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Review

The Antitumor Effect of Singlet Oxygen

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Abstract. *Tumor cells are protected against intercellular apoptosis-inducing signaling through expression of membrane-associated catalase and superoxide dismutase. Exogenous singlet oxygen derived from activated photosensitizers or from cold atmospheric plasma causes local inactivation of protective catalase which is followed by the generation of secondary extracellular singlet oxygen. This process is specific for tumor cells and is driven by a complex interaction between H_2O_2 and peroxynitrite. Secondary singlet oxygen has the potential for autoamplification of its generation, resulting in optimal inactivation of protective catalase and reactivation of intercellular apoptosis-inducing signaling. An increase in the endogenous NO concentration also causes inactivation of catalase and autoamplificatory generation of secondary singlet oxygen. This principle is essential for the antitumor activity of secondary plant products, such as cyanidins and other inhibitors of NO dioxygenase. It seems that the action of the established chemotherapeutic taxol and the recently established antitumor effect of certain azoles are based on the same principles.*

This review summarizes the role of reactive oxygen/nitrogen species (ROS/RNS) during tumor progression and presents a coherent picture of the multiple activities of membrane-associated catalase of tumor cells at the crossing point of ROS/RNS chemical biology. These activities of catalase enable tumor cells to establish a tight control system of intercellular ROS/RNS-dependent apoptosis-inducing signaling. It will then be shown that the application of exogenous singlet oxygen or the enhancement of the cellular level of NO leads to the inactivation or inhibition of membrane-associated catalase. This leads to the generation of secondary singlet oxygen and establishment of an autoamplificatory, selfperpetuating system of singlet oxygen generation, catalase inactivation and reactivation of intercellular apoptosis-inducing signaling. Finally the role of these processes in established and novel therapeutic approaches is elucidated with the focus on the action of singlet oxygen.

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1. ROS/RNS Signaling and Tumor Progression

Tumor development and progression are causally related to the expression of membrane-associated NADPH oxidase 1 (NOX1) (Figure 1) which ensures sustained generation of extracellular superoxide anions. Their spontaneous or catalytic dismutation product H_2O_2 is required for the maintenance of the transformed state and for the control of proliferation of malignant cells (1-3). Tumor progression selects for cells that maintain a sufficiently high production rate of extracellular superoxide anions and H_2O_2 but that at the same time are protected against the HOCl and the NO/peroxynitrite signaling pathways (2-6). Both pathways induce apoptosis selectively in malignant cells as they are controlled by extracellular superoxide anions (2,3, 6-8). HOCl signaling (reaction steps #1, 2, 8, 9 in Figure 1) (2, 7) is based on H_2O_2-dependent HOCl synthesis by the peroxidase (POD) domain of dual oxidase (DUOX) that is released from membrane-associated DUOX through matrix metalloproteases (9). HOCl then interacts with extracellular superoxide anions, yielding hydroxyl radicals that cause lipid peroxidation. This triggers the mitochondrial pathway of apoptosis (10, 11). NO/peroxynitrite signaling (steps #10 - 13, 15-19 in Figure 1) is based on the formation of peroxynitrite through NO/superoxide anion interaction (7, 8, 12-14), followed by protonation of peroxynitrite to peroxynitrous acid (6). Protonation of peroxynitrite is favoured in the vicinity to the membrane of malignant cells due to the presence of proton pumps, whereas peroxynitrite distant of the membrane preferentially reacts with CO_2 (6).

Peroxynitrous acid decomposes into NO_2 and apoptosis-inducing hydroxyl radicals (13, 15).

The classical work by Deichman's group showed that tumor progression requires the selection of cells with increased resistance to exogenous H_2O_2 (16, 17). In line with their findings, we confirmed that transformed cells (early stages in tumor development) are subject to efficient elimination by the HOCl and the NO/peroxynitrite signaling pathways but *bona fide* tumor cells, *i.e.* late stages of tumor progression, are strongly protected against intercellular signaling (4-6). The protective system on the outside of tumor cells consists primarily of membrane-associated catalase ($CATFe^{III}$, ferricatalase) that interferes with HOCl signaling through decomposition of H_2O_2, and with NO/peroxynitrite signaling through oxidation of NO and decomposition of peroxynitrite (5, 18, 19). Decomposition of H_2O_2 and peroxynitrite is mediated by a two-step mechanism that involves the formation of compound I ($CATFe^{IV}=O^{\bullet+}$) as intermediate (5) (reaction steps #6, 7, 16, 17 in Figure 1). Oxidation of NO by compound I of catalase (reaction step #14 in Figure 1) is catalyzed by two subsequent one-electron transfers (reaction steps #6-8 in Figure 2) (6, 19, 20). Catalase is released by tumor cells and is then attached to the tumor cells through transglutaminase (21). In this way, transglutaminase becomes an integral part of the protective system of tumor cells.

As superoxide anions inhibit catalase through direct generation of the inactive compound III ($CATFe^{III}O_2^{\bullet-}$) and through reduction of the active intermediate compound I to the inactive compound II ($CATFe^{IV}=O$), followed by H_2O_2-dependent generation of compound III (reaction steps #3 - #5 in Figure 1) (22-24), catalase cannot be functional in the close vicinity of superoxide anion-generating NOX1. Therefore, in order to establish protection towards intercellular ROS/RNS-mediated apoptosis-inducing signaling, tumor cells need to express membrane-associated superoxide dismutase (SOD) as an essential part of their protective system (3, 25). The local concentration of membrane-associated SOD must be sufficiently high to remove free superoxide anions below a level that is inhibitory for catalase (reaction step #2 in Figure 1).

Figure 2 underlines that catalase is not merely a highly specialized, H_2O_2-decomposing enzyme but rather controls the crossing point between ROS and RNS chemical biology. *Vice versa*, catalase itself is controlled by defined ROS and RNS. Ferricatalase ($CATFe^{III}$) reacts with H_2O_2 or alternatively with peroxynitrite and thereby generates the active intermediate compound I (reaction steps # 3 and #5 in Figure 2) (5). Compound I then oxidates NO in a two-step reaction with compound II as intermediate (reaction steps #6-#8 in Figure 2) (6, 19, 20). Formation of compound I as prerequisite for the oxidation of NO is constantly warranted on the membrane of tumor cells due to their generation of extracellular H_2O_2 and peroxynitrite. Oxidation of NO by compound I interferes with the reaction between compound I and either H_2O_2 or peroxynitrite (not shown in Figure 2). As oxidation of NO leads to the recovery of ferricatalase after a transitient generation of compound II, it slows down the catalase cycle but does not inhibit it completely. However, beyond a certain concentration (Ki=0.18 μM) (18), NO inhibits catalase (reaction step #13 in Figure 2) and thus prevents decomposition of H_2O_2 and peroxynitrite, as well as oxidation of NO. Superoxide anions can revert NO-mediated inhibition of catalase (reaction step #14 in Figure 2) (26), whereas NO can convert compound II and III to ferricatalase. In this way, NO abrogates the inhibitory effect of superoxide anions on catalase (reaction step #10 in Figure 2) (27). This complex picture shows that the balance of ROS and RNS determines the reaction profile and the activity of catalase, and at the same time, catalase determines the profile of intercellular ROS/RNS signaling.

The interdependency between the membrane-associated SOD/catalase-based protective system of tumor cells and active NOX1, which is characteristic of malignant cells and is located at the same site as SOD and catalase, offers the unique chance to utilize two tumor cell-specific features when the protective system is inhibited or inactivated and ROS/RNS signaling is reactivated. Apoptosis induction achieved in this way is therefore highly selective for tumor cells *in vitro*, and potentially also *in vivo*.

