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Cold Atmospheric Plasma and Plasma-Activated Medium: Antitumor Cell Effects with Inherent Synergistic Potential

Georg Bauer

Institute of Virology, Medical Center and Faculty of Medicine, University of Freiburg, Germany; Tel.: 0049 761 203 6618, Fax: 0049 761 203 6562, E-mail: georg.bauer@uniklinik-freiburg.de

ABSTRACT: Nitrite and H_2O_2, long-lived molecular species from cold atmospheric plasma (CAP) and plasma-activated medium (PAM), reach tumor target cells *in vitro* and *in vivo*. Through several steps, the interaction between nitrite and H_2O_2 leads to generation of singlet oxygen ($^1O_2$). $^1O_2$ then interacts with a specific biochemical switchboard on tumor cells that is composed of catalase, superoxide dismutase (SOD), first aptosis signal (FAS) receptor, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. As a result, local inactivation of catalase by minute concentrations of primary singlet oxygen opens a strong autoamplificatory sustained process of secondary singlet oxygen generation and catalase inactivation. This process is driven by tumor cell–specific NADPH oxidase-1 and spreads within the tumor cell population. The concerted action of singlet oxygen interaction with catalase, SOD, and FAS receptor causes an efficient mode of synergistic interaction. Defined reactive oxygen and reactive nitrogen species (ROS/RNS) such as H_2O_2 and nitrite have multiple functions in this process. Catalase-mediated oxidation of nitrite enhances generation of nitrogen dioxide, which is rate limiting for singlet oxygen generation. Before singlet oxygen–mediated inactivation of catalase and, subsequently, reactivated intercellular ROS/RNS signaling can activate the mitochondrial pathway of apoptosis, counteraction of glutathione to lipid peroxidation must be abrogated through aquaporin-mediated influx of H_2O_2 into cells. CAP- and PAM-dependent immunogenic cell death triggers a strong immune response that finalizes antitumor action *in vivo*. Thus, the high efficiency of CAP and PAM seem to depend on concerted action of several dominant steps and their autoamplificatory potential.

KEY WORDS: cold atmospheric plasma, plasma-activated medium, singlet oxygen, catalase, immunogenic cell death

I. INTRODUCTION

The complexity of the physics and chemistry of cold atmospheric plasma (CAP) and plasma-activated medium (PAM) is paralleled by their striking biological effects, ranging from antimicrobial action to stimulating wound healing and impressive antitumor effects *in vitro* and *in vivo*.[1–19] CAP and PAM represent a reactive oxygen and reactive nitrogen species (ROS/RNS)-dependent system that may be applicable to an impressively wide range of tumors that are derived from different tissues and show a high degree of selective action in most systems studied. CAP and PAM are unique with respect to their mode of initial interaction with tumor cells and tumor tissue: Initial interaction of ROS and RNS with tumor cells occurs primarily on the outside of the cells. No other experimental antitumor system has this specific feature. Even photodynamic therapy that uses the power of one defined initial ROS, that is, singlet oxygen, does not apply its key molecule to the

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outside of malignant cells, because the photosensitizer is given sufficient time to enter cells before illumination triggers intracellular production of singlet oxygen.

Novel insights into the chemical biology of malignant cells during tumor progression, rich literature on CAP- and PAM-dependent chemistry and biology, and data on the interaction of defined ROS and RNS with malignant and nonmalignant cells allow investigators to propose a potential mechanism of CAP- and PAM-dependent antitumor effects.[20,21] The high efficiency and impressive selectivity of CAP- and PAM-mediated antitumor effects have served to inspire us to search for evidence-based potential synergistic effects that may be inherent to CAP and PAM action. The biochemical basis of these synergistic effects is discussed in this conceptional review. Detailed knowledge about these synergistic effects may be valuable for further optimization of CAP or PAM treatment. This may become especially relevant under conditions allowing only the application of lower doses, such as future endoscopic application of CAP or PAM to tumors in internal organs.

II. CONCEPTIONAL REVIEW

A. Basic Principles of CAP and PAM Action

1. Physical Plasma and Plasma Medicine

The gaseous and liquid phases of CAP are a rich source of radical and nonradical ROS/RNS.[1–19] Due to variable lifetimes, ranges of action, and multiple potentials of interaction, these species represent a unique scenario in ROS/RNS chemical biology. The treatment of media with CAP results in generation of PAM that maintains the major biological effects of CAP, although it only contains long-lived species from CAP such as nitrite, nitrate, and H_2O_2.[22–25]

The study of the effects of exogenous ROS/RNS in CAP and PAM on biological systems has opened the exiting new field of plasma medicine.[2,3,6,8,9,26–35] CAP and PAM cause impressive antibacterial[36–38] and antiviral effects[39] and benefit wound healing[40–43] and treatment of actinic keratosis.[44–46] A particular focus is on promising *in vitro* and *in vivo* antitumor effects on a broad variety of tumor systems.[8,9,12,29,30,47–55] Clinical application of CAP in tumor therapy yielded the first encouraging results without severe side effects.[31–34,56] Although CAP and PAM seem to target a rather general principle regarding tumor cells, the mechanisms underlying their antitumor action are still a matter of scientific debate.

2. CAP- and PAM-Mediated Antitumor Effects

In most studies, antitumor effects of CAP and PAM are found to be selective with respect to the malignant phenotype of target cells *in vitro* and *in vivo*.[22,57–68] Only a few reports claim to have found nonselective apoptosis-inducing effects of CAP or PAM.[69–73] Further studies, including those for standardizing CAP and PAM doses and composition,

may clarify whether this discrepancy might simply be explained by a conceivable correlation between doses and selectivity or nonselectivity of action.

a. Initial Effects of CAP and PAM on Tumor Cells

General agreement exists that initial CAP effects on tumor cells are mediated by CAP-derived ROS/RNS and amplified by intracellular ROS.[8,9,13,29,30,47,48,50,51,52–55,74] Because apoptosis is induced in areas of treated tumors that are not directly reached by constituents of CAP, a self-perpetuating signaling system was postulated.[12,13,20,47,53] Tumor treatment with CAP and PAM therefore seems to have a strong conceptual and mechanistic overlap with other ROS/RNS-dependent treatments, such as photodynamic therapy and modulating the endogenous NO level of tumor cells for subsequent ROS/RNS-driven processes that lead to selective apoptosis induction in tumor cells.[75,76] Therefore, the well-characterized ROS/RNS composition of CAP and PAM and the chance to modulate their composition offer great analytical potential for research on antitumor action of CAP and PAM and their application. Analogous to photodynamic therapy that requires interaction between an initial singlet oxygen–dependent step and a subsequent immunological process,[77] CAP and PAM action may also be connected to strong immunological processes.

b. Immunogenic Cell Death Induced by CAP and PAM

It seems that CAP- and PAM-dependent selective apoptosis induction in tumor cells has characteristics of immunogenic cell death; that is, stimulation of dendritic cells that thus provokes subsequent activation of cytotoxic T cells.[78–85] In addition, CAP gives rise to activation of cytotoxic macrophages.[86] The findings that CAP- and PAM-induced cell death may trigger an additional strong and selective immunological antitumor effect, that is far ranging within the tumor and the body, is in perfect agreement with the general concept that immunological cell death is a necessary element in finalizing tumor treatment by various antitumor agents.[77,87–91]

3. Common Principles of CAP and PAM Action in Tumor Cells

It is intriguing that PAM mimics antimicrobial and antitumor effects of CAP,[22–24,38,58,92–98] although its composition seems to be restricted to H_2O_2, nitrite, and nitrate.[22–25] However, it is important to consider that the spectrum of species contained in the liquid phase of CAP that can actually reach tumor cells *in vitro* or *in vivo* depends essentially on volume and composition of medium or biological material above or around the target cells. Highly reactive CAP species may likely be filtered out before they can react with target cells, whereas long-lived and far-ranging species such as H_2O_2, nitrite, and nitrate have a good chance of reaching target cells. Model experiments show that CAP-derived molecular species that can actually reach a tumor *in vivo* may be the same as those found

in PAM.[99–103] How can relatively simple species such as H_2O_2, nitrite, and nitrate mediate such efficient, complex, and selective effects on tumor cells?

4. Divergent Working Hypotheses for CAP and PAM Action

a. Classical Concept for CAP and PAM: Direct Action on Target Cells

Working hypotheses of most investigators were previously based on assumptions that the molecular species acting selectively on tumor cells are (1) already present in CAP or PAM itself, and (2) present at sufficiently high concentrations to allow direct induction of apoptotic or necrotic cell death. However, this conceptional approach has not yet led to the establishment of an experimentally determined concise model for CAP- and PAM-dependent signaling chemistry that is in perfect agreement with existing central biological and chemical observations.

H_2O_2 in particular had often been assumed to act as a direct, selective apoptosis inducer of CAP and PAM. However, this assumption disagrees with experimental assessments showing that tumor cells are less sensitive to exogenous H_2O_2 than nonmalignant cells.[104,105] This is due to the expression of membrane-associated catalase on the tumor cell membrane.[20,21,105–114] Therefore, H_2O_2 by itself cannot cause selective apoptosis induction in tumor cells.

b. Novel Concept for CAP and PAM Action: Triggering Specific Cellular and Intercellular Signaling Pathways

An alternative model to explain selective antitumor action of CAP and PAM was proposed by Bauer and Graves.[20,21] This model is based on the concept that an initially low concentration of singlet oxygen contained in or generated by CAP and PAM is insufficient to induce cell death directly but triggers tumor cells to generate higher concentrations of secondary singlet oxygen in an autoamplificatory process. This leads to inactivation of protective catalase in the membrane of the initially targeted cell, followed by a bystander signaling-like spread of catalase inactivation on neighboring cells. Finally, this allows reactivation of intercellular ROS/RNS-dependent apoptosis-inducing signaling within the population of tumor cells. This model is based on experimental evidence that was obtained using multiple approaches in which singlet oxygen–dependent effects on tumor cells were studied.[75,76,109,115–117]

The initially determining switching-on effect by CAP- or PAM-derived singlet oxygen allows strong subsequent and continuous generation of secondary singlet oxygen by tumor cells. This is based on their active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and NO synthase (NOS), with H_2O_2 and peroxynitrite acting as intermediates.[109,115–117] Selectivity of CAP and PAM action for tumor cells is thus warranted by the (1) initial singlet oxygen/catalase interaction, (2) potential of tumor cells (but not nonmalignant cells) to drive NOX1- and NOS-dependent generation of secondary singlet oxygen, and (3) NOX1-driven intercellular ROS/RNS-dependent apoptosis-inducing signaling after catalase inactivation.

c. Role of Aquaporins during CAP and PAM Action

Yan et al. presented strong evidence for a dominant controlling function of aquaporins related to the action of CAP and PAM.[118,119] These investigators also suggested that aquaporins are primary interaction partners for CAP- or PAM-derived H_2O_2. In their model, selectivity of CAP and PAM action for tumor cells is explained by the higher concentration of aquaporins in tumor cell membranes. However, this model did not take into account that membrane-associated catalase on tumor cells may decompose exogenous H_2O_2 before it can pass through aquaporins. Therefore, Bauer suggested that the contribution of aquaporins to the sensitization of tumor cells by CAP and PAM requires preceding catalase inactivation.[21] Recently, this concept was experimentally verified in kinetics studies showing that aquaporin action was required *after* catalase inactivation due to singlet oxygen derived from CAP or generated by PAM (Bauer, manuscript in preparation). Because glutathione depletion substitutes for aquaporin function, the significance of aquaporin-dependent influx of H_2O_2 seems to be related to sensitization of tumor cells for exogenous ROS effects through reduced endogenous glutathione levels.

Completed experimental work using a bottom-up experimental approach with compounds that are found in PAM confirmed central predictions of our previous models. A portion of these data was presented at the 7th International Conference on Plasma Medicine (ICPM-7) in Phildadelphia.

B. Chemical Biology of CAP and PAM Interaction with Tumor Cell Surface

Evaluation of possible interactions of CAP and PAM with the surface of tumor cells requires (1) detailed knowledge of the complex nature of the membrane of tumor cells,[21,76,108,111,112] (2) composition of CAP and PAM,[1–19] (3) free diffusion path length of CAP- and PAM-derived ROS/RNS, and (4) scavenging effect of biological materials for CAP- and PAM-derived molecular species.[99–103]

1. Biochemistry of Malignant and Nonmalignant Cell Surfaces

Essential aspects of the surface of tumor cells compared to nonmalignant cells are shown in Fig. 1. These central aspects include active NOX1 (the hallmark of malignant cells)[120–124,111,112] and membrane-associated catalase that protects tumor cells from NOX1-driven intercellular ROS/RNS signaling. Because NOX-derived superoxide anions have the potential to inhibit catalase,[125–128] catalase function requires the parallel expression of membrane-associated superoxide dismutase (SOD) that reduces the concentration of superoxide anions. Whereas the concentration of membrane-associated catalase is sufficiently high to completely prevent intercellular ROS/RNS signaling,[105,108] SOD concentration on the surface does not allow complete removal of free superoxide anions but ensures catalase function by reducing concentration of superoxide anions to a level that does not lead to inhibition of catalase.[110,129]

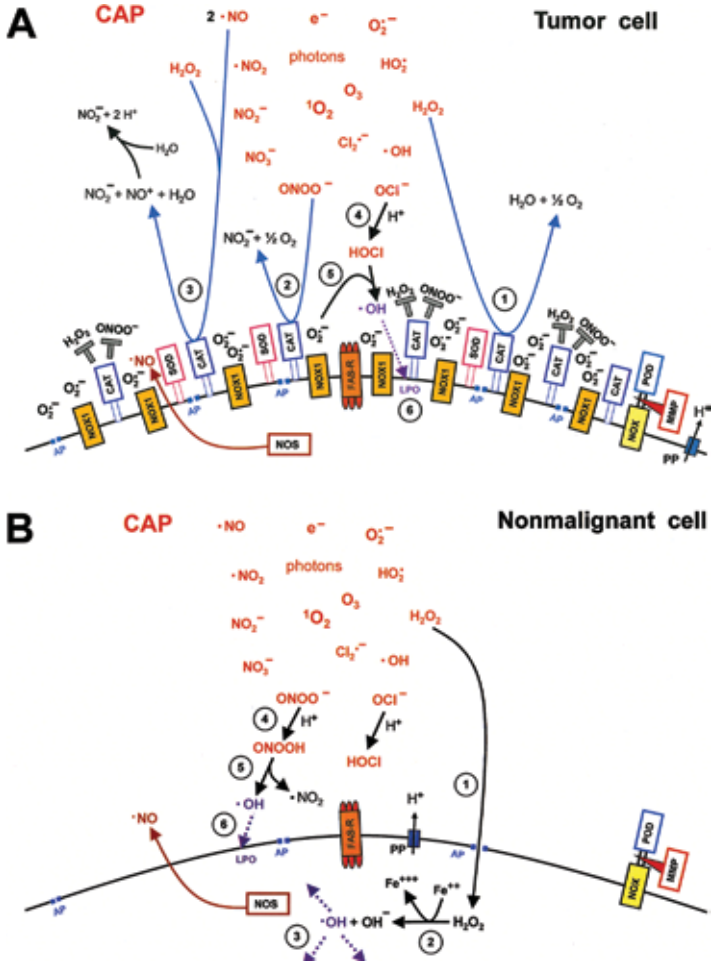

FIG. 1: Interaction of CAP with tumor cells and nonmalignant cells. (A) Tumor cells. The membrane of bona fide tumor cells contains superoxide anion–generating NOX1 and dual oxidase (DUOX), which consists of a peroxidase domain (POD) and a NOX domain. POD is removed from DUOX through matrix metalloprotease (MMP) action. Catalase (CAT) is attached to the outside of the membrane and decomposes the H_2O_2 and peroxynitrite that is generated by tumor cells. SOD that is attached to the membrane prevents superoxide anion–mediated inhibition of catalase. The membrane contains aquaporins (AP), proton pumps (PP), and FAS receptor. CAP-derived H_2O_2 (1) and peroxynitrite (2) is decomposed by catalase, whereas NO is oxidized by catalase (3). Oxidation of NO requires the formation of compound I through catalase/H_2O_2 interaction. Protonation of hypochloride anions leads to the formation of HOCl (4). The interaction of HOCl with superoxide anions (5) causes generation of hydroxyl radicals that induce lipid peroxidation (6). (B) Nonmalignant cells. H_2O_2 passes the membrane through aquaporins (1) and is subject to intracellular Fenton chemistry (2),(3), resulting in damage and apoptosis-inducing hydroxyl radicals. Peroxynitrite causes lipid peroxidation (LPO) and apoptosis through (4)–(6).

Further membrane elements of high significance for signaling effects are aquaporins that control the influx of H_2O_2 into cells and proton pumps that allow conversion of peroxynitrite to peroxynitrous acid in close vicinity to the membrane.

2. Conceivable Interactions between CAP and PAM ROS/RNS and the Cell Surface

CAP contains a plethora of defined ROS/RNS, electrons, and photons, as summarized in Fig. 1(A).[1–19] Biological material surrounding the tumor and the layer of medium above tumor cells *in vitro* most likely consumes most of the highly reactive species derived from CAP or generated through CAP/liquid-phase interaction. This process thus selects for a few long-lived species. Therefore, CAP-derived electrons, photons, hydroxyl radicals, and superoxide anions seem to have no chance to reach tumors *in vivo* or tumor cells *in vitro* at concentrations that might potentially induce biological effects.[99–103]

Because the surface of tumor cells expresses catalase and SOD, CAP- or PAM-derived species with strong apoptosis-related potential such as H_2O_2, peroxynitrite, or NO are efficiently decomposed (H_2O_2, peroxynitrite) or oxidized (NO) by tumor cell protective catalase.[21,108,124] Thus, they have no chance to induce apoptosis in tumor cells, unless they are applied at very high concentrations. Hypochloride anions are potentially able to generate HOCl after approaching proton pumps in the membrane, followed by hydroxyl radical generation after HOCl/superoxide anion interaction.[124] But HOCl also likely reacts with biological material surrounding the tumor. In addition, its apoptosis-inducing reaction does not have obvious potential to trigger self-perpetuation of the process. Therefore, the role of HOCl is rather limited for triggering a minor apoptotic response. This limitation might also be due to the absence of HOCl catalase inhibition, preventing influx of H_2O_2 into tumor cells. Therefore, the counteraction of intracellular glutathione peroxidase-4/glutathione for lipid peroxidation impedes the effect of HOCl-derived hydroxyl radicals.

Because nonmalignant cells do not express NOX1, and in particular have no catalase or SOD on their surface [Fig. 1(B)], CAP-derived hydrogen peroxide and peroxynitrite at sufficiently high concentrations may induce apoptosis in nonmalignant cells.[104,105] Because nonmalignant cells are more highly affected by exogenous H_2O_2 and peroxynitrite than are catalase-bearing tumor cells, the concentration of these two species in CAP and PAM must be below an apoptosis-inducing concentration for nonmalignant cells, when CAP- and PAM-mediated selective action toward tumor cells and sparing of nonmalignant accompanying cells would occur. Recent experimental evidence (Bauer, manuscript in preparation) shows that H_2O_2 concentrations that are too low to induce apoptosis in nonmalignant cells are sufficient to allow enough singlet oxygen generation to trigger cascades that lead to tumor cell death when combined with nitrite. Under these conditions, generation of low concentrations of singlet oxygen is ensured and seems to drive the subsequent biochemical steps that lead to tumor cell apoptosis.

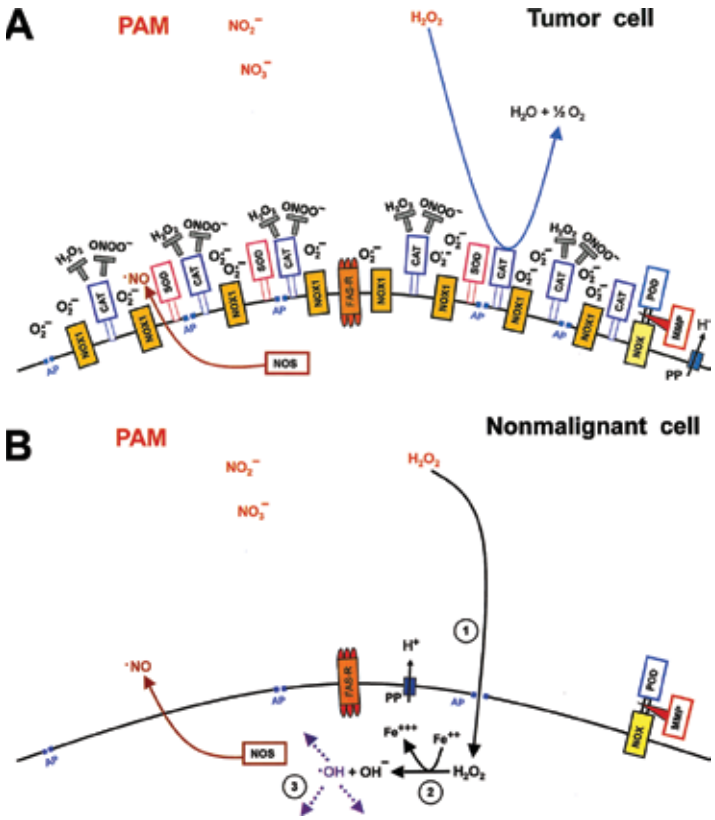

FIG. 2: Interaction of PAM with tumor cells and nonmalignant cells. PAM essentially contains H_2O_2, nitrite, and nitrate. H_2O_2 is decomposed by membrane-associated catalase of tumor cells (A) and may induce apoptosis in nonmalignant cells through (1)–(3) (B), provided that it is present at sufficiently high concentrations. Selective apoptosis induction in tumor cells therefore requires the concentration of H_2O_2 to be too low to affect nonmalignant cells, and the reaction between H_2O_2 and nitrite must lead to generation of singlet oxygen, as outlined in Fig. 3.

It is obvious in Fig. 2 that H_2O_2, which is contained in PAM, is most likely decomposed by tumor cells, unless its concentration is extremely high. Very high concentrations of H_2O_2 can lead to SOD inactivation, followed by superoxide anion–mediated inhibition of neighboring catalase and onset of singlet oxygen–generation of H_2O_2.[117] However, the high concentrations of H_2O_2 that are required for these effects do not allow selective apoptosis induction in tumor cells, because nonmalignant cells are affected by H_2O_2 even more strongly than tumor cells.[105] Therefore, we can conclude that establishment of a selective antitumor effect requires that H_2O_2 in PAM must be below an apoptosis-inducing concentration for nonmalignant cells. It is also obvious that the biological effect of PAM on tumor cells requires generation of a signaling species from its constituents to achieve specific apoptosis induction in tumor cells.

3. Signaling Effects through $^1O_2$ Derived from CAP and PAM

Chemical biological effects of singlet oxygen that are generated by an illuminated photosensitizer shows that extracellular singlet oxygen efficiently mediates apoptosis induction in tumor cells, without affecting their nonmalignant counterparts.[115] The selectivity of apoptosis induction by singlet oxygen is strictly dependent on its generation outside of the cells; it was lost when singlet oxygen was generated inside cells.[115] It is thus safe to assume that as singlet oxygen that is derived from CAP or generated by PAM approaches target cells from the outside, it may exert the same specific antitumor effects as those established in model experiments with an extracellular photosensitizer. Figure 3(A) illustrates that singlet oxygen that is contained in CAP has the potential to cause local inactivation of a few catalase molecules on the surface of tumor cells, in accordance with recent findings.[115,130,131] Figure 3(A) also shows that the CAP liquid phase, in addition to singlet oxygen, contains various constituents that may lead to the generation of further singlet oxygen molecules through multiple, interconnected pathways. It becomes obvious that the sequence of reactions starting with reaction (4) in Fig. 3(A) may create a pathway that causes PAM to generate $^1O_2$ through the scheme shown in Fig. 3(B). The potential relevance of reactions (1)–(3) in Fig. 3(B) had been already recognized by Girard et al.,[24] Kurake et al.,[23] and Jablonowski and von Woedtke.[132] Due to the relatively low reaction rate of (1), the importance of this scenario was initially underestimated by the plasma community. In light of the novel concept that PAM generates signaling molecules rather than directly damaging tumor cells, the slow reaction 1 can rather be seen as an advantage, because it ensures long-lasting generation of low concentrations of signaling molecules. It also ensures the availability of H_2O_2 for (4) after (1) is accomplished. Recent experimental work (Bauer, manuscript in preparation) has confirmed the reaction scheme for PAM action, as shown in Fig. 3(B).

Multiple completed model experiments that used either direct application of exogenous singlet oxygen[115] or NO-mediated inhibition of catalase[75,109,116] demonstrate that local inactivation of membrane-associated tumor cell catalase provokes a subsequent autoamplificatory generation of secondary singlet oxygen that is driven by free H_2O_2 and peroxynitrite at the site of inactivated catalase. Due to the activity of NOX1 and NOS, tumor cells continuously generate H_2O_2 and peroxynitrite. Thus, the sustained generation of secondary singlet oxygen is ensured. This is followed by inactivation of large amounts of tumor cell protective catalase molecules on the tumor cell membrane. As a result, intercellular apoptosis-inducing ROS/RNS signaling is subsequently reactivated. We may therefore conclude that low concentrations of singlet oxygen from CAP or PAM are sufficient to trigger secondary singlet oxygen generation and catalase inactivation in an autoamplificatory mode. This scenario is summarized in Fig. 4(A) and has been confirmed by direct experimental approach (Bauer, manuscript in preparation). In contrast to tumor cells, nonmalignant cells are unaffected by extracellular singlet oxygen at low to moderate concentrations, because they lack both catalase and NOX1 [Fig. 4(B)].

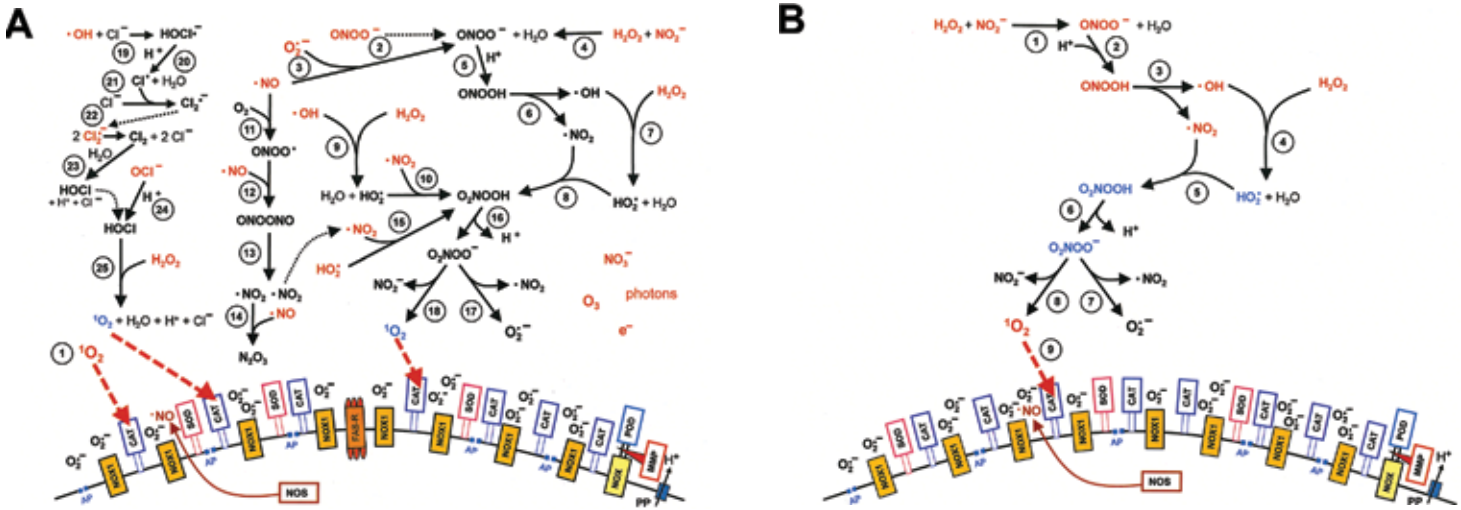

FIG. 3: Singlet oxygen in CAP and PAM. (A) Multiple sources of singlet oxygen in CAP. CAP contains singlet oxygen ($^1O_2$) (reaction 1). In addition, most of the reactive species contained in CAP (labeled in red) have the chemical potential to contribute to generation of singlet oxygen. Peroxynitrite that is present in CAP (2) or generated through the interaction between superoxide anions and NO (3) or between H_2O_2 and nitrite (4) may lead to the formation of peroxynitric acid (O_2NOOH) through (6)–(8), involving CAP-contained H_2O_2. Peroxynitric acid may alternatively be generated through (9), (10), or (15). Reaction (15) requires NO_2 to be present in CAP or generated through oxidation of NO (11)–(14). Peroxynitric acid leads to the formation of singlet oxygen through (16) and (18). Reactions (19)–(25) summarize alternative pathways for the generation of HOCl and singlet oxygen generation through the interaction between H_2O_2 and HOCl. Singlet oxygen from all of these pathways has the potential to inactivate catalase through reaction with histidine at the active center, provided that it is generated sufficiently close to the enzyme. (B) Singlet oxygen generation in PAM. H_2O_2 and nitrite are sufficient to generate singlet oxygen through (1)–(9), in analogy to the reaction described for CAP (4)–(8),(16)–(18) in (A). In this way, PAM has the potential for local inactivation of catalase molecules by singlet oxygen.

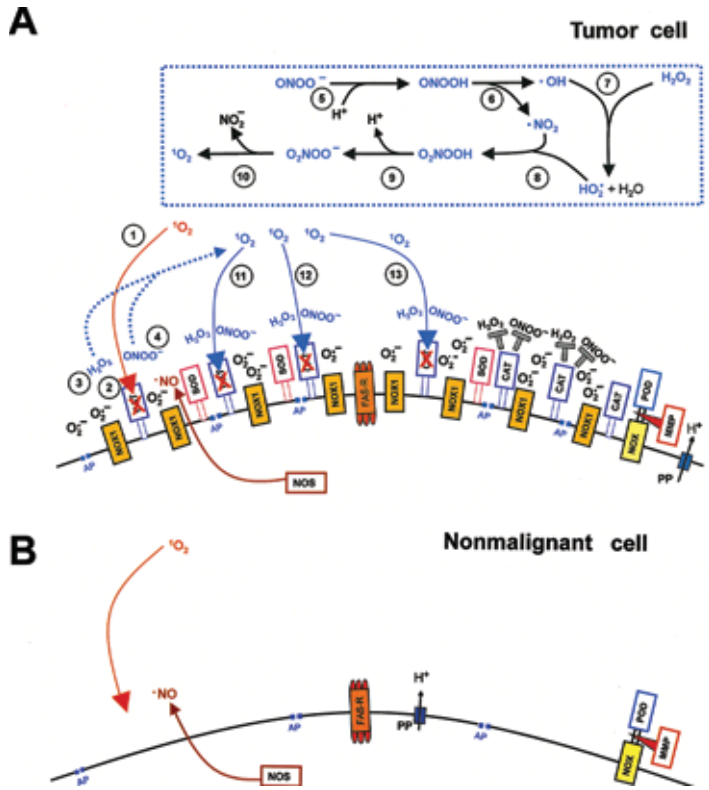

FIG. 4: Singlet oxygen from CAP or PAM triggers the generation of secondary singlet oxygen selectively by tumor cells. (A) Tumor cells. Singlet oxygen from CAP or PAM leads to local inactivation of catalase, followed by free H2O2 and peroxynitrite in close vicinity of the inactivated enzyme (1). Free H2O2 (3) and peroxynitrite (4) allow for the formation of secondary singlet oxygen (details in [5]–[10]). Secondary singlet oxygen causes inactivation of further catalase molecules (11)–(13) that will then allow further generation of singlet oxygen in an autoamplificatory process. It is important to note that singlet oxygen generation at the site of inactivated catalase is continuously propagated through a new supply with H2O2 and peroxynitrite that are derived from NOX1 and NOS. (B) Nonmalignant cells. Singlet oxygen from CAP or PAM finds no biologically relevant target structure on nonmalignant cells and therefore produces no effect (at up to moderately high concentrations).[115]

4. Additional Potential Signaling Elements

Ozone has been found to inactivate catalase,[133,134] so CAP-derived ozone may potentially contribute to initial inactivation of tumor cell protective catalase. This would lead to generation of secondary singlet oxygen, analogous to the scenario triggered by initial singlet oxygen. Therefore, ozone might act in concert with singlet oxygen to induce the same signaling process, provided it can reach malignant target cells at sufficiently high concentrations.

Free electrons from CAP are not likely to reach the surface of tumors *in vivo* or tumor cells *in vitro* that are covered by a layer of medium. Therefore, electron-dependent reduction of catalase compound I ($CATFe^{IV}=O^{\cdot+}$) to compound II ($CATFe^{IV}=O$), followed by H_2O_2–mediated generation of the terminally inactive compound III ($CATFe^{III}O_2^{\cdot-}$), is solely a theoretical possibility, although catalase inactivation through electron transfer has been observed in electron donors such as methyldopa.[116]

C. Potential Synergistic Effects Inherent to CAP and PAM

1. Effects Induced by Singlet Oxygen

a. Synergistic Effects through Parallel Singlet Oxygen–Dependent Inactivation of Membrane-Associated Catalase and SOD

Due to its higher concentration on the cell membrane, catalase is more likely to react with the limited concentration of singlet oxygen derived from CAP or PAM than that from SOD. However, as soon as secondary singlet oxygen is generated through the complex interaction between H_2O_2 and peroxynitrite at the site of inactivated catalase, chances of singlet oxygen–dependent inactivation of SOD obviously increase [Fig. 5(A)].[130,131] This then results in a local increase in concentration of NOX-1–derived superoxide anions at the site of inactivated SOD. Because superoxide anions can inhibit catalase,[125–128] superoxide anion–dependent inhibition of catalase in the neighborhood of inactivated SOD should then trigger higher generation of secondary singlet oxygen through the reaction between free H_2O_2 and peroxynitrite. One might assume that singlet oxygen–dependent inactivation of SOD thus merely contributes additively to the biochemical effects of inactivated catalase. But experimental analysis of specific neutralizing single-domain antibodies directed toward either catalase or SOD showed that parallel targeting of SOD and catalase causes a remarkable synergistic effect.[110] Compared to single application of antibodies, the necessary concentration of the two antibodies used in the synergy experiments could be reduced to < 1% of the value required to achieve the same effect with single antibodies. It is justified to assume that parallel inactivation of catalase and SOD by singlet oxygen would cause a similar synergistic effect as that for parallel targeting with neutralizing antibodies. This mechanism may therefore significantly contribute to the synergistic potential that is inherent in CAP and PAM and at least partially explain the striking biological efficiency.

b. Synergistic Effects through Singlet Oxygen–Mediated Activation of First Apoptosis Signal (FAS) Receptor

Because FAS receptor can be activated by singlet oxygen (even in the absence of a genuine FAS ligand),[135] secondary singlet oxygen generated after initial CAP- or PAM-dependent singlet oxygen effects on tumor cell catalase can very likely have a chance to activate FAS receptor in target cells [Fig. 5(B)]. Usually, tumor cells do not express

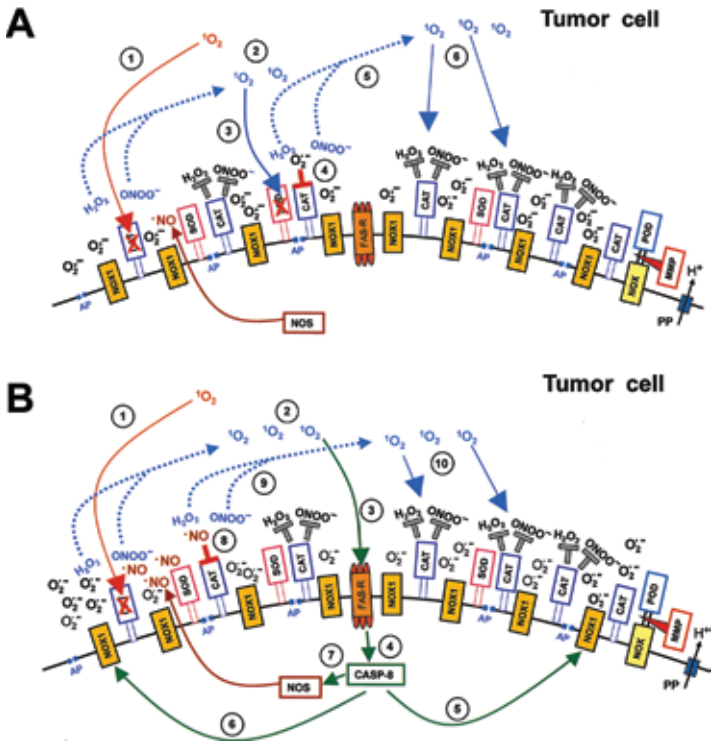

FIG. 5: Additional targets for secondary singlet oxygen on tumor cells. (A) Targeting of SOD. Secondary singlet oxygen (2) generated after the initial action of CAP- or PAM-derived primary singlet oxygen (1) may hit membrane-associated SOD and inactivate the enzyme through interaction with histidine at its active center.[130,131] As a consequence, the concentration of NOX-derived superoxide anions may increase in close vicinity to the inactivated enzyme and cause inhibition of neighboring catalase molecules.[125–128] This then triggers further amplification of secondary singlet oxygen generation. (B) Targeting of FAS receptor. Secondary singlet oxygen may activate FAS receptor (2),(3).[135] As a consequence, caspase-8 is activated by the receptor and enhances activities of NOX1 and NOS (4)–(7). High local concentrations of NO may reversibly inhibit catalase (8). This allows further continuous generation of secondary singlet oxygen (9),(10).

sufficient FAS receptor to allow direct death receptor–dependent cell death through the canonical FAS pathway.[75] However, FAS receptor present even at low concentrations on tumor cells causes a dramatic increase in NOX1 and NOS activities.[75,116,136–138] This leads to a valuable synergistic effect on singlet oxygen generation and subsequent catalase inactivation. Synergistic enhancement does not occur at very low concentrations of initially present singlet oxygen.[115] Likewise, intercellular apoptosis-inducing ROS/RNS signaling after inactivation of catalase may take advantage of the increased concentrations of superoxide anions, NO, and their derivatives after singlet oxygen–mediated activation of FAS receptor.

These established scenarios allow us to conclude that in the case where primary singlet oxygen that is derived from CAP or PAM, rather than inactivating catalase, would inactivate a SOD molecule or activate a FAS receptor, secondary singlet oxygen generation in an autoamplificatory mode would also be ensured (Fig. 6). Thus, the final outcome of the treatment would be same as that after initial targeting of catalase by singlet oxygen. These homologies of action might well contribute to the overall efficiency of CAP and PAM action.

2. Potential Enhancing Effects of Nitrite

Nitrite is essential as a reaction partner for H_2O_2 to generate peroxynitrite [Fig. 7(A)]. Nitrite has no direct apoptosis-inducing potential up to concentrations of 1 mM. Although (2) and (3) in Fig. 7(A) that lead to singlet oxygen generation through further reactions of peroxynitrite can be counteracted by the efficient reaction of peroxynitrite with CO_2

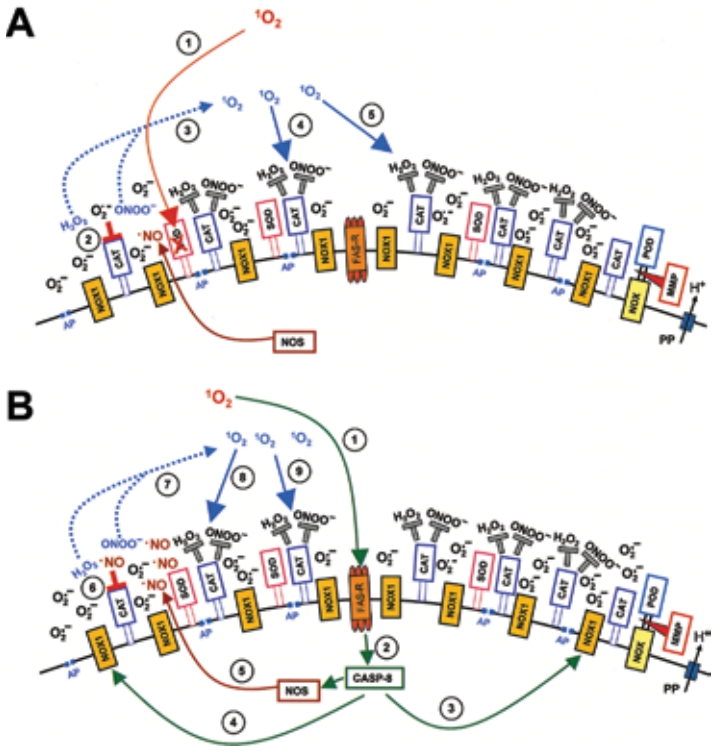

FIG. 6: Initial targeting of SOD or FAS receptor by primary singlet oxygen derived from CAP or PAM. When SOD (A) or FAS receptor (B) are targeted by primary singlet oxygen instead of abundant catalase [as shown in Fig. 4(A)], autoamplification of secondary singlet oxygen generation and catalase inactivation are nevertheless warranted. This is achieved through (1)–(4) in (A) and (1),(7) in (B).

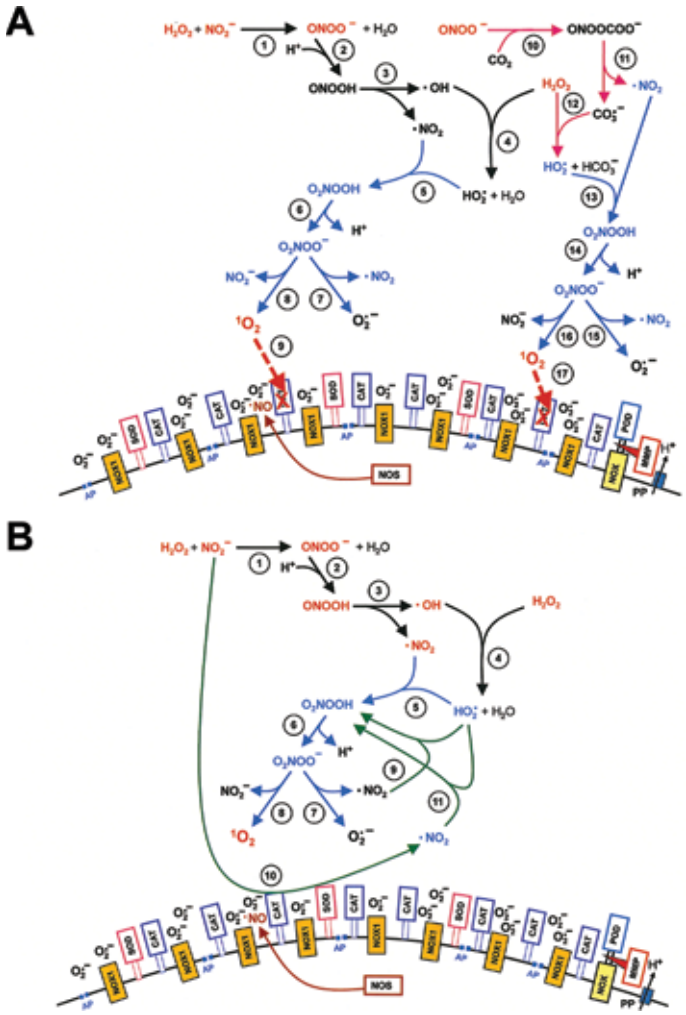

FIG. 7: Plasticity of singlet oxygen generation through interaction between the long-lived species H_2O_2 and nitrite. (A) Role of peroxynitrite. Peroxynitrite is centrally involved in generation of singlet oxygen through the interaction between H_2O_2 and nitrite, following (1)–(9), as shown earlier. This pathway is more likely to occur close to the membrane, due to the presence of proton pumps. As negative side effect, this pathway is minimized by active catalase on tumor cells. Because it is farther away from the cells, peroxynitrite is more likely to interact with CO_2 (10). Decