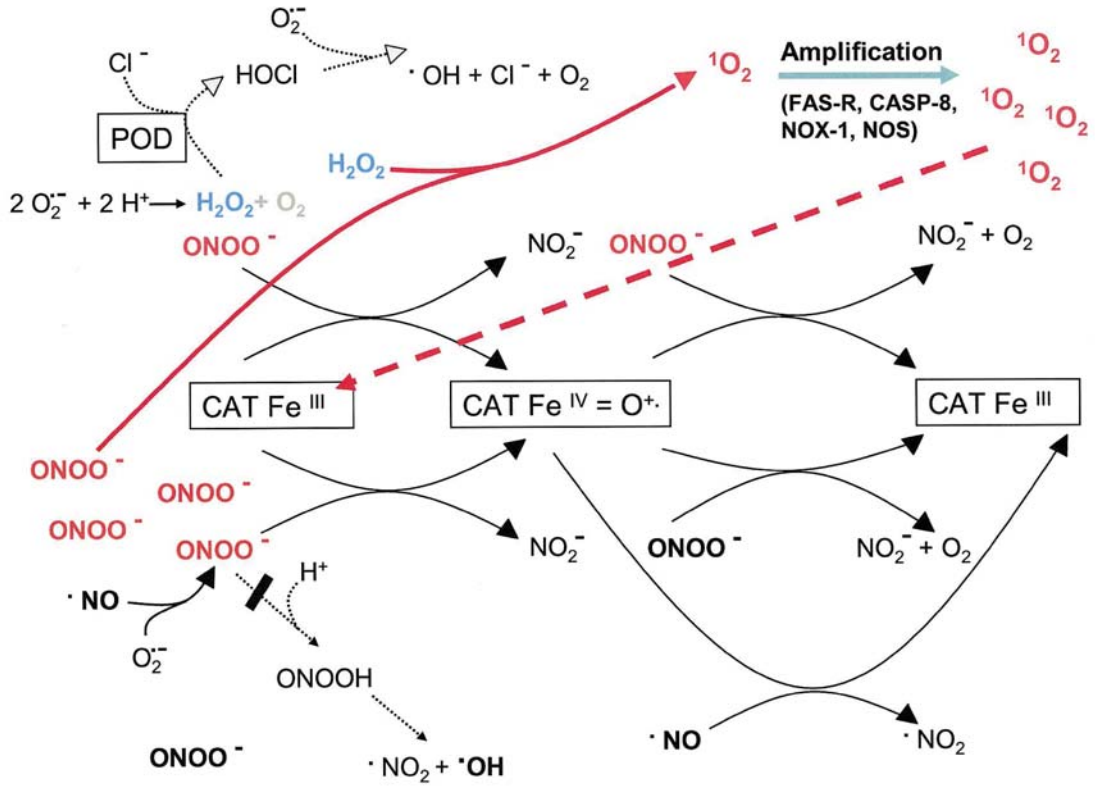

Figure 10. *Excess peroxynitrite causes singlet oxygen generation through displacement of H_2O_2 from catalase. Addition of exogenous peroxynitrite to tumor cells causes displacement of H_2O_2 from catalase and subsequent singlet oxygen generation through H_2O_2/peroxynitrite interaction. The subsequent steps, amplification of singlet oxygen generation, catalase inactivation and re-establishment of intercellular ROS-mediated apoptotic signaling, are identical to those described in Figure 9.*

apoptosis induction (53). The inhibitory effect of the NO donor sodium nitroprusside (SNP) on HOCl signaling is explained by the consumption reaction between NO and hydrogen peroxide. Apoptosis induction at both concentrations of 3-AT was dependent on the activity of caspase-3 and caspase-9, whereas caspase-8 inhibitor had no signficant inhibitory effect. This finding is in good agreement with apoptosis induction through the mitochondrial pathway of apoptosis. The singlet oxygen scavenger histidine had no effect under the conditions of this experiment. These data demonstrate that a low degree of catalase inhibition can be sufficient to reactivate NO/peroxynitrite signaling, whereas a higher degree of inhibition is necessary to establish HOCl signaling. This sequence of events is reasonable, as protection against peroxynitrite, which is directly decomposed at the membrane, requires a high local concentration of protective catalase and therefore reacts sensitively to even low degrees of catalase inhibition. In contrast, the HOCl signaling pathway depends on sufficient concentrations of hydrogen peroxide and therefore requires a higher degree of catalase inhibition than does the NO/peroxynitrite pathway. The reverse sequence of events was seen when intercellular ROS-dependent apoptosis induction in sarcoma oncogene (SRC)-transformed cells was gradually inhibited by addition of increasing concentrations of exogenously added catalase (54): Low concentrations of catalase efficiently blocked intercellular induction in a concentration-dependent mode. Signaling was initially dependent on the sole action of the HOCl signaling pathway, but switched to NO/peroxynitrite signaling with increasing concentrations of exogenous catalase. At defined catalase concentrations, HOCl signaling was completely blocked and NO/peroxynitrite signaling was enhanced due to interference of catalase with the consumption between hydrogen peroxide and NO. Further increase in catalase concentration finally, also abrogated NO/peroxynitrite signaling, but rather high concentrations of catalase were necessary to achieve complete inhibition. This is a reasonable finding, as it requires high concentrations of soluble catalase to mimic the high local concentration at the cell membrane that is a prerequisite for protection from peroxynitrite. These data point to the significance of site-specific interactions in this complex intercellular signaling system and its control. The exact

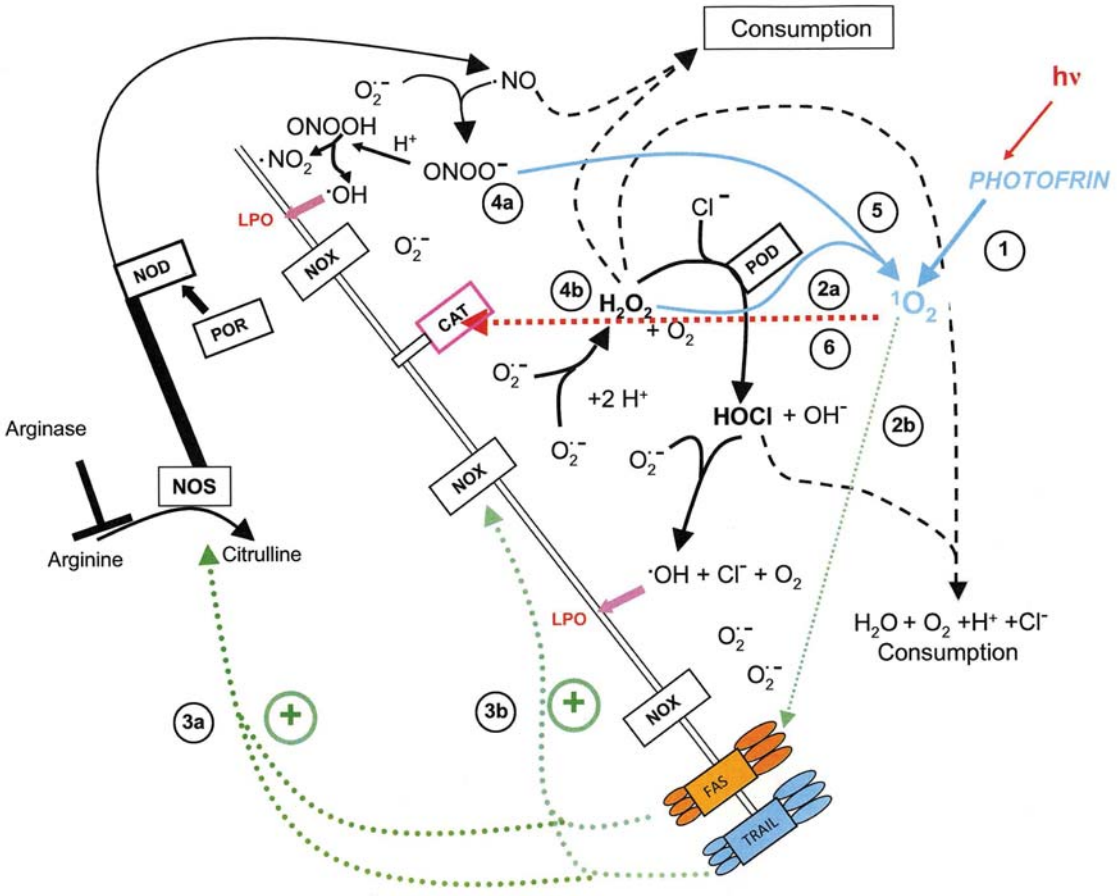

Figure 11. *The exogenous singlet oxygen generator photofrin sensitizes tumor cells to intercellular reactive oxygen species (ROS)-mediated apototic signaling through inactivation of protective catalase. Addition of high concentrations (10 μM) photofrin and application of visible light causes sufficient singlet oxyxgen generation for direct inactivation of catalase (step 2a) and subsequent ROS-mediated apoptotic signaling. Addition of lower photofrin concentrations requires amplification of singlet oxygen generation through APO/FAS receptor activation (step 2b), NADPH oxidase (NOX1) and NO synthase (NOS) stimulation (steps 3a and 3b), reaction between peroxynitrite (step 4a) and hydrogen peroxide (step 4b) which results in singlet oxygen formation (step 5), before catalase is inactivated and ROS-mediated signaling is re-established.*

signaling profile of malignant cells depends on the relative ratios between superoxide anions, peroxidase and NO generated by the tumor cells. In certain tumor cell systems, such as neuroblastoma cells and Ewing sarcoma cells, a relative abundance of NO may be responsible for exclusive signaling through the NO/peroxynitrite pathway, at all 3-AT concentrations tested (54). In these cells, apoptosis induction by 3-AT up to 100 mM was exclusively dependent on superoxide anions (inhibition by AEBSF) and NO (inhibition by the nNOS-specific inhibitor 3-bromo-7-nitroindazole), but was not affected by the HOCl-scavenger taurine, nor by the singlet oxygen-scavenger histidine (54). A similar signaling profile has been found for mammary and ovarian carcinoma cells (Bauer, unpublished results). Work in progress shows that the signaling profiles of human tumor cells in the presence of a catalase inhibitor can be modulated in a predictable way, through modulation of the central players of intercellular ROS signaling. The increase in superoxide anion production shifts signaling towards HOCl signaling, whereas reduction of superoxide anion production and an increase in NO production causes dominant NO/peroxynitrite signaling.

Reestablishment of Intercellular ROS Signaling through Addition of Signaling Components or Deferoxamine (DFO)-dependent Prevention of Side Reactions

ROS-dependent induction of apoptosis in tumor cells can also be achieved by the addition of exogenous HOCl (Figure 8), mimicking a situation in which tumor cells are confronted with activated neutrophils that generate HOCl

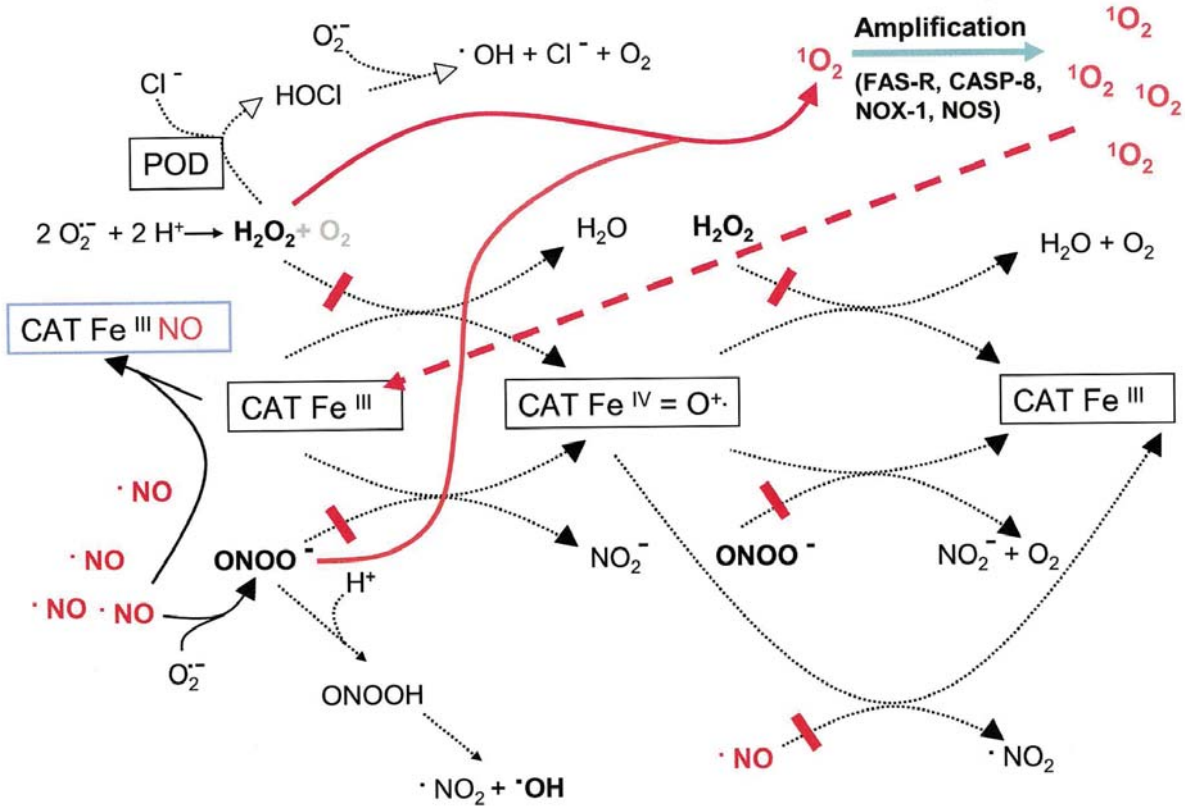

Figure 12. *NO-mediated catalase inhibition causes subsequent singlet oxygen-dependent catalase inactivation. The increase of free NO in tumor cells causes a transient and reversible inhition of catalase through formation of CATFe^{III}NO. As a consequence, the decomposition of H_2O_2 and peroxynitrite by catalase is reduced and these two molecules can interact and form singlet oxygen that triggers its own amplification and subsequent catalase inactivation in analogy to the mechanisms described in Figures 9-11.*

through activated NADPH oxidase and myeloperoxidase (118-120). Apoptosis induction in tumor cells by exogenous HOCl requires free superoxide anions for the interaction with HOCl, leading to the formation of apoptosis-inducing hydroxyl radicals (31). Apoptosis induction by HOCl is more efficient in the presence of active catalase which removes hydrogen peroxide that otherwise might consume HOCl. Addition of DFO, a chelator of ferric ions, causes apoptosis induction in tumor cells. This is explained by the interference of DFO with nondirected Fenton chemistry of hydrogen peroxide and HOCl distant from the cell membrane. As hydrogen peroxide and HOCl are rather far-ranging molecular species, hydroxyl radicals derived from their interaction with ferrous ion are too far away to reach the membrane of the target cells. Therefore, these two central signaling components are used up in a cycle that is driven by superoxide anion-dependent reduction of ferric ions back to reactive ferrous ions. Removal of ferric ions by DFO, thus, causes a dramatic net concentration of signaling components in the milieu of the tumor cells and subsequent apoptosis induction. This system is challenging for analytical goals but does not seem to be too promising for therapeutic approaches, as DFO might interfere with the synthesis of other metal-containing essential enzymes in normal tissue. DFO has been used in tumor therapy (121) and the effects have been discussed in relation to the role of iron for the growth of neuroblastoma cells (122). It is possible that the mechanism described here also contributed to the outcome of the study by Blatt (121). As protection of endogenous hydrogen peroxide and HOCl proved to be sufficient for apoptosis induction in tumor cells, addition of excess hydrogen peroxide (*e.g.* through steady generation by glucose oxidase), alone or in combination with additional peroxidase (*e.g.* myeloperoxidase), should allow restoration of intercellular ROS signaling despite the presence of protective catalase in the cell membrane. This prediction was indeed confirmed in model experiments (52, 53), and allowed to draw the conclusion that an excess of the substrate H_2O_2 might have overrun catalase and thus allowed subsequent ROS-mediated apoptosis. Control

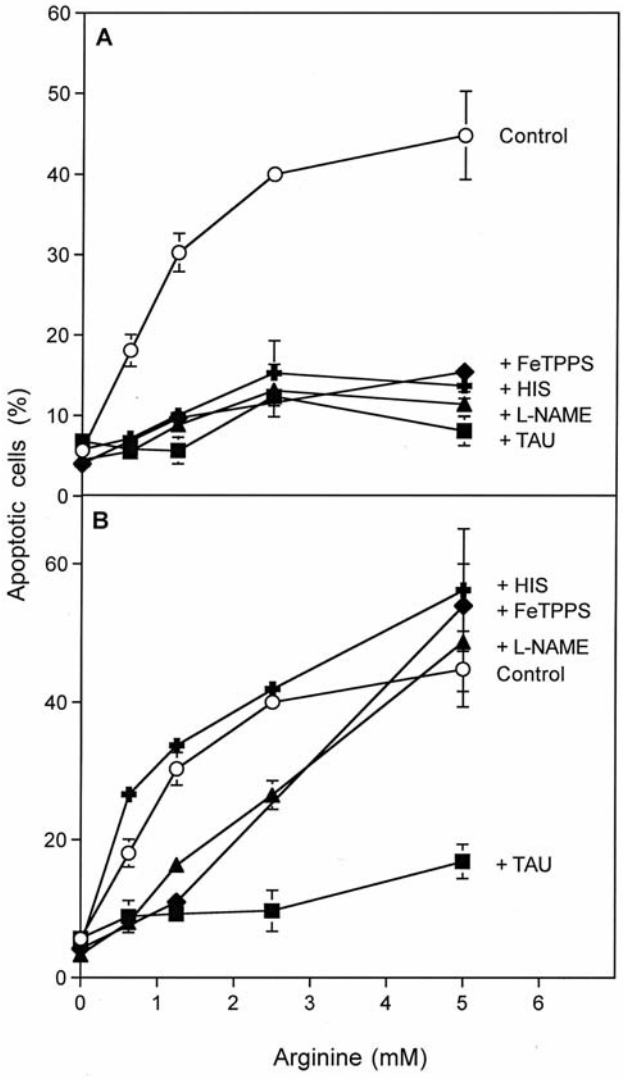

Figure 13. *Increase in endogenous NO after arginine addition causes singlet oxygen-dependent catalase inactivation and ROS-mediated intercellular signaling. A total of 25,000 human lymphoma Gumbus cells in 100 μl medium containing 10% fetal bovine serum (FBS) were treated with the indicated concentrations of arginine. Assays received either no inhibitors or 2 mM of the singlet oxygen scavenger histidine, 2.4 mM of the NOS inhibitor N-ω-nitro-L-arginine methylester hydrochloride (L-NAME), 25 μM of the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) or 50 mM of the HOCl-scavenger taurine. Inhibitors were added either immediately before (A) or 1 h after arginine application. The percentages of apoptotic cells were determined 3 h after (B) arginine addition. Please find details for cell culture, inhibitors and quantitation of apoptosis in (52) and (54). Gumbus cells were a generous gift of Dr. G. Dölken, University of Greifswald, Germany.*

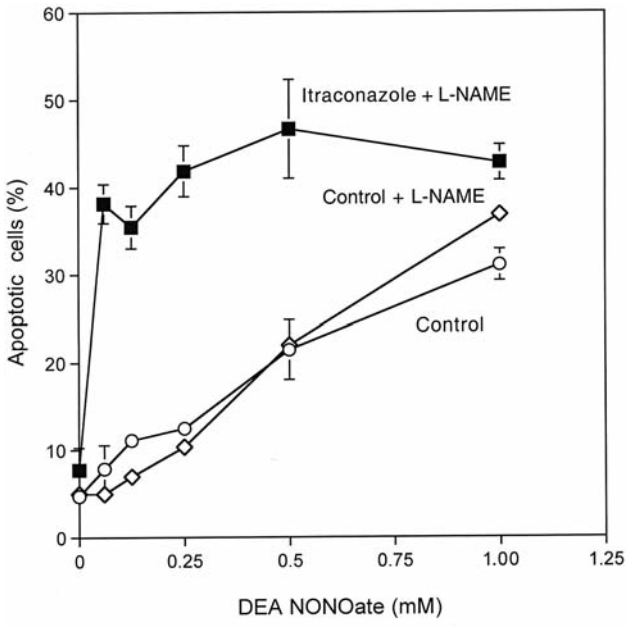

Figure 14. *Inhibition of NOD enhances peroxynitrite-dependent effects. A total of 12,500 transformed 208Fsrc3 cells in 100 μl medium, supplemented with 5% fetal bovine serum (FBS) were treated with increasing concentrations of the NO donor diethylamine NONOate (DEA NONOate), in the presence and absence of the NO synthase (NOS) inhibitor N-ω-nitro-L-arginine methylester hydrochloride (L-NAME), which had been added 10 min before the NO donor. Parallel assays received 20 μg/ml itraconazole plus L-NAME 10 min before addition of DEA NONOate. The percentages of apoptotic cells were determined 3.5 h after addition of the NO donor. Induction of apoptosis was solely dependent on the concentration of exogenously added NO. Endogenously produced NO did not contribute to the reaction measured under these conditions. In the presence of itraconazole (and the NOS inhibitor) apoptosis induction was efficiently enhanced at lower concentrations of the NO donor. When 1 mM DEA NONOate was applied, the enhancement by itraconazole was only marginal. This finding is in agreement with stabilization of the free NO concentration through inhibition of NOD that converts NO to nitrate. Control experiments (data not shown) confirmed that apoptosis induction measured in this experiment was dependent on superoxide anions and had been caused by peroxynitrite formation.*

experiments ensured that the effect of glucose oxidase alone, and in combination with peroxidase, was indeed due to HOCl/superoxide anion interaction leading to the generation of apoptosis-inducing hydroxyl radicals.

Singlet Oxygen-dependent Modulation of Intercellular ROS-dependent Apoptotic Signaling

Although experimental addition of glucose oxidase to tumor cells re-establishes intercellular ROS signaling (Figure 8; references 52 and 53), the initial steps after generation of excess H_2O_2 by glucose oxidase are far more complex than originally anticipated (Bauer, manuscript in preparation). The use of additional inhibitors revealed that singlet oxygen-dependent reactions were also involved in this scenario. Thereby, the formation of singlet oxygen through peroxynitrite/H_2O_2 interaction (123) and the potential of singlet oxygen to inactivate catalase (124, 125), possibly also through

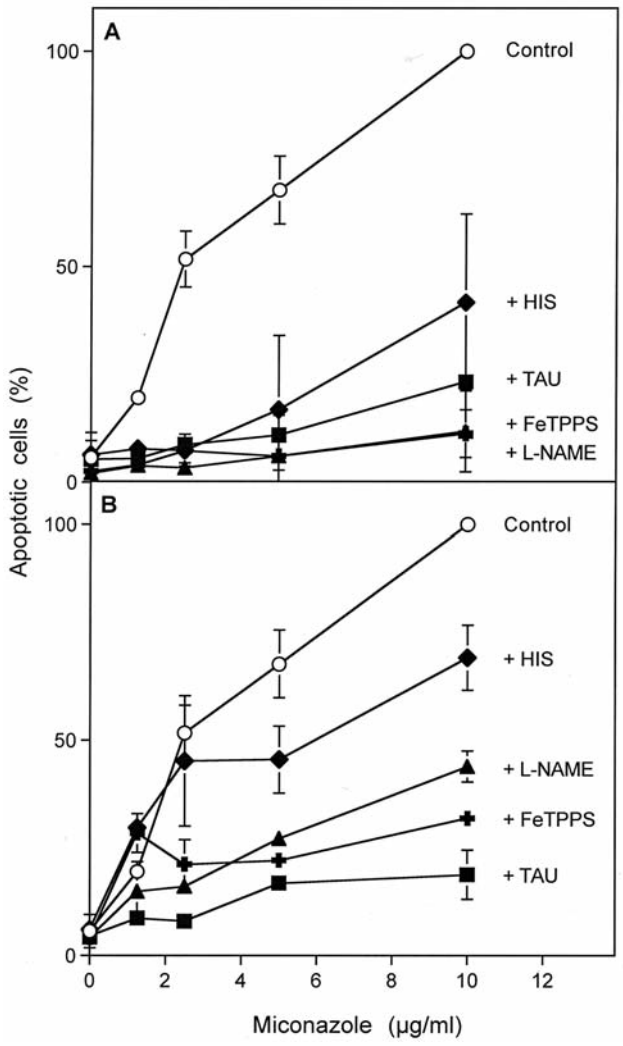

Figure 15. *ROS-mediated apoptosis induction in the human lymphoma cell line Gumbus, by application of the NOD inhibitor miconazole. A total of 25,000 human lymphoma Gumbus cells in 100 µl RPMI-1640 medium containing 10% fetal bovine serum (FBS) were treated with the indicated concentrations of miconazole. Assays received either no inhibitor or 2 mM of the singlet oxygen-scavenger histidine, 2.4 mM of the NO synthase (NOS) inhibitor N-ω-nitro-L-arginine methylester hydrochloride (L-NAME), 25 µM of the peroxynitrite decomposition catalyst 5-,10-,15-,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) or 50 mM of the HOCl-scavenger taurine. Inhibitors were added either immediately before (A) or 1 h after (B) miconazole application. The percentage of apoptotic cells was determined 10 hs after miconazole addition.*

interaction with the essential histidine at position 74 (126), are of crucial importance. The ability of singlet oxygen to induce the death receptor APO/FAS in a ligand-independent mode (127) and the ability of the APO/FAS receptor to stimulate NOX1 activity (128, 129) and NOS expression (130) proved to be additional components in this autoamplificatory signaling cascade, whose details are summarized in Figure 9. The figure visualizes that by competition, the presence of an excess of H_2O_2 excludes peroxynitrite from destruction by catalase. Peroxynitrite now has a greater chance of interacting with one of the abundant H_2O_2 molecules not yet decomposed by catalase and thus to generate singlet oxygen. The concentration of singlet oxygen generated this way may not be sufficiently high to inactivate the number of catalase molecules necessary to release signaling from negative control. However, if singlet oxygen hits an APO/FAS receptor (127), the subsequent caspase-8-dependent induction of NOX1 (128, 129) and NOS activity (130) results in overall increased concentrations of hydrogen peroxide, as well as of peroxynitrite. This allows for subsequent increased formation of singlet oxygen (123), at a concentration to cause optimal catalase inactivation and subsequent apoptotic ROS signaling. As soon as lipid peroxidation has been induced in the cell membrane by hydroxl radicals derived from ROS signaling, a further loop of singlet oxygen generation is conceivable, as peroxynitrite also forms singlet oxygen in the reaction with biological hydroperoxides (131), based on the reaction with the model compound tert-butyl hydroperoxide (132). The reaction steps with amplificatory potential have been elucidated by kinetic addition of inhibitors and by the use of specific siRNA-mediated knockdown of certain players; this study is to be published in detail elsewhere. The kinetic study of inhibitor action revealed that APO/FAS-triggered caspase-8 was only required during the first minutes for activation of NOX1 and NOS, but not for the subsequent induction of apoptotic cell death which was controlled by caspases 9 and 3. If the reaction scheme proposed in Figure 9 was correct, the opposite approach, *i.e.* outcompeting of H_2O_2 by a vast excess of peroxynitrite, should lead to the same effect as generation of relatively high concentrations of H_2O_2. Recently completed experiments (Bauer, manuscript in preparation) show this assumption to be correct. As summarized in Figure 10, an excess of exogenous peroxynitrite seems to outcompete H_2O_2, leading to singlet oxygen generation, amplification of singlet oxygen generation through singlet oxygen-dependent APO/FAS activation, increased NOX1 activity and NOS enzyme concentration. This allows for subsequent catalase inactivation by singlet oxygen formed through the interaction between excess hydrogen peroxide and peroxynitrite. For both scenarios (Figures 9 and 10), early (but not late) requirement of APO/FAS and caspase-8 activity, as well as catalase inactivation (enabling late intercellular ROS signaling) have been experimentally verified (data not shown). When synergistic conditions with parallel enhancement of peroxynitrite and H_2O_2 generation were experimentally established, the amplification step by the APO/Fas receptor and caspase-8 was no longer required. The central role of singlet oxygen during a complex set of biochemical steps leading to ROS-driven catalase inactivation, prompted testing of whether the generation of extracellular

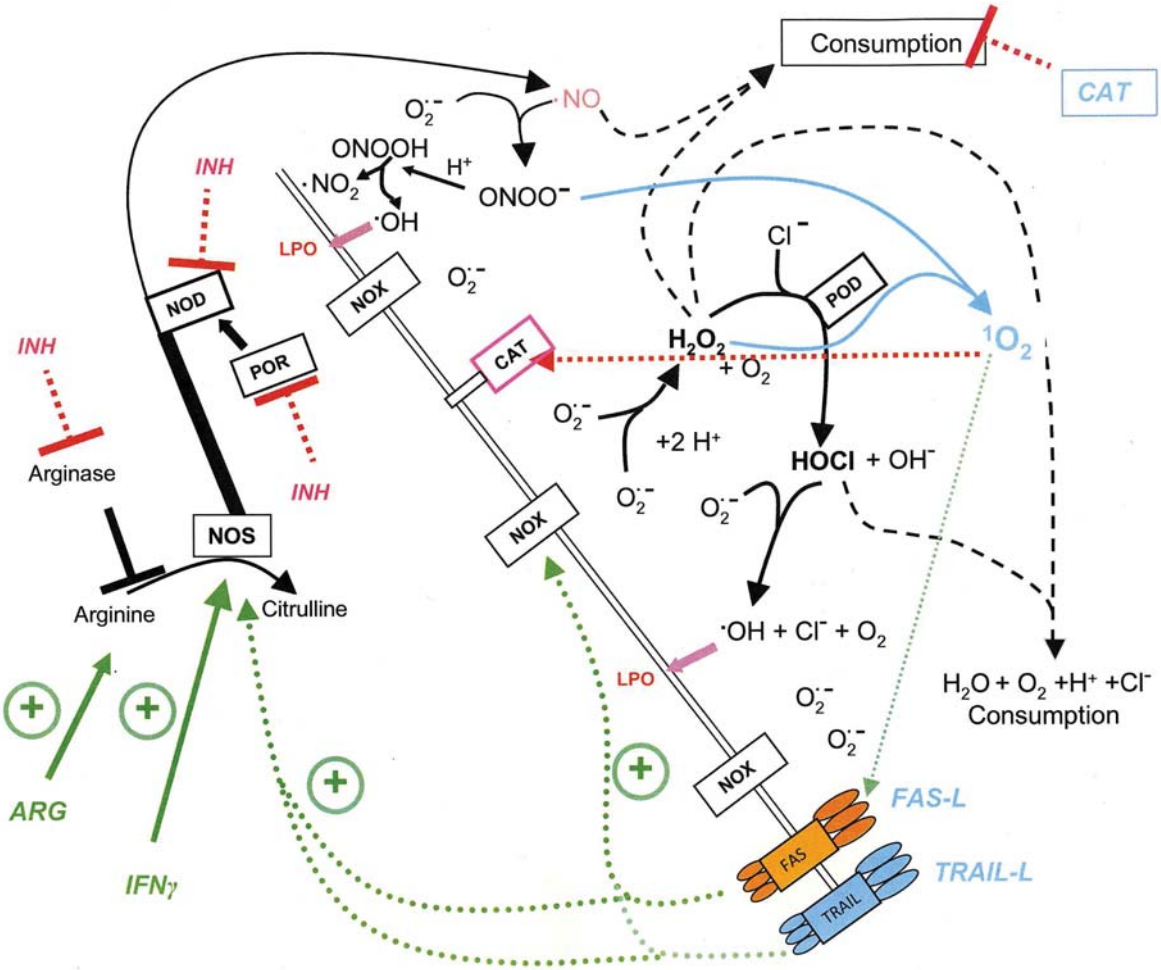

Figure 16. *Distinct ways to generate cell-derived catalase-inactivating singlet oxygen by modulation of free NO. Experimental evidence obtained in a comprehensive survey showed a remarkable variety of strategies using modulation of NO availability for apoptosis induction in tumor cells, based on preceeding singlet oxygen generation and catalase inactivation. Inhibition of arginase or addition of arginine cause singlet-oxygen-mediated catalase inactivation in many different tumor cell systems. Likewise, induction of NOS expression by interferon γ, APO/FAS ligand/receptor interaction and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) ligand/TRAIL receptor interaction caused an analogous effect. Death receptor effects were most prominent in those cell systems that did not show apoptosis by activation of the receptor alone. In addition to the previously reported inhibitors of NO dioxygenase (NOD) (111), a large number of secondary plant compounds were found to inhibit either NOD directly or cytochrome P450 oxido-reductase (POR), that is interconnected with NOD. Intriguingly, certain established chemotherapeutics were also found to act at this step, unexpectedly (data not shown). Finally, a finely tuned addition of exogenous catalase abrogated the consumption reaction between H_2O_2 and NO, but allowed for peroxynitrite/H_2O_2-dependent singlet oxygen generation, catalase inactivation and ROS-mediated apoptotic signaling. The knowledge of these biochemical interactions should allow for establishment of novel therapeutic approaches that are based on the re-establishment of tumor cell apoptotic ROS signaling after catalase inactivation. A large body of knowledge on potentially interesting synergistic effects within the signaling network presented here has been worked out and will be presented elsewhere. The establishment of synergistic effects should allow the concentrations of the interactive compounds to be reduced to a small percentage of the concentration required for application of any single compound alone. Application of synergistic effects might optimize the specific antitumor effects and thereby mimimize unwanted side-effects on normal tissue.*

singlet through application of a singlet oxygen generator such as photofrin might lead to the inactivation of tumor cell-protective catalase and subsequent specific ROS signaling. When normal cells and tumor cells were incubated with photofrin in the dark, and visible light was applied after the photosensitizer had reached the cytoplasm, both cell types died readily through apoptotic and necrotic cell death. However, when visible light was applied immediately after the addition of photofrin to the cells, before the photosenitizer had a chance to enter the cells, the non-transformed cells were not affected,

but the tumor cells died through specific ROS signaling, as shown by the effects of inhibitor application (Riethmüller and Bauer, in preparation). Detailed studies revealed that singlet oxygen by itself was not sufficient to induce apoptosis directly under these conditions, but singlet oxygen-dependent catalase inactivation was necessary to allow intercellular apoptotic ROS signaling based on tumor cell-specific extracellular superoxide formation and re-establishment of NO/peroxynitrite and/or HOCl signaling (data not shown). When high concentrations of photofrin were applied, the generated concentration of singlet oxygen was sufficient to directly inactivate catalase (step 2a) and to allow for immediate intercellular ROS signaling. Potential effects of singlet oxygen on the cell membrane were not suffient for apoptosis induction and the requirement for catalase inactivation (through singlet oxygen/histidine interaction) was directly proven by kinetic inhibitor experiments. When low concentrations of photofrin were applied (Figure 11), an amplification step through activation of the APO/Fas receptor, and potentially also of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, was necessary (step 2 b), leading to the same biochemical amplification machinery as outlined in Figures 9 and 10. The generation of sufficient singlet oxygen after these amplification steps, caused sufficient catalase inactivation that allowed for ROS-dependent signaling by the known signaling pathways. Under suboptimal inactivation conditions, the NO/peroxynitrite pathway dominated, whereas optimal inactivation of catalase enabled re-establishement of HOCl signaling. These data demonstrate the central importance of singlet oxygen for the inactivation of catalase. A modification of photodynamic therapy (which is based on the generation of singlet oxygen) in a way that cell-impermeable photosensitizers are used for selective apoptosis induction in tumor cells is thus proposed. As yet, photodynamic therapy depends on an increased uptake of photofrin and related compounds into the tumor, but not on a selective action at the cellular level. Photosensitizers acting strictly in the extracellular space, thereby inactivating tumor cell catalase and establishing intercellular ROS signaling, might improve selectivity and efficiency of photodynamic compounds. In parallel, this approach might reduce unwanted side-effects on nonmalignant tissue.

NO-Mediated Catalase Inactivation Based on Singlet Oxygen Generation

The knowledge on the multiple functions of catalase and the multitude of chemical interactions related to singlet oxygen, as outlined in the previous figures, allowed speculations on NO-mediated induction of tumor cell sensitivity for intercellular signaling, despite the fact that the NO/peroxynitrite pathway is efficiently controlled by catalase. As summarized in Figure 12, an increase in the free NO concentration (Ki=0.18 μM) causes a transient inhibition of catalase (115). This situation should lead to less decomposition of H_2O_2 and peroxynitrite and therefore these two molecules now may interact and form singlet oxygen. Experimental biochemical evidence (as outlined in Figures 9-11) allows the prediction that this should lead to APO/FAS receptor and caspase-8 activation, induction of NOX1 and NOS, and the subsequent generation of more singlet oxygen, based on the relative abundance of H_2O_2 and peroxynitrite. As a result, catalase should be sufficiently inactivated to allow ongoing intercellular ROS–mediated apoptotic signaling. The following figures demonstrate essential parts from the experimental verification of this concept. The addition of arginine represents a simple way to increase the synthesis of NO in tumor cells. As shown in Figure 13, addition of arginine to the human lymphoma cell line Gumbus causes apoptosis in a concentration-dependent mode. Addition of the peroxynitrite decomposition catalyst FeTPPS, the singlet oxygen-scavenger histidine, the NOS inhibitor L-NAME, or the HOCl-scavenger taurine immediately before arginine addition, caused nearly complete inhibition of arginine-mediated induction of apoptosis (Figure 13A). When histidine was added one hour after arginine, there was no inhibition at all, indicating that the singlet oxygen-dependent step was restricted to the initial phase of this process (Figure 13B). Addition of L-NAME or FeTPPS one hour after arginine resulted in strong inhibition of apoptosis in the low concentration range of applied arginine and decreasing inhibition at higher arginine concentrations. This finding is consistent with the interpretation that there is an early NO- and peroxynitrite-dependent step at all arginine concentrations and a later NO/peroxynitrite-dependent step only in the assays containing lower concentrations of arginine initially. Finally, the inhibitory effect of taurine was independent of the time of addition of the inhibitor, indicating that HOCl-dependent signaling represented a late effect at all applied arginine concentrations. Together with further control experiments that directly illuminated the inactivation of catalase in this scenario, the following model can be suggested: The increase of NO after arginine addition causes transient inhibition of catalase and subsequent peroxynitrite-dependent singlet oxygen generation through peroxynitrite/H_2O_2 interaction. This rather fast reaction causes inactivation of protective membrane-bound catalase and subsequent intercellular ROS-mediated apoptotic signaling. When lower concentrations of arginine were applied, the NO/peroxynitrite and the HOCl pathway acted co-operatively in the induction of apoptosis. Therefore, inhibition of one of the two pathways alone, even at one hour after the addition of arginine, caused nearly complete inhibition of apoptosis. At higher concentrations of initial arginine, late intercellular ROS signaling leading to cell death was nearly completely dependent on HOCl signaling. As indicated before (Figure 3),

the level of available NO is also controlled by NOD, an enzyme that convertes NO into nitrate (109-112). Initially found as a microbial enzyme with profound effects on the resistance of microbes to NO-mediated attack by monocytes and neutrophils, NOD has been also described to be present in mammalian cells, including tumor cells (109-111). Several inhibitors have been described for NOD, including certain azoles (some of them with antifungal activity), quercetin and garlic extract (111). Figure 14 demonstrates that addition of itraconazole to transformed cells in the presence of an NO donor (DEA NONOate) and an inhibitor of NOS (L-NAME), caused a dramatic increase in the efficiency of NO-mediated, peroxynitrite-dependent apoptosis induction. This effect is well explained by the stabilizing effect on NO through inhibition of NOD. It is definitely not due to a modulation of intracellular NO synthesis, as it depends on exogenously added NO and not on active NOS. An increase in the activity of NOX1 rather than an increase in free NO, through NOD inhibition, would also cause an increase in the efficiency of peroxynitrite-mediated apoptosis induction. However, the curves obtained through NOD inhibition, compared to superoxide anion induction are quite different: at low concentrations of the NO donor, NOD inhibition results in marked enhancement of apoptosis induction, whereas superoxide anion induction may result in a decrease of apoptosis induction due to overwhelming consumption of NO by hydrogen peroxide. At high concentrations of the NO donor, NOD inhibition only caused marginal effects as seen in Figure 14, whereas induction of superoxide anion synthesis resulted in a substantial increase in apoptosis induction. NOD inhibition by azoles seems to be sufficient to induce apoptosis in human tumor cells, as shown for the human lymphoma cell line Gumbus, treated with the antifungal azole miconazole (Figure 15). Apoptosis induction was dependent on the concentration of miconazole. Again, the inhibitor profile and the window of action of certain inhibitors, indicates that miconazole causes an early, NO–, peroxynitrite- and singlet oxygen-dependent step (directly defined in parallel control experiments as catalase inactivation) and a subsequent intercellular signaling reaction that is characterized by the co-operation between the NO/peroxynitrite and the HOCl signaling pathways. The knowledge on ROS-mediated interactions during singlet-oxygen formation and singlet oxygen-dependent catalase inactivation, in combination with the multiple pathways induced by the molecular players whose action potentials are no longer suppressed after catalase inactivation, allowed several alternative pathways to be experimentally defined. They are based on the increase in free NO concentration. These pathways cause ROS-related amplification steps that finally lead to singlet oxygen-dependent catalase inactivation. This scenario is summarized in Figure 16: The concentration of NO can be alternatively increased by the addition of arginine, inhibition of arginase, induction of NOS (*e.g.* by interferon γ), inhibition of NOD or cytochrome P450-dependent oxido-reductase and even through abrogation of the consumption reaction between hydrogen peroxide and NO. The latter mechanism requires a fine-tuned catalase concentration that is sufficiently high to abrogate NO consumption, but still too low to successfully decompose peroxynitrite site-specifically, at the cell membrane. All of these experimental procedures that cause an increase of NO also cause initial singlet oxygen formation, autoamplification of singlet oxygen formation (through APO/FAS receptor, caspase-8, NOX1 and NOS), as demonstrated in the previous figures, throughout this article. They finally lead to catalase inactivation and subsequent apoptotic cell death due to release of intercellular ROS signaling from negative control by catalase.

The Significance of ROS-driven Catalase Inactivation and Subsequent Apoptotic ROS Signaling: Potential for a Highly Specific Approach in Tumor Therapy

Targeting tumor cell-specific membrane-associated catalase for selective apoptosis induction should allow establishment of very selective and efficient therapeutic strategies that are dependent on two specific phenotypic features of tumor cells, namely extracellular superoxide anion production, through NOX1 and the presence of membrane-associated catalase. The therapeutic use of antibodies directed towards catalase allows for targeting specifically membrane-associated catalase, the characteristic feature of tumor cells, and should have no effect on normal tissue as the antibodies are not cell-permeable. This first selective step in the use of an antibody against catalase is linked to a second selective step, as only malignant cells with sustained NOX1 activity have the potential to respond with apoptotic ROS signaling, driven by their own superoxide anions and subsequent HOCl and/or NO/peroxynitrite signaling that causes their own cell death. The suggested therapeutic use of compounds that increase free NO concentration (Figures 12-16), has three levels of control with respect to selectivity for tumor cells: i) the initial step of extracellular singlet oxygen generation requires sustained superoxide anion production and is therefore restricted to malignant cells with activated oncogenes; ii) singlet oxygen generation takes place on the outside of the cells and therefore preferentially affects membrane-associated catalase, the major controlling factor of tumor cells in apoptotic ROS signaling. Intracellular catalase (essential in malignant and nonmalignant cells) with its protective function against hydrogen peroxide generated by mitochondria or certain metabolic pathways is not affected; iii) singlet oxygen-mediated inactivation of catalase only has an apoptosis-mediating effect on cells that show sustained NOX1 activity, which is characteristic of malignant cells with

activated oncogenes. Based on the established knowledge about the interconnected levels of ROS signaling between malignant cells, several modes of synergistic interactions have been recently defined. These may be instrumental for further optimization and reduction of the necessary concentrations of individual compounds, thus mimimizing the risk of unwanted side effects on normal tissue. These aspects will be presented elsewhere.

Dynamic Aspects of ROS-driven Catalase Inactivation and Subsequent Apoptotic ROS Signaling

Targeting tumor cell-specific catalase for selective apoptosis induction not only represents a mechanism-based, rational approach for selective destruction of malignant cells, it also has a valuable dynamic aspect. Ongoing experiments indicate that the inactivation of catalase in a small group of cells within a population of tumor cells causes a bystander effect-like spread of catalase inactivation and subsequent apoptotic ROS signaling. This process is driven by peroxynitrite/H_2O_2 interaction in the vicinity of the catalase-negative cells, leading to singlet oxygen generation and subsequent action of singlet oxygen on neighboring catalase-positive tumor cells (Bauer, in preparation). In this way, catalase inactivation and ROS signaling, finally occur in the whole population of cells. The knowledge of the exact biochemical parameters of this enhancing effect may be useful to optimize future therapeutic approaches based on ROS-dependent signaling in tumor cells.

ROS Signaling and Tumor Cell Dormancy

Tumor cell metastasis followed by dormancy, enharboring the potential for later reactivation of tumor cell proliferation represents one of the challenging problems of tumor therapy. The established knowledge of ROS-mediated signaling in malignant cells, as presented here, allows a rather concise working hypothesis: Suppression of NOX1 activity or scavenging of NOX1-derived superoxide anions and H_2O_2 by a dominant antioxidant milieu might prevent tumor cell proliferation and expression of the malignant phenotype. As catalase expression in the membrane of tumor cells seems to be regulated by constant H_2O_2 signals (Bauer, unpublished findings), these dormant tumor cells most probably would not exhibit strong catalase expression in situ and thus would neither be easily detectable by specific anti-catalase staining nor be subject to therapy based on targeting catalase. Elimination of dormant tumor cells of this phenotype would require enhancement of NADPH oxidase activity and reduction of antioxidant control. As a result, dismutation of superoxide anions might generate sufficient H_2O_2 for positive feedback on catalase expression. This would then allow targeting of tumor cells with an antibody directed towards catalase, or with ROS-driven singlet oxygen generation, followed by apoptotic ROS signaling. The resulting positive therapeutic effect of this complex scenario would however be counterbalanced by the H_2O_2-dependent proliferation stimulus of the tumor cells. This counterbalance needed to be outweighed by optimal ROS signaling for an overall removal of malignant cells through apoptosis induction.

ROS Signaling and Tumor Prevention

The focus of this article is on the utilization of specific ROS signaling of malignant cells for novel therapeutic approaches. Nevertheless, the tumor-preventive potential of intercellular ROS-mediated apoptotic signaling should not be completely neglected in this context. As outlined in Figure 1, ROS signaling of transformed cells (without sufficient catalase protection) has been suggested as representing an early control step in oncogenesis (21, 30). This ROS-driven control step thus might counterbalance the multiple pro-carcinogenic effects of ROS in tumor initiation and promotion. Mathematical modeling is in favour of this conclusion (85). The results obtained *in vivo* by the group of Deichman (12-16) are also in favor of this assumption, as they are best explained by selection of malignant cells with protective catalase and parallel elimination of catalase-negative transformed cells. The regular finding of membrane-associated catalase in human tumor cell lines further supports this concept. Based on the signaling chemistry presented in Figures 12–16, it may be concluded that ROS-dependent elimination of malignant cells *in vivo* should not be necessarily restricted to early stages of transformed cells without sufficient catalase protection, but might still become effective on microtumors with established catalase protection. Natural compounds that modulate the concentration of free NO might induce singlet oxygen generation, catalase inactivation and ROS-mediated apoptosis, in analogy to the mechanisms described in Figures 12-16. An ongoing survey in our laboratory has shown that many secondary plant compounds indeed exhibit the potential to modulate free NO concentration and do show the predicted effects on tumor cells *in vitro*. Further characterization of these effects may be useful for our understanding of tumor prevention by nutrition and also may aid in drug development for the establishment of novel therapeutic approaches.

Conclusion

In this review article, multiple pro-carcinogenic effects of ROS, related to tumor initiation, induction of genomic variation, oncogene activity, proliferation control, maintenance of the transformed state, tumor cell motility, angiogenesis and metastasis are summarized. These pro-carcinogenic effects are counterbalanced by ROS-mediated apoptotic signaling with the

potential for selective elimination of malignant cells. This ROS-dependent counterbalance against oncogenesis is antagonized through expression of catalase in the cell membrane of tumor cells. Catalase-mediated resistance to ROS signaling represents a necessary step during tumor progression, in line with the classical work by Deichman's group (12-16).

The reversion of this principle, *i.e.* catalase inhibition (*e.g.* by specific antibodies), or catalase inactivation (*e.g.* through singlet oxygen generated by the tumor cells themselves upon adequate stimuli), followed by re-established ROS-mediated apoptotic signaling, specifically in tumor cells, represents the major issue of this article.

This complex, but well-established ROS-dependent signaling chemistry should be translated into novel approaches for experimental therapy *in vivo*, utilizing i) the regular occurrence of catalase in the membrane of tumor cells from different tissues, ii) the specific ROS-related features of malignant cells, and iii) the multiple interconnections of ROS signaling chemistry and apoptosis induction. This approach is based on and related to the impressive work of many colleagues who have previously outlined various specific ways to use ROS and RNS of malignant cells for therapeutic approaches (133-144). Some of these approaches suggest targeting NOX1, whereas others establish ways to utilize NO for selective apoptosis induction in tumor cells. The approach suggested in this article is in line with and extending these concepts, defining tumor cell membrane-associated catalase as a novel and promising target, and utilizing superoxide anions and NO i) as basic players for the generation of singlet oxygen, and ii) as a driving force for apoptotic ROS signaling after catalase inactivation by singlet oxygen. First initial experiments in animal models support the view that this goal might be achievable.

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References

1 Weinberg RA: Oncogenes, Anti-oncogenes and the molecular basis of multistep carcinogenesis. Cancer Res *49*: 3713-3721, 1989.

2 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.

3 Vogelstein B and Kinzler KW: Cancer genes and the pathways they control. Nature Med *10*: 789-799, 2004.

4 Temin HM: Control by factors in serum of multiplication of uninfected and cells infected and converted by avian sarcoma viruses. *In*: Growth Regulatory Substances for Animal Cells in Culture. Vol. 7. Defendi V and Stoker M (eds.). Philadelphia, The Wistar Symposium Monograph, Wistar Institute Press, pp. 103-116, 1967.

5 Sporn MB and Todaro GJ: Autocrine secretion and malignant transformation. New Engl J Med *303*: 878-880, 1980.

6 Heldin CH and Westermark B: Growth factors as transforming proteins. Eur J Biochem *184*: 487-496, 1989.

7 Stoker MGP, Shearer M and O'Neill C: Growth inhibition of polyoma-transformed cells by contact with static normal fibroblasts. J Cell Sci *1*: 297-310, 1966.

8 Delinassios JG: Fibroblasts against cancer cells *in vitro*. Anticancer Res *7*: 1005-1010, 1987.

9 Trosko JE, Chang CC, Madhukar BV and Klaunig JE: Chemical, oncogene and growth factor inhibition of gap junctional intercellular communication: an integrative hypothesis of carcinogenesis. Pathobiology *58*: 265-278, 1990.

10 Barcellos-Hoff MH: It takes a tissue to make a tumor. Epigenetics, cancer and microenvironment. J Mammary Gland Biol Neoplasia *6*: 213-221, 2001.

11 Flaberg E , Markasz L, Petranyi G, Stuber G, Dicso F, Alchihabi N, Olah E, Csizy I, Jozsa T, Andren O, Johansson JE, Andersson SO, Klein G and Szekely L: High-throughput live-cell imaging reveals differential inhibition of tumor cell proliferation by human fibroblasts. Int J Cancer *128*: 2793-2802, 2011.

12 Deichman GI and Vendrov EL: Characteristics of *in vitro* transformed cells essential for their *in vivo* survival, selection and metastatic activity. Int J Cancer *37*: 401-409, 1986.

13 Deichman GI, Kluchareva TE, Matveeva VA, Kushlinsky NE, Bassalyk LS and Vendrov EL: Clustering of discrete cell properties essential for tumorigenicity and metastasis. I. Studies of syrian hamster embryo fibroblasts spontaneously transformed *in vitro*. Int J Cancer *44*: 904-907, 1989.

14 Deichman G, Matveeva VA, Kashkina LM, Dyakova NA, Uvarova EN, Nikiforov MA and Gudkov AV: Cell transforming genes and tumor progression: *in vivo* unified secondary phenotypic cell changes. Int J Cancer *75*: 277-283, 1998.

15 Deichman G: Natural selection and early changes of phenotype of tumor cells *in vivo*: Acquisition of new defense mechanisms. Biochem (Mosc) *65*: 78-94, 2000.

16 Deichman G: Early phenotypic changes of *in vitro* transformed cells during *in vivo* progression: possible role of the host innate immunity. Sem Cancer Biol *12*: 317-326, 2002.

17 Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW and Giaccia AJ: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature *379*: 88-91, 1996.

18 Kinzler KW and Vogelstein B: Life (and death) in a malignant tumour. Nature *379*: 19-20, 1996.

19 Folkman J: Fundamental concepts of the angiogenic process. Current Mol Med *3*: 643-651, 2003.

20 Bauer G: Resistance to TGF-β-induced elimination of transformed cells is required during tumor progression. Int J Oncol *6*: 1227-1229, 1995.

21 Bauer G: Elimination of transformed cells by normal cells: a novel concept for the control of carcinogenesis. Histol Histopathol *11*: 237-255, 1996.

22 Bauer G; Reactive oxygen and nitrogen species: efficient, selective and interactive signals during intercellular induction of apoptosis. Anticancer Res *20*: 4115-4140, 2000.

23 Bauer G: Signaling and proapoptotic functions of transformed cell-derived reactive oxygen species. Prostagl Leukotri Essent Fatty Acid *66*: 41-56, 2002.

24 Bauer G: Low-dose radiation and intercellular induction of apoptosis: potential implications for the control of oncogenesis. Int J Radiation Biol *83*: 887-902, 2007.

25 Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T and Goldschmidt-Clermont PJ: Mitogenic signalling by oxidants in Ras-transformed fibroblasts. Science *275*: 1649-1652, 1997.

26 Irani K and Goldschmidt-Clermont PJ: Ras, superoxide and signal transduction. Biochem Pharmacol 55: 1339-1346, 1998.

27 Suh Y-A, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK and Lambeth JD: Cell transformation by the superoxide-generating oxidase Mox1. Nature *401*: 79-82, 1999.

28 Bittinger F, Gonzalez-Garcia JL, Lein CL, Brochhausen C, Offner F and Kirkpatrick CJ: Production of superoxide by human malignant melanoma cells. Melanoma Res *8*: 381-387, 1998.

29 Yang JQ, Li S, Domann FE, Buettner G and Oberley LW: Superoxide generation in v-Ha-ras-transduced human keratinocyte HaCaT cells. Mol Carcinogenesis *26*: 180-188, 1999.

30 Herdener M, Heigold S, Saran M and Bauer G: Target cell-derived superoxide anions cause efficiency and selectivity of intercellular induction of apoptosis. Free Rad Biol Med *29*: 1260-1271, 2000.

31 Engelmann I, Dormann S, Saran M and Bauer G: Transformed target cell-derived superoxide anions drive apoptosis induction by myeloperoxidase. Redox Report *5*: 207-214, 2000.

32 Schwieger A, Bauer L, Hanusch J, Sers C, Schäfer R and Bauer G: *Ras* oncogene expression determines sensitivity for intercellular induction of apoptosis. Carcinogenesis *22*: 1385-1392, 2001.

33 Ivanovas B and Bauer G: Selective and nonselective apoptosis induction in transformed and nontransformed fibroblasts by exogenous reactive oxygen and nitrogen species. Anticancer Res *22*: 841-856, 2002.

34 Brar SS, Kennedy TP, Sturrock AB, HueckseadtTP, Quinn MT, Whorton AR and Hoidal Jr.: An NADPH oxidase regulates growth and transcription in melanoma cells. Am J Physiol *282*: C1212-C1224, 2002.

35 Heigold S, Sers C, Bechtel W, Ivanovas B, Schäfer R and Bauer G: Nitric oxide mediates apoptosis induction selectively in transformed fibroblasts compared to nontransformed fibroblasts. Carcinogenesis *23*: 929-941, 2002.

36 Chamulitrat W, Schmidt R, Tomakidi P, Stremmel W, Chunglok W, Kawahara T and Rokutan K: Association of GP91PHOX homolog NOX1 with anchorage-independent growth and MAP kinase activation of transformed human keratinocyte. Oncogene *22*: 6045-6053, 2003.

37 Mitsushita J, Lambeth JD and Kamata T: The superoxide-generating oxidase Nox1 is functionally required for Ras oncogenic transformation. Cancer Res *64*: 3580-3585, 2004.

38 Alexandrova AY, Kopnin PB, Vasilev JM and Kopnin PB: Ros up-regulation mediates Ras-induced changes of cell morphology and motility. Exp Cell Res *312*: 2066-2073, 2006.

39 Laurent E, McCoy JW, Maccina RA, Liu W, Cheng GJ, Robine S, Papkoff J and Lambeth JD: Nox1 is overexpressed in human colon cancers and correlates with activating mutations in *K-Ras*. Int J Cancer *123*: 100-107, 2008.

40 Ma Q, Cavallin LE, Yan B, Zhu S, Duran EM, Wang H, Hala LP, Dong C, Cesarman E, Mesri EA and Goldschmidt-Clermont PJ: Antitumorigenesis of antioxidants in a transgenic Rac1 model of Kaposi's sarcoma. Proc Natl Acad Sci USA *106*: 8683-8688, 2009.

41 Kim E-Y, Seo J-M, Kim C, Lee J-E, Lee K-M and Kim J-H: BLT2 promotes the invasion and metastasis of aggressive bladder cancer through a reactive oxygen species-linked pathway. Free Rad Biol Med *49*: 1072-1081, 2010.

42 Du J, Liu J, Smith BJ, Tsao MS and Cullen J: Role of rac-1-dependent NADPH oxidase in the growth of pancreatic cancer. Cancer Gene Therapy *18*: 135-143, 2011.

43 Behrend L, Henderson G and Zwacka RM: Reactive oxygen species in oncogenic transformation. Biochem Soc Trans *31*: 1441-1444, 2003.

44 Geiszt M and Leto L: The nox family of NAD(P)H oxidases: host defense and beyond. J Biol Chem *279*: 51715-51718, 2004.

45 Bedard K and Krause KH: The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev *87*: 245-313, 2007.

46 Lambeth JD: Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. Free Rad Biol Med *43*: 332-347, 2007.

47 Lopez-Lazaro M: Excessive superoxide anion generation plays a key role in carcinogenesis. Int J Cancer *120*: 1378-1380, 2007.

48 Kamata T: Roles of Nox1 and other Nox isoforms in cancer development. Cancer Sci *100*: 1382-1388, 2009.

49 Weinberg F and Chandel NS: Reactive oxygen species-dependent signaling regulates cancer. Cell Mol Life Sci *66*: 3663-3673, 2009.

50 Engelmann I, Eichholtz-Wirth H and Bauer G: *Ex vivo* tumor cell lines are resistant to intercellular induction of apoptosis and independent of exogenous survival factors. Anticancer Res *20*: 2361-2370, 2000.

51 Engelmann I and Bauer G: How can tumor cells escape intercellular induction of apoptosis? Anticancer Res *20*: 2297-2306, 2000.

52 Bechtel W and Bauer G: Catalase protects tumor cells against apoptosis induction by intercellular ROS signaling. Anticancer Res *29*: 4541-4557, 2009.

53 Bechtel W and Bauer G: Modulation of intercellular ROS signaling of human tumor cells. Anticancer Res *29*: 4559-4570, 2009

54 Heinzelmann S and Bauer G: Multiple protective functions of catalase against intercellular apoptosis-inducing ROS signaling of human tumor cells. Biol Chem *391*: 675-693, 2010.

55 Lopez-Lazaro M: Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. Cancer Lett *252*: 1-8, 2007.

56 Cheng G, Diebold BA, Hughes Y and Lambeth JD: Nox1-dependent reactive oxygen generation is regulated by Rac1. J Biol Chem *281*: 17718-17726, 2006.

57 Arnold RS, Shi J, Murad E, Whalen AM, Sun CQ, Palavarapu R, Parthasarathy S, Petros JA and Lambeth JD: Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc Natl Acad Sci USA *98*: 5550-5555, 2001.

58 Shinohara M, Shang W-H, Kubodera M, Hanada S, Mitsushita J, Kato M, Miyazaki H, Suminoto H and Kamata T: Nox1 redox signaling mediates oncogenic *Ras*-induced disruption of stress fibers and focal adhesions by down-regulating *Rho*. J Biol Chem *282*: 17640-17648, 2007.

59 Sadok A, Bourgarel-Rey V, Gattacceca F, Penel C, Lehmann M and Kovacic H: Nox1-dependent superoxide production controls colon adenocarcinoma cell migration. Biochem Biophys Acta *1783*: 23-33, 2008.

60 Tominaga K, Kawahara T, Sano t, Toida K, Kuwano Y, Sasaki H and Kawai T: Evidence for cancer-associated expression of NADPH oxidase 1 (Nox1)-base oxidase system in the human stomach. Free Rad Biol Med *43*: 1627-1638, 2007.

61 Liu SY, Yen CY, Yang SC anc Chiang WF: Overexpression of Rac-1 small GTPase-binding protein in oral squamous cell carcinoma. J Oral Maxillofac Surg *62*: 702-707, 2004.

62 Hwang SL, Hong YR, Sy WD, Lieu AS, Lin CL, Lee KS and Howng SL: Rac1 gene mutations in human brain tumors. Eur J Surg Oncol *30*: 68-72, 2004.

63 Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS and Lambeth JD: Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Acad Natl Acad Sci USA *99*: 715-720, 2002.

64 Blanchetot C and Boonstra J: The ROS-NOX connection in cancer and angiogenesis. Crit Rev Euk Gene Expr *18*: 35-45, 2008.

65 Ferraro D, Corso S, Fasano E, Panieri E, Santangelo R, Borrelo S, Giordano S, Pani G and Galeotti T: Pro-metastatic signaling by *c-Met* through *Rac-1* and reactive oxygen species. Oncogene *25*: 3689-3698, 2006.

66 Lopez-Lazaro M: Why do tumors metastasize? Cancer Biol Ther *6*: 141-144, 2007.

67 Chiera F, Meccia E, Degan P, Aquilina G, Pietraforte D, Minetti M, Lambeth D and Bignami M: Overexpression of human NOX1 complex induces genome instability in mammalian cells. Free Rad Biol Med *44*: 332-342, 2008.

68 Jürgensmeier J, Schmitt CP, Viesel E, Höfler P and Bauer G: TGF-β-treated normal fibroblasts eliminate transformed fibroblasts by induction of apoptosis. Cancer Res *54*: 393-398, 1994.

69 Jürgensmeier J, Höfler P and Bauer G: TGF-β-induced elimination of transformed fibroblasts by normal cells: independence of cell-to-cell contact and dependence on reactive oxygen species. Int J Oncology *5*: 525-531, 1994.

70 Schaefer D, Jürgensmeier J and Bauer G: Catechol interferes with TGF-β-induced elimination of transformed cells by normal cells: implications for the survival of transformed cells during carcinogenesis. Int J Cancer *60*: 520-526, 1995.

71 Langer C, Jürgensmeier JM and Bauer G: Reactive oxygen species act both at TGF-β-dependent and -independent steps during induction of apoptosis of transformed cells by normal cells. Exp Cell Res *222*: 117-124, 1996.

72 Hipp M-L and Bauer G: Intercellular induction of apoptosis in transformed cells does not depend on p53. Oncogene *15*: 791-797, 1997.

73 Jürgensmeier J and Bauer G: Interference of Bcl-2 with intercellular control of carcinogenesis. Int J Cancer *71*: 698-704, 1997.

74 Panse J, Hipp M-L and Bauer G: Fibroblasts transformed by chemical carcinogens are sensitive for intercellular induction of apoptosis: implications for the control of oncogenesis. Carcinogenesis *18*: 259-264, 1997.

75 Beck E, Schäfer R and Bauer G: Sensitivity of transformed fibroblasts for intercellular induction of apoptosis is determined by their transformed phenotype. Exp Cell Res *234*: 47-56, 1997.

76 Zucker B and Bauer G: Intercellular induction of apoptosis of transformed cells is modulated by their intracellular glutathione concentration. Int J Oncol *10*: 141-146, 1997.

77 Eckert S and Bauer G: TGF-β isoforms and fibroblast growth factor exhibit analogous indirect antioncogenic activity through triggering of intercellular induction of apoptosis. Anticancer Res *18*: 45-52, 1998.

78 Dormann S and Bauer G: TGF-β and FGF trigger intercellular induction of apoptosis: analogous activity on non-transformed but differential activity on transformed cells. Int J Oncol *13*: 1247-1252, 1998.

79 Von Eynatten K and Bauer G: Central and ambivalent role of hydrogen peroxide during intercellular induction of apoptosis. Int J Oncol *18*: 1169-1174, 2001.

80 Bauer G, Chatgilialoglu C, Gebicki JL, Gebicka L, Gescheidt G, Golding BT, Goldstein S, Kaizer J, Merenyi G, Speier G and Wardman P: Biologically relevant small radicals. Chimia *62*: 1-9, 2008.

81 Saran M and Bors W: Oxygen radicals as chemical messengers: a hypothesis. Free Rad Res Comm *7*: 213-220, 1989.

82 Steinmann M, Moosmann N, Schimmel M, Gerhardus C and Bauer G: Differential role of extra- and intracellular superoxide anions for nitric oxide-mediated apoptosis induction. In Vivo *18*: 293-310, 2004.

83 Steinebach C and Bauer G: An alternative signalling pathway based on nitryl chloride during intercellular induction of apoptosis. In Vitro Appl Mol Toxicol *14*: 107-120, 2001.

84 Schimmel M and Bauer G: Proapoptotic and redox state-related signalling of reactive oxygen species generated by transformed fibroblasts. Oncogene *21*: 5886-5896, 2002.

85 Kundrát P, Bauer G, Jacob P and Friedland W:. Mechanistic modelling suggests that the size of preneoplastic lesions is limited by intercellular induction of apoptosis in oncogenically transformed cells. Carcinogenesis *33*: 253-259, 2012.

86 Teoh MLT, Sun W, Smith BJ, Oberley LW and Cullen AJ: Modulation of reactive oxygen species in pancreatic cancer. Clin Cancer Res *13*: 7441-7450, 2007.

87 Sarsour EH, Venkataraman S, Kalen AL, Oberley LW and Gowami PC: Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. Aging Cell *7*: 405-417, 2008.

88 Policastro L, Molinari B, Larcher F, Blanco P, Podhajcer OL, Costa CS, Rojas P and Durn H: Imbalance of antioxidant enzymes in tumor cells and inhibition of proliferation and malignant features by scavenging hydrogen peroxide. Mol. Carcinogenesis *39*: 103-113, 2004.

89 Burdon RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation Free Rad Biol Medicine *18*: 775-794, 1995.
90 Droge W: Free radicals in the physiological control of cell function. Physiol Rev *82*: 47-95, 2002.
91 Ammendola R, Ruocchio MR, Chirico G, Russo L, De Felice C, Esposito F, Russo T and Cimino F: Inhibition of NADH/NADPH oxidase affects signal transduction by growth factor receptors in normal fibroblasts. Arch Biochem Biophys *397*: 253-257, 2002.
92 Long CA and Bielski BH: Rate of reaction of superoxide radical with chloride-containing species. J Phys Chem *84*: 555-557, 1980.
93 Candeias LP, Patel KB, Stratford MRL and Wardmann P: Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. FEBS *333*: 151-153, 1993.
94 Folkes LK, Candeias LP and Wardman P: Kinetics and mechanisms of hypochlorous acid reactions. Arch Biochem Biophys *323*: 120-126, 1995.
95 Saran M and Bors W: Signalling by O_2- and NO: How far can either radical, or any specific reaction product, transmit a message under *in vivo* conditions? Chem Biol Interact *90*: 35-45, 1994.
96 Saran M, Michel C and Bors W: Radical functions *in vivo*: a critical review of current concepts and hypotheses. Zeitschr Naturforsch *53* c: 210-227, 1998.
97 Moncada S and Higgs EA: The L-arginine-nitric oxide pathway. New Engl J Med *329*: 2002-2012, 1993.
98 Dawson TM and Dawson VL: Nitric oxide: action and pathological roles. Neuroscientist *1*: 17-18, 1995.
99 Saran M, Michel C and Bors W: Reaction of NO with O_2-. Implication for the action of endothelium-derived relaxing factor (EDRF). Free Rad Res Comm *10*: 221-226, 1990.
100 Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H and Beckman JS: Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol *5*: 834-842, 1992.
101 Huie RE and Padmaja S: The reaction of NO with superoxide. Free Rad Res Comm *18*: 195-199, 1993.
102 Pryor WA and Squadrito GL: The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol *268*: L699-L722, 1995.
103 Gatti RM, Alvarez B, Vasquez-Vivar J, Radi R and Augusto O: Formation of spin trap adducts during the decomposition of peroxynitrate. Arch Biochem Biophys *349*: 36-46, 1998.
104 Merenyi G and Lind J: Free radical formation in the peroxynitrous acid (ONOOH)/peroxynitrite (ONOO-) system. Chemical Res Toxicol *11*: 243-246, 1998.
105 Edens WA, Sharling L, Cheng G, Shapira R, Kinkade J, Lee T, Edens H, Tang X, Sullards C, Flaherty DB, Benian GM und Lambeth JD: Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. J Cell Biol *154*: 879-891, 2001.
106 Lambeth JD: NOX enzymes and the biology of reactive oxygen. Nature Rev Immunol *4*: 181-189, 2004.
107 Donkó A, Zalán P, Sum A, Leto T und Geiszt M: Dual Oxidases. Phil Trans R Soc *360*: 2301-2308, 2005.
108 Santiskulvong C and Rozengurt E: Galardin (GM 6001), a broad-spectrum matrix metalloproteinase inhibitor, blocks bombesin- and LPA-induced EGF receptor transactivation and DNA synthesis in rat-1 cells. Exp Cell Res *290*: 437-446, 2003.
109 Gardner PR, Martin LA, Hall D and Gardner AM: Dioxygen-dependent metabolism of nitric oxide in mammalian cells. Free Rad Biol Med *31*: 191-204, 2001
110 Schmidt K and Mayer B. Consumption of nitric oxide by endothelial cells: Evidence for the involvement of a NAD(P)H-, flavin and heme-dependent dioxygenase reaction. FEBS Lett *577*: 199-204, 2004.
111 Hallstrom CK, Gardner AM and Gardner PR: Nitric oxide metabolism in mammalian cells: substrate and inhibitor profiles of a NADPH-cytochrome P450 oxidoreductase-coupled microsomal nitric oxide dioxygenase. Free Rad Biol Med *37*: 216-228, 2004.
112 Gardner PR: Assay and characterization of the NO dioxygenase activity of flavohemoglobins. *In*: Globins and Other Nitric Oxide-reactive Proteins. Meth Enzymol *436*: 217-237, 2008.
113 Gebicka, L. and Didil J: Catalytic scavenging of peroxynitrite by catalase. Int J Inorg Biochem *103*: 1375-1379, 2009.
114 Kono Y, Yamasaki T, Ueda A and Shibata H: Catalase catalyzes of peroxynitrite-mediated phenolic nitration. Biosci Biotechnol Biochem *62*: 448-452, 1998.
115 Brown GC: Reversible binding and inhibition of catalase by nitric oxide. Eur J Biochem *232*: 188-191, 1995.
116 Brunelli L, Yermilov V and Beckman JS. Modulation of catalase peroxidatic and catalytic activity by nitric oxide. Free Rad Biol Med *30*: 709-714, 2001.
117 Ophoven SJ and Bauer G: Salen-manganese complexes: sophisticated tools for the study of intercellular ROS signaling. Anticancer Res *30*: 3967-3980, 2010.
118 Clark RA, Klebanoff SJ, Einstein AB and Fefer A: Peroxidase - H_2O_2- halide system: cytotoxic effect on mammalian tumor cells. Blood *45*: 161-170, 1975.
119 Clark RA and Klebanoff SJ: Neutrophil-mediated tumor cell cytotxicity – role of peroxidase system. J Exp Med *141*: 1442-1447, 1975.
120 Okajima T, Onishi M, Hayama E, Motoji N and Momose Y: Cytolysis of B-16 melanoma tumor cells mediated by the myeloperoxidase and lactoperoxidase systems. Biol Chem *377*: 689-693, 1996.
121 Blatt J: Deferoxamine in children with recurrent neuroblastoma. Anticancer Res *14*: 2109-2112, 1994.
122 Blatt J and Wharton V: Stimulation of growth of neuroblastoma cells by ferritin *in vitro*. J. Lab Clin Med *119*: 139-143, 1992.
123 Di Mascio P, Bechara EJH, Medeiros MHG, Briviba K and Sies H: Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide. FEBS Lett *355*: 287-289, 1994.
124 Escobar JA, Rubio A and Lissi EA: SOD and catalase inactivation by singlet oxygen and peroxyl radicals. Free Rad Biol Med *20*: 285-290, 1996.
125 Kim YK, Kwon OJ and Park J-W: Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye. Biochimie *83*: 437-444, 2001.
126 Fita I and Rossmann MG: The active center of catalase. J Mol Biol *185*: 21-37, 1985.
127 Zhuang S, Demir JT and Kochevar IE: Protein kinase C inhibits singlet oxygen-induced apoptosis by decreasing caspase-8 activation. Oncogene *20*: 6764-6776, 2001.
128 Suzuki Y, Ono Y and Hirabayashi Y: Rapid and specific reactive oxygen species generation *via* NADPH oxidase activation during FAS-mediated apoptosis. FEBS Lett *425*: 209-212, 1998.

129 Reinehr R, Becker S, Eberle A, Grether-Beck S and Häussinger D: Involvement of NADPH oxidase isoforms and src family kinases in CD95-dependent hepatocyte apoptosis. J Biol Chem *280*: 27179-27194, 2005.

130 Selleri C, Sato T, Raiola AM, Rotoli B, Young NS and Maciejewski JP. Induction of nitric oxide synthase is involved in the mechanism of FAS-mediated apoptosis in hematopoietic cells. Br J Hematol *99*: 481-489, 1997.

131 Miyamoto S, Ronsein GE, Prado FM, Uemi M, Correa TC, Toma IN, Bertolucci A, Oliveira MCB, Motta FD, Medeiros MHG and Di Mascio P: Biological hydroperoxides and singlet molecular oxygen generation. IUBMB LIFE *59*: 322-331, 2007.

132 Di Mascio P, Briviba K, Sasaki ST, Catalani LH, Medeiros MHG, Bechara EJH and Sies H: The reaction of peroxynitrite with tert-butyl hydroperoxide produces singlet molecular oxygen. Biol Chem *376*: 1071-1074, 1997.

133 Pelicano H, Carney D and Huang P: ROS stress in cancer cells and therapeutic implications. Drug Resist Updates *7*: 97-110, 2004.

134 Bonavida B, Khineche S, Huerta-Ypez S and Garban H: Therapeutic potential of nitric oxide in cancer. Drug Resist Updates *9*: 157-173, 2006.

135 Hirst D and Robson T: Targeting nitric oxide for cancer therapy. J Pharm Pharmacol *59*: 3-13, 2006.

136 Hirst DG and Robson T: Nitrosative stress in cancer therapy. Front Biosci *12*: 3406-3418, 2007.

137 Halliwell B: Oxidative stress and cancer: Have we moved forward? Biochem J *401*: 1-11, 2007.

138 Lambeth JD, Krause KH and Clark RA: NOX enzymes as novel targets for drug development. Sem Immunopathol *30*: 339-363, 2008.

139 Ushuio-Fukai M and Nakamura Y: Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. Cancer Lett *266*: 37-52, 2008.

140 Mocellin S: Nitric oxide: Cancer target or anticancer agent? Curr Cancer Drug Targ *9*: 214-236, 2009.

141 Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nature Rev Drug Discov *8*: 579-591, 2009.

142 Wondrak GT: Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid Redox Signal *11*: 3013-3069, 2009.

143 Maciag AE, Chaprapani H, Saavedra J, Morris NL, Holland RJ, Kosak KM, Shami P, Anderson LM and Keefer LK: The nitric oxide prodrug JS-K is effective against non-small-cell lung cancer cells *in vitro* and *in vivo*: Involvement of reactive oxygen species. J Pharmacol Exp Ther *336*: 313-320, 2011.

144 Kim MY: Nitric oxide triggers apoptosis in A375 human melanoma cells treated with capsaicin and resveratrol. Mol Med Reports *5*: 585-591, 2012.

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