2. Inactivation of Membrane-associated Catalase by Singlet Oxygen

Singlet oxygen is one of the most interesting and versatile molecules within the ROS family (28). Its potential for the control of oncogenesis and in a variety of therapeutic antitumor approaches has essentially been underestimated. Singlet oxygen has been shown to inactivate catalase through reaction with histidine at the active center of the enzyme (29, 30) and thus to abrogate the antioxidant activity of one of the central molecules of tumor cells.

Model experiments showed that low concentrations of extracellular singlet oxygen, generated through illumination of the photosensitizer photofrin, cause local inactivation of few catalase molecules within the protective belt of catalase molecules on the membrane of tumor cells (reaction step # 1 in Figure 3A) (31). As a result, in the vicinity of inactivated catalase, H_2O_2 and peroxynitrite (which are constantly generated on the outside of tumor cells) are no longer decomposed (reaction steps #2, 3), and NO is no longer oxidated (reaction step #4). Due to the proton pumps in the membrane, peroxynitrite is protonated and the resultant peroxynitrous acid generates $^{\bullet}NO_2$ and hydroxyl radicals (reaction steps #5,6):

$ONOO^- + H^+ \rightarrow ONOOH$ (6)

$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH$ (13, 15)

The interaction between H_2O_2 and hydroxyl radicals leads to the formation of hydroperoxide radicals that seem to react with NOX1-derived superoxide anions and form singlet oxygen (reaction steps # 7,8):

$^{\bullet}OH + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O$ (32, 33)

$HO_2. + O_2^{\bullet} - + H^+ \rightarrow H_2O_2 + {}^1O_2$

(reaction rate 9.7×10^7 M^{-1} s^{-1}) (34-38).

This sequence of reactions is in line with the original finding by Di Mascio *et al*. (39) on the formation of singlet oxygen through the interaction between H_2O_2 and peroxynitrite. It resolves the discrepancies between the findings of Di Mascio *et al*. (39) and Alvarez *et al*. (40). As discussed by Riethmüller *et al*. (31), both groups demonstrated the generation of singlet oxygen after combining H_2O_2 with peroxynitrite, but differed in the suggested mechanism of reaction. Whereas Di Mascio *et al*. (39) suggested a direct interaction between both compounds, followed by the generation of singlet oxygen through the Russels mechanism, Alvarez *et al*. (40) found that two molecules of peroxynitrite were necessary for the generation of one molecule of singlet oxygen. The findings by Alvarez *et al*. do not support the mechanistic conclusions by Di Mascio *et al*., but might be explained by reaction steps #5-8 in Figure 3A. As Alvarez *et al*. did not have a source for superoxide anions in their experimental system, their proposed stoichiometry would imply the reaction of hydroxyl radicals derived from two molecules of peroxynitrous acid with two molecules of H_2O_2, yielding two hydroperoxide radicals that allowed the formation of one molecule of H_2O_2 and singlet oxygen ($k=8.6\times10^5$ M^{-1} s^{-1}) (38). As the generation of singlet oxygen through the interaction between H_2O_2 and peroxynitrite in model experiments was found to be dependent on the generation of hydroxyl radicals (31), we conclude that this reaction chain is the basis for the generation of secondary singlet oxygen in our experimental system. As this process takes place in close vicinity to the cell membrane, the final step of singlet oxygen generation might be due to the interaction of one hydroperoxyl radical with one superoxide anion, in a reaction that is about 100 times faster than the reaction between two hydroperoxyl radicals ($k=9.7\times10^7$ M^{-1} s^{-1}) (38).

The generation of singlet oxygen through the reaction between superoxide anions/hydroperoxide radicals has been a matter of dispute but is supported by the analysis by Tarr and Valenzeno (38). This conclusion was mainly based on the findings by Steinbeck *et al*. (37). The data obtained by Steinbeck *et al*. (37), Di Mascio *et al*. (39), Alvarez *et al*. (40), Miyamomto *et al*. (41) and the model experiments by Riethmüller *et al*. (31) strongly support the reaction scheme for the generation of singlet oxygen through the interaction between H_2O_2 and peroxynitrite, involving hydroxyl radicals and hydroperoxyl radicals, as presented in Figure 3A. An alternative explanation, such as direct generation of singlet oxygen from peroxynitrous acid (42), has been disproven by Martinez *et al*. (43), in line with the findings of Merenyi *et al*. (44). Furthermore, a possible mechanism based on lipid peroxidation mediated by hydroxyl radicals derived from peroxynitrous acid and subsequent singlet oxygen generation through the interaction between peroxynitrite and lipid hydroperoxides (41, 45) can be excluded in the context discussed here, as it would not require the presence of H_2O_2, in contrast to the findings by Di Mascio *et al*. (39), Alvarez *et al*. (40) and Riethmüller *et al*. (31).

'Secondary singlet oxygen' that is generated after inactivation of catalase has the chance to either inactivate more catalase molecules or to activate the FAS receptor (FASR), a member of the tumor necrosis factor receptor family, in a ligand-independent mode (46) (#1, 2 in Figure 3B). As a result of FAS receptor activation, NOX1 activity is enhanced (#3) and NO synthase (NOS) expression is induced (47-49) (#4). This results in a local increase in superoxide anions, H_2O_2, NO and peroxynitrite (#5). Especially at sites with primarily inactivated catalase, this mixture leads to a massive increase in secondary singlet oxygen (#7-13), followed by inactivation of a sufficient proportion of protective catalase molecules (#14) to allow reactivation of intercellular ROS/RNS-mediated apoptosis-inducing signaling. Most likely, not only inactivation of catalase by singlet oxygen will trigger the generation of secondary singlet oxygen, but also the local increase in free NO during this process may cause reversible inhibition of catalase at other sites (#6), followed by the generation of additional secondary singlet oxygen (#17).

When relatively high concentrations of exogenous singlet oxygen are applied (#1 in Figure 4), the generation of secondary extracellular singlet oxygen is also necessary to obtain an optimal degree of catalase inactivation and subsequent apoptosis-inducing intercellular ROS/RNS signaling of tumor cells (31) (reaction steps #2-9). However, under these conditions, the amplification step by the FAS receptor is not necessary and catalase inactivation and intercellular ROS/RNS-dependent signaling occur even if the FAS receptor or caspase-8 had been knocked out by small interfering ribonucleic acid or when caspase-8 was inhibited (31).

It seems to be more likely that low concentrations of singlet oxygen have a higher chance to initially hit catalase, which is present in abundance on the membrane, rather than the FAS receptor. But even if the FAS receptor were hit first, the final outcome would be the same as that after initial attack of catalase, as FAS receptor activation by singlet oxygen causes NOS induction and an increase in NO (49) (#1-3 in Figure 5A). As a result, catalase molecules would be reversibly inhibited (18, 19) (#4, 5) and subsequent local formation of secondary singlet oxygen would be possible (#6-12). This would trigger an autoamplificatory cascade of generation of secondary singlet oxygen, inactivation of catalase and reactivation of intercellular ROS/RNS-mediated apoptosis-inducing signaling. The feasibility of this FAS receptor-

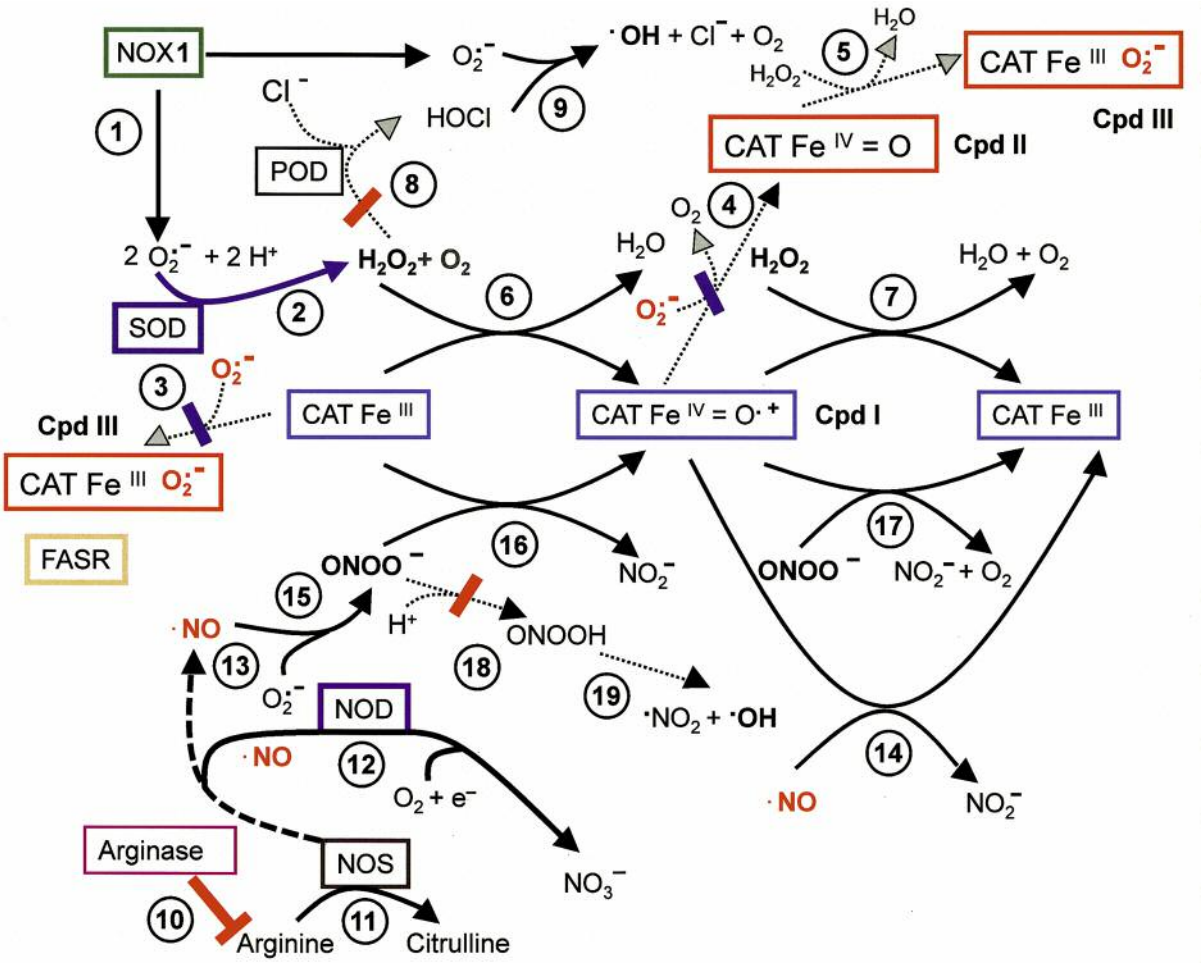

Figure 1. *The protective system of tumor cells towards intercellular reactive oxygen species/reactive nitrogen species (ROS/RNS)-dependent apoptosis-inducing signaling. NADPH oxidase-1 (NOX1), superoxide dismutase (SOD) and catalase (CAT) and its intermediates, as well as the FAS receptor (FASR), a member of the tumor necrosis factor receptor family, are membrane-associated on the outside of tumor cells. Peroxidase (POD) has been removed from membrane-associated dual oxidase (DUOX) by matrix metalloproteases and is in the extracellular space. Arginase, NO synthase (NOS) and NO dioxygenase (NOD) are located intracellulary. The reaction of catalase (ferricatalase, $CATFe^{III}$) with its substrates involves the formation of the active intermediate compound I (Cpd I, $CATFe^{IV}{=}O^{\bullet+}$). Compound II (Cpd II, $CATFe^{IV}{=}O$) and compound III (Cpd III, $CATFe^{III}O_2^{\bullet-}$) are inactive. The figure illustrates the protective effect of catalase towards the HOCl signaling pathway (steps #1, 2, 8, 9) and the NO/peroxynitrite signaling pathway (steps # 10-13, 15, 18, 19) and the co-modulatory effect of SOD which prevents superoxide anion-dependent inhibition of catalase. Details are described in section 1.*

dependent pathway has been proven, as activation of the FAS receptor in tumor cells with suboptimal concentration of FAS receptor for death receptor-mediated cell death causes autoamplification of singlet oxygen generation, catalase inactivation and reactivation of intercellular ROS/RNS-mediated apoptosis-inducing signaling through the mitochondrial pathway of apoptosis (Figure 5B) (6). In contrast to FAS receptor-dependent NOS induction, enhancement of NOX1 activity through the activated FAS receptor seems not to be sufficient to trigger catalase inactivation and subsequent reactivation of intercellular ROS/RNS-dependent apoptosis-inducing signaling. This conclusion is supported by the finding that the alternative strong and selective stimulation of NOX1 activity by low-dose irradiation (50) does not reactivate signaling, despite its potential to act synergistically with other reactivating compounds (6, 51).

3. Modulation of NO Metabolism and the Generation of Singlet Oxygen

An increase in the endogenous NO concentration (#5 in Figure 6A) by inhibition of arginase (#1), addition of arginine (#29,

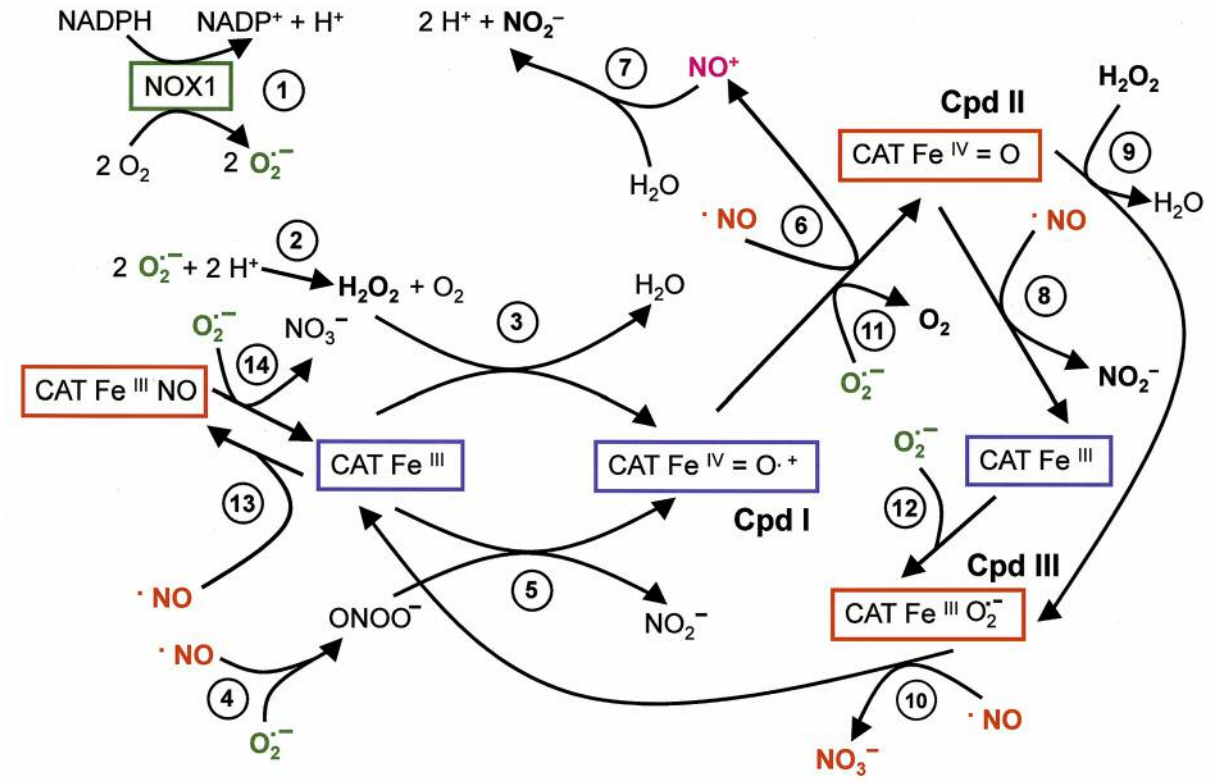

Figure 2. *Membrane-associated catalase of tumor cells, an enzyme at the crossing point of reactive oxygen species (ROS) and reactive nitrogen species (RNS). This figure summarizes the multiple interactions between H_2O_2, peroxynitrite, NO and superoxide anions with catalase and its intermediates. In addition to compound I (Cpd I), Cpd II and Cpd III of catalase defined in Figure 1, this figure also depicts the inactive complex between catalase and NO (CAT Fe^{III}•NO). The reactions are described in detail in section 1.*

induction of NOS expression by interferons (not shown) or inhibition of NO dioxygenase (NOD) (#3) by anthocyanidins, various flavonoids, antifungal azoles, diallyldisulfide, artemisinine, taxol and many other agents, causes local reversible inhibition of catalase molecules on the surface of tumor cells (#5) (6, 11; Bauer, unpublished data), as NO readily passes the cell membrane. This leads to a first round of formation of extracellular secondary singlet oxygen (#6-12) and amplification of singlet oxygen generation through singlet oxygen-dependent activation of the FAS receptor (Figure 6B). Subsequently, inactivation of a sufficient concentration of membrane-associated catalase allows for sufficient subsequent intercellular ROS/RNS signaling, followed by induction of the mitochondrial pathway of apoptosis. In particular, the inhibition of NOD by many secondary plant compounds (6, 11, 52) gives insight into the mechanism of the tumor preventive effect of secondary plant compounds but also opens a new field for novel antitumor drugs. This approach is particularly interesting as the knowledge of the signaling system allows to instrumentalize synergistic effects that can be obtained by triggering the system at distinct molecular sites.

4. The Potential Role of Singlet Oxygen in Tumor Prevention and Therapy

4.1 Photodynamic therapy. It has been shown that intracellular generation of singlet oxygen causes cell death in non-malignant and malignant cells with equal efficiency, whereas extracellular singlet oxygen generation causes a selective effect on tumor cells as it targets catalase and reactivates intercellular ROS/RNS-dependent apoptosis-inducing signalling (31). Extracellular singlet oxygen at concentrations that caused apoptosis induction in tumor cells thereby had no effect on non-malignant cells (31).

Classical photodynamic therapy is not selectively targeting the catalase/SOD-based protective system of tumor cells through the action of singlet oxygen action and thus provoking selective apoptosis induction in tumor cells. Rather, due to the established method and the chemical nature of photosensitizers presently used, classical photodynamic therapy is believed to be based on preferential uptake of photosensitizers by tumor tissue compared to non-malignant tissue (53, 54). At the time of photoactivation

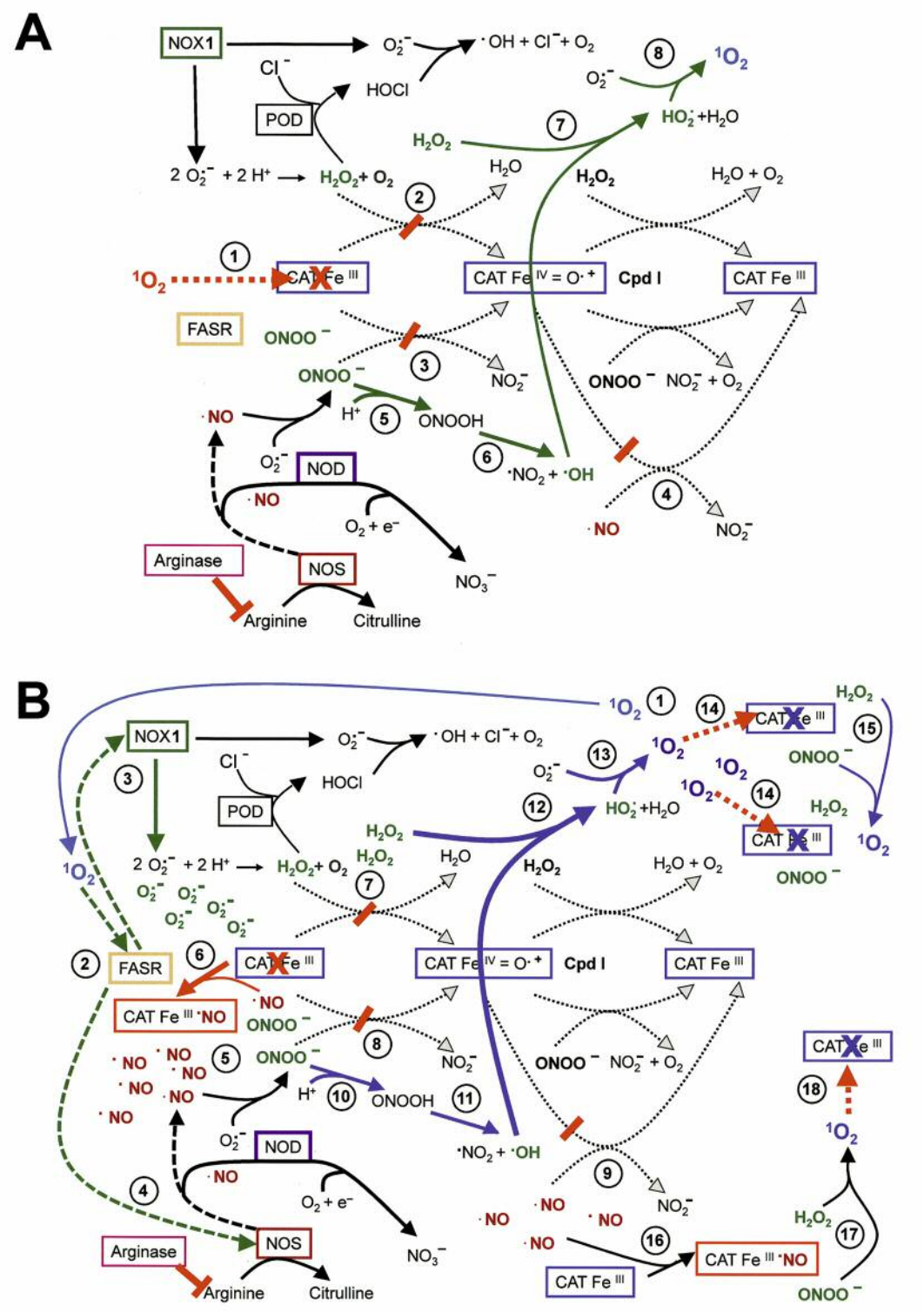

Figure 3. *Inactivation of catalase by low concentrations of singlet oxygen: an autoamplificatory mechanism based on FAS receptor (FASR)-mediated enhancement of NADPH oxidase-1 (NOX1) and NO synthase (NOS) activities and generation of secondary singlet oxygen. The FASR is a member of the tumor necrosis factor receptor family. A: Low concentrations of exogenous singlet oxygen cause local inactivation of membrane-associated catalase, followed by an interaction between H_2O_2 and hydroxyl radicals derived from peroxynitrite, generation of hydroperoxyl radicals ($HO_2^{\cdot}$) and finally of singlet oxygen ($^1O_2$). B: Secondary singlet oxygen generated through the reactions described in (A) activates the FASR. Subsequent enhancement of NOX1 and induction of NOS expression contributes to increased generation of singlet oxygen and catalase inactivation. See section 2 for details.*

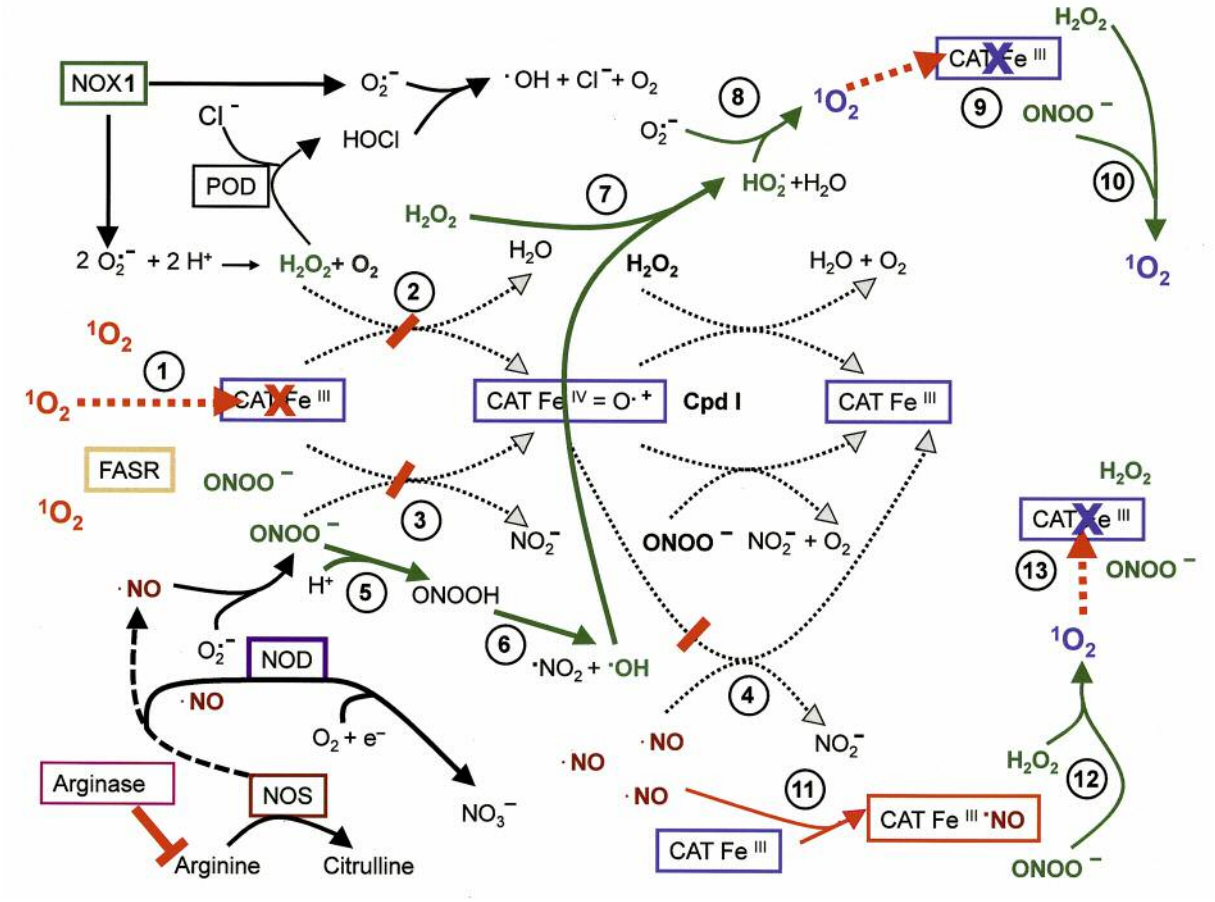

Figure 4. *Inactivation of catalase by high concentrations of singlet oxygen: an autoamplificatory mechanism based on the generation of secondary singlet oxygen. High concentrations of exogenous singlet oxygen causes multiple events of local inactivation of membrane-associated catalase on tumor cells. This allows for optimal generation of singlet oxygen through complex interaction between H_2O_2 and peroxynitrite, followed by catalase inactivation that allows the reactivation of intercellular reactive oxygen species (ROS)-dependent apoptosis-inducing signalling. See section 2 for details.*

through illumination, the photosensitizers are located inside the cells and thus generate intracellular singlet oxygen ($^1O_2$) which may hit a multitude of intracellular targets and thus induces apoptosis or necrosis. Therefore, the beneficial effect of present photodynamic is explained by selective transport to and action in tumor tissue rather than by selective activity directed towards malignant cells.

However, Rapozzi *et al*. reported on induction of iNOS by photodynamic therapy and presented evidence on a dual role of the resultant NO on tumor cell survival. Importantly, apoptosis induction by photodynamic therapy was inhibited in their experiments when the activity of NOS was blocked (54, 55). Therefore, it is obvious that under the conditions of the test system described by Rapozzi *et al*., the concentration of singlet oxygen was not sufficiently high to cause direct apoptosis induction. It is, therefore, attractive to speculate that induction of NOS by photodynamic therapy might have caused a sufficiently high concentration of NO for the transient inhibition of membrane-associated catalase, in analogy to the induction by the FAS system (6) or by interferons (52). As a result, the generation of secondary singlet oxygen, FAS receptor involvement, catalase inactivation and reactivation of intercellular ROS/RNS signalling might have been the dominant cause of apoptosis induction, in addition to the other effects of NO characterized by Rapozzi *et al*. (54), Thus the selective uptake of the photosensitizer might have led to NO-mediated catalase inhibition, followed by selective inactivation of catalase by secondary singlet oxygen and subsequent ROS/RNS-dependent apoptosis-inducing signaling.

This aspect awaits further clarification and might be useful for further improvement and optimization of photodynamic therapy. In addition, the development of cell-impermeable photosensitizers that might specifically act on tumor cells through selective singlet oxygen-dependent inactivation of membrane-associated catalase and/or SOD is suggested.

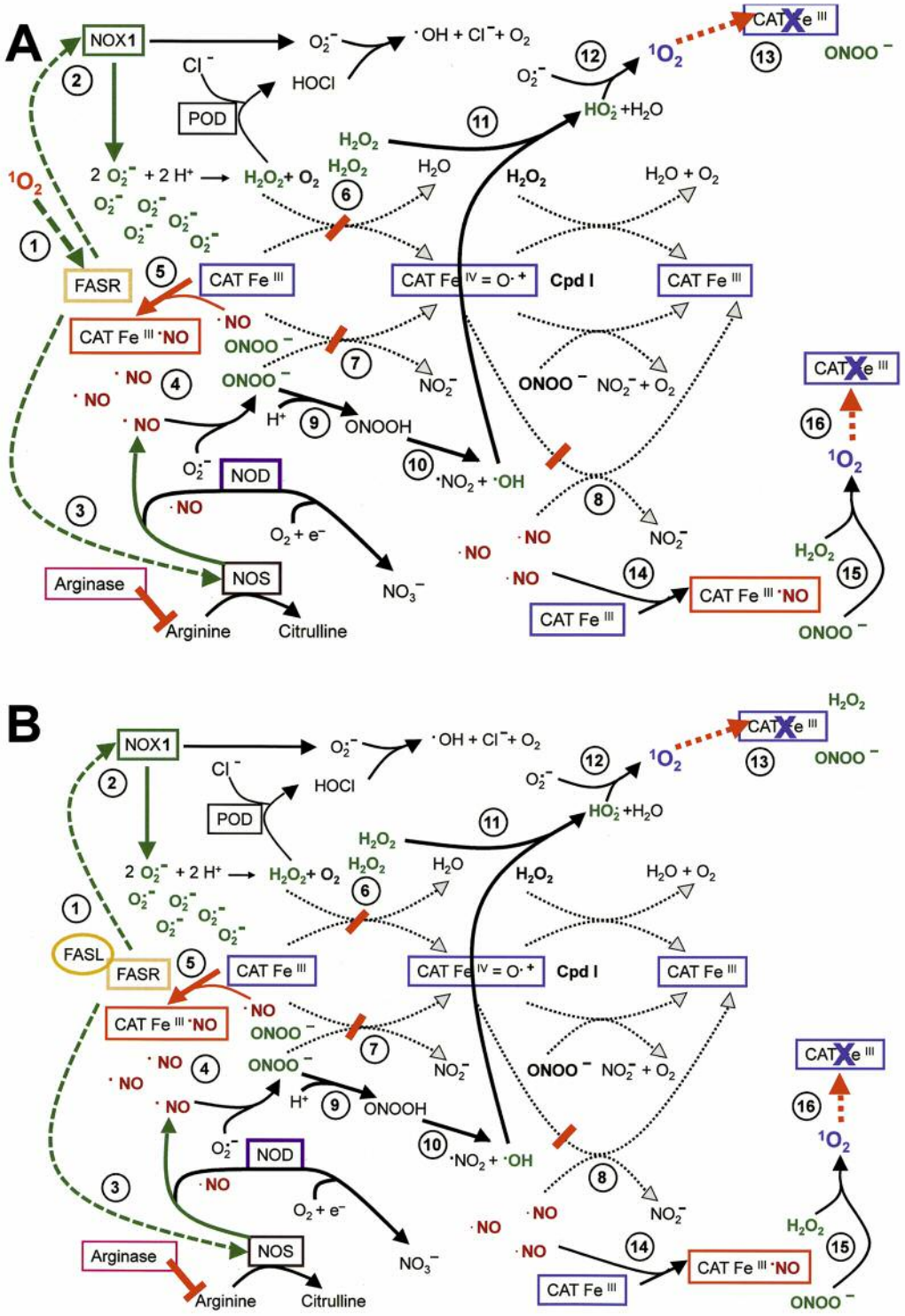

Figure 5. *Direct activation of the FAS receptor (FASR), a member of the tumor necrosis factor receptor family, by singlet oxgen or its ligand triggers singlet oxygen-mediated catalase inactivation. A: Activation of the FASR by singlet oxygen (#1) leads to activation of NADPH oxidase-1 (NOX1) (#2) and induction of NO synthase (NOS) expression (#3). The increased concentration of free NO (#4) causes reversible inhibition of catalase (#5), which interferes with reactions #6-#8 and allows for the formation of hydroperoxide radicals (reactions #9-11), the generation of singlet oxygen (#12) and inactivation of more catalase molecules (#13). Finally, intercellular reactive oxygen species/reactive nitrogen species (ROS/RNS)-dependent apoptosis-inducing signaling is reactivated. B: Activation of the FASR by its ligand causes the same sequence of events as shown in (A) for the activation of the FASR by singlet oxygen.*

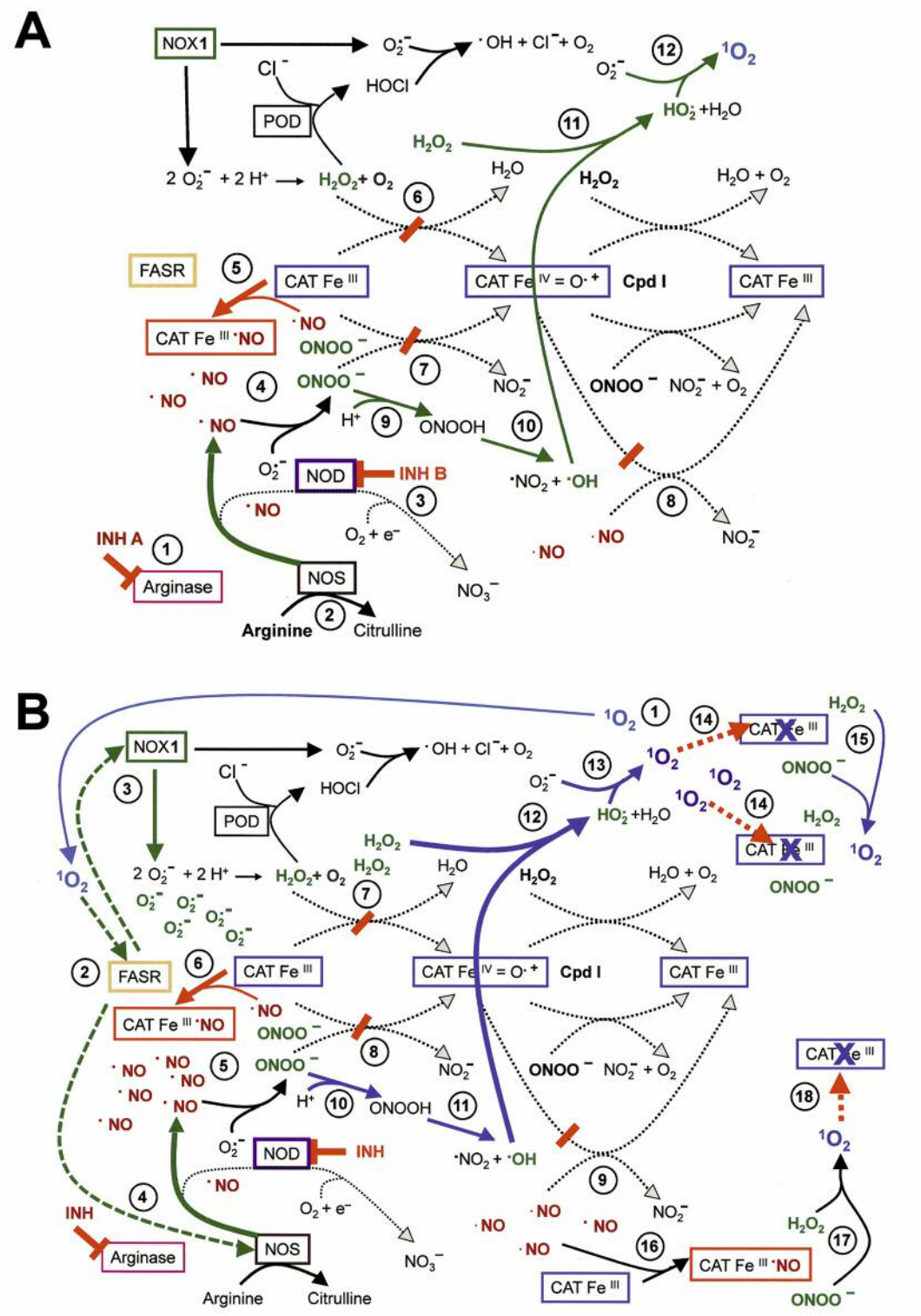

Figure 6. *Increase in free NO causes singlet oxygen-dependent inactivation of tumor cell protective catalase and reactivation of intercellular reactive oxygen species/reactive nitrogen species (ROS/RNS)-dependent signaling. A: Inhibition of arginase (#1) or NO dioxygenase (NOD) (#3) (or an increase in arginine or induction of NO synthase (NOS) expression by interferons, not shown in the figure) lead to an increase in free NO (#4) that reversibly inhibits catalase (#5). As a consequence of catalase inhibition, H_2O_2 and peroxynitrite are not decomposed (#6, 7) and NO is not oxidated (#8). Therefore hydroperoxyl radicals (#11) and secondary singlet oxygen (#12) are generated. B: Secondary singlet oxygen (#1) generated through the reactions described under A amplifies the generation of singlet oxygen in analogy to the reactions described in Figure 5A.*

4.2 Cold atmospheric plasma (CAP). One of the most exciting recent developments in experimental tumor therapy is the biomedical application of CAP, which might open promising new ways for tumor treatment at low costs (56-58). CAP devices generate a multitude of biologically active ROS/RNS such as superoxide anions ($O_2^{\bullet-}$), H_2O_2, hydroxyl radicals ($^{\bullet}OH$), NO, nitrogen dioxide ($^{\bullet}NO_2$), peroxynitrite ($ONOO^-$), nitrite (NO_2^-), nitrate (NO_3^-), $^1O_2$, hypochloride anion (OCl^-) and dichloride anion radicals ($Cl_2^{\bullet-}$) (59-62). The versatility of ROS/RNS and their interactions in biological processes seems to be utilized by treatment with CAP (6, 59, 63). Keidar *et al.* (64, 65), Schlegel *et al.* (66), Barekzi and Laroussi (67), Laroussi (68), Graves (61) and Ratovitski *et al.* (69) have recently reviewed the literature related to treatment of tumor cells by CAP *in vitro* and *in vivo* and point out that i) CAP treatment inhibits proliferation and induces apoptosis selectively in malignant cells; ii) CAP-derived ROS/RNS seem to be the trigger of tumor cell apoptosis, and iii) CAP treatment triggers the generation of intracellular ROS. It was also noted that CAP treatment must trigger a self-perpetuating process that reaches areas of the tumor that have not been directly reached by constituents of CAP.

A recent analysis (70) connected the data on ROS/RNS constituents in CAP with the established knowledge of site-specific ROS/RNS interactions and with experimental data on ROS/RNS interactions with tumor cells and nonmalignant cells (5, 7, 8, 21, 31, 71). Importantly, model experiments (31) have shown that $^1O_2$ can act selectively against membrane-bound catalase of tumor cells, thus restoring their sensitivity to intercellular induction of apoptosis. As singlet oxygen is present in CAP at the time of treatment, and as it can be generated from defined CAP constituents, Bauer and Graves concluded that CAP-derived singlet oxygen seems to be the prime compound to act on tumor cells *in vitro* and *in vivo* (70). The available data did not support a central direct role for any of the other ROS/RNS in CAP. In addition, a mechanism through which $^1O_2$ signaling can result in self-perpetuating apoptotic signaling from cell-to-cell was experimentally confirmed (70).

Therefore, CAP treatment of tumors seems to represent a promising singlet oxygen-driven approach for a rational tumor therapy that utilizes the selectivity of ROS/RNS signaling of malignant cells. Further work along these lines has been initiated.

4.3 Plasma-activated medium (PAM). Another very important finding in plasma medicine is the antitumor effect of plasma-treated medium (plasma-activated medium; PAM). PAM has selective antitumor effects *in vitro* (72-80) and *in vivo* (73) even hours after treatment of medium with CAP. This stability over time excludes hydroxyl radicals, superoxide anions, singlet oxygen, peroxynitrite and peroxynitrous acid as possible mediators of the PAM effect on tumor cells and tumors, as these species are short-lived. More stable compounds such as nitrite, nitrate, H_2O_2 were therefore in the focus of PAM-related research (81). Kurake *et al.* (79) and Girard *et al.* (80) recently reported that PAM contains nitrite in the millimolar concentration range and H_2O_2 in the tens of micromolar concentration range. They also showed that reconstituting the medium with the same concentrations of nitrite and H_2O_2 as found in PAM, fully mimicked the selective antitumor effect of PAM. AIthough Kurake *et al.* and Girard *et al.* defined the essential compounds in PAM, they did not propose a mechanism that explains the selectivity of antitumor action of PAM.

Bauer (unpublished data) suggests that the antitumor effect of nitrite and H_2O_2 in PAM as described by Kurake *et al.* (79) and Girard *et al.* (80) might be explained by a sequence of reactions that is initiated by peroxynitrite formation through the interaction between nitrite and H_2O_2, according to the reaction described by Lukes *et al.* (82). Peroxynitrite formed in the vicinity of the tumor cells, but at sufficient distance to be out of reach by the membrane-bound catalase, might then be either protonated through H^+ derived from proton pumps in the membrane or react with CO_2. Both pathways finally lead to the formation of hydroperoxyl radicals, either through the reaction between hydroxyl radicals and H_2O_2 or carbonate radicals and H_2O_2. Hydroperoxyl radicals may then generate singlet oxygen through their reaction with NOX1-derived superoxide anions. The proposed mechanism is completely in line with the findings by Kurake *et al.* (79) and Girard *et al.* (80), the known reaction potentials of the involved radical and nonradical species and with the results obtained in recently performed model experiments (31).

PAM treatment, therefore, also might represent a novel therapeutic strategy in which singlet oxygen is generated by stable precursor molecules and the selective autoamplificatory potential of tumor cells is used for the generation of secondary singlet oxygen. After singlet oxygen-mediated catalase inactivation, apoptosis is selectively induced in tumor cells through reactivated intercellular ROS/RNS-dependent signaling.

CAP and PAM not only use singlet oxygen as a final effector molecule, but both principles might also potentially interact during classical CAP treatment. It is not unlikely that CAP-derived species first establish the conditions of PAM generation and the effect on tumor cells is then mediated through PAM, under conditions where tumor cells are separated from the CAP source through fluid or biological materials.

4.4 NO shifts direct inhibition of catalase by salicylic acid to a singlet oxygen-dependent process. Salicylic acid and its derivatives have been recently shown to directly inhibit tumor cell-protective catalase and thus to trigger reactivation of

intercellular ROS/RNS-dependent signalling independently of the action of singlet oxygen (6, 11). This activity might explain the remarkable tumor-preventive role of acetyl salicylic acid (83-86). The frequently used explanation for the tumor-preventive effect of salicylic acid and its derivates based on the inhibition of cyclo-oxygenase (COX) is not conclusive as i) salicylic acid has been shown also to affect COX-negative tumor cells, and ii) as some other effective COX inhibitors do not show an antitumor effect [summarized in (11)].

The combination of *N*-acetyl salicylic acid and the NO donor diethylamine NONOate resulted in an impressive synergistic effect with respect to catalase inactivation and apoptosis induction of tumor cells (6). This synergistic effect targeted membrane-associated catalase in a very fast reaction that was mediated by singlet oxygen. The synergistic effect between *N*-acetyl salicylic acid and an NO donor correlated very well with the biological effect of NCX 4040, a compound with covalent binding of NO to aspirin (6). NCX 4040 caused rapid singlet oxygen-dependent inactivation of tumor cell catalase that was followed by reactivated intercellular ROS/RNS-dependent apoptosis-inducing signalling. Application of the singlet oxygen scavenger histidine in parallel with NCX 4040 abrogated the singlet oxygen-dependent synergistic effect and shifted the concentration-dependency curve of apoptosis induction for about two log steps towards the curve obtained with *N*-acetyl salicylic acid alone. Importantly, nitric oxide-donating *N*-acetyl salicylic acid prevented pancreatic cancer in a hamster tumor model, whereas *N*-acetyl salicylic acid applied alone was not effective (87). This finding demonstrates the antitumor potential of singlet oxygen and points to the role of catalase as antitumor target.

4.5 Singlet oxygen promotes the pro-oxidative effect of certain plant-derived tumor-preventive antioxidants. Tumor prevention by secondary antioxidative plant compounds such as flavonoids, or garlic compounds like diallyldisulfide and many others, is a major area of research (88, 89). Scavenging of mutagenic ROS and RNS is one of the concepts that has been discussed as basis for this finding. However, the recognition of multiple synergistic effects between plant compounds has led to the conclusion that mechanisms distinct from direct scavenging activities might be triggered by certain plant compounds (89). In addition, it was found that anthocyanidins induce a strong effect against tumor cell lines (11, 90-96) and tumors *in vivo* (92) These findings cannot be explained by ROS/RNS scavenging, but rather require to consider other mechanisms as underlying principles.

Anthocyanidins, despite their antioxidant nature, provoked a strong prooxidative and proapoptotic response selectively in tumor cells (11). This effect was based on the inhibition of NOD, an enzyme that consumes NO through conversion into NO_3^- (97, 98). The increased concentration of NO after inhibition of NOD allowed for the inactivation of membrane-associated catalase, followed by secondary singlet oxygen production and reactivation of intercellular ROS/RNS-dependent apoptosis-inducing signalling as described in Figure 6. This mechanism therefore might explain the antitumor activity of cyanidins *in vivo* (92), as well as their tumor-preventive potential (96).

4.6 Singlet oxygen-dependent antitumor effects of antifungal azoles. Antifungal azoles such as miconazole, ketoconazole, itraconazole and others have been shown to inhibit NOD (98). In line with their potential to increase the local concentration of free NO in tumor cells, and with the model on singlet oxygen generation by tumor cells after an increase in free NO as outlined in Figure 6 (11), these azoles have been shown to cause apoptosis selectively in tumor cells (2, 6). The cell biological effect of azoles was undistinguishable from that of other NOD inhibitors and inducers of singlet oxygen generation such as cyanidin.

The antitumor cell effect exerted by azoles *in vitro* correlates well with discovered antitumor effect *in vivo* that is already in the process of being translated into clinical application (99-101). As azoles also seem to affect tumor angiogenesis and defined cellular signalling pathways such as hedgehog, the contribution of the singlet oxygen dependent pathway is not clarified yet *in vivo* (such as the contributions of the other azole-dependent effects). However, as scavenging of singlet oxygen, inhibition of NOS or NOX1 completely inhibited apoptosis induction by azoles *in vitro*, a dominant role of these ROS/RNS-related processes in the antitumor activity of azoles seems to be most likely.

4.7 Singlet oxygen generation through inducers of NOS expression. Interferons and the FAS receptor are known to induce expression of NOS and thus cause an increase in the available concentration of NO [summarized in (6) and (52)]. This leads to the generation of singlet oxygen, catalase inactivation and reactivation of intercellular ROS/RNS-dependent apoptosis-inducing signalling (6, 52). It remains to be determined whether these processes play a role during the attack of cytotoxic T-cells on tumor cells, where interferon gamma and the FAS ligand play roles in addition to perforin/granzyme.

4.8 Singlet oxygen-related aspects related to the action of taxol. Although the classical explanation for the antitumor action of the established chemotherapeutic taxol focuses on the stabilization of microtubules by taxanes and the resultant impact on cell division, direct experimental proof for the impact of this mechanism in taxol-based tumor therapy has not been convincingly shown, whereas an early and crucial role of ROS for the action of taxol has been experimentally

demonstrated *in vitro* and *in vivo* (102). This alternative explanation for taxol action is in perfect agreement with the findings by Song *et al*. (103) and Torres and Horwitz (104) who have shown that different mechanisms can be activated, dependent on the concentration of taxol applied. In line with this finding, Lin *et al*. have shown that low concentrations of taxol cause cell death without affecting microtubules and mitosis (105).

Ongoing experiments have shown that taxol affects tumor cells through modulation of NO metabolism, singlet oxygen-dependent inactivation of catalase and reactivation of intercellular ROS/RNS-dependent apoptosis-inducing signaling analogously to the effects described for anthocyanidins and summarized in Figure 6 (Bauer unpublished data). Thereby, the selective antitumor effect of taxol requires sustained expression of NOX1 of the target cells. In analogy to anthocyanidins (6, 11, 52), taxol interacts synergistically interacts with catalase inhibitors and with enhancers of NOX1 activity (Bauer, unpublished data). The evaluation and utilization of these synergistic effects might allow establishment of novel strategies that reduce the required doses of taxol for therapy with the aim of reducing side-effects and costs of treatment in the future.

5. Concluding Remarks

Sustained generation of extracellular superoxide anions by NOX1 and the control of the potentially resultant intercellular ROS/RNS-dependent apoptosis-inducing signalling through membrane-associated catalase are hallmarks of the phenotype of tumor cells. Membrane-associated catalase of tumor cells therefore represents a rational and unique target for therapeutic approaches that utilize tumor cell-specific ROS chemistry for their selective destruction.

These approaches for tumor therapy may be based on the application of extracellular singlet oxygen through novel strategies such as treatment with CAP or PAM. As outlined here, extracellular singlet oxygen inactivates membrane-associated catalase and thus enables the generation of secondary singlet oxygen which triggers autoamplification of catalase inactivation and subsequent reactivation of intercellular ROS/RNS signalling.

Classical photodynamic therapy does not use this principle but is based on the preferential uptake of singlet oxygen-generating photosynthesizers into tumor tissue. The generation of cell-impermeable photosynthesizers in the future might add specific targeting of membrane-associated catalase to this approach and thus increase its selective action on tumor cells.

Alternatively, the enhancement of intracellular NO levels through inhibitors of NOD (such as many secondary plant compounds *e.g*. anthocyandins or taxol) or enhancers of NOS expression (such as activated FAS receptor or interferon gamma) causes transient inhibition of catalase through formation of an inactive $CATFe^{III}{}^{\bullet}NO$ complex, followed by generation of secondary singlet oxygen, autoamplification of catalase inactivation and reactivation of intercellular apoptosis-inducing signalling. The knowledge of the chemical biology of singlet oxygen and its precursors should allow establishment of strategies for the utilization of synergistic effects that are based on rational application of the established signal chemistry of ROS/RNS and that may increase efficiency while reducing side-effects and costs.

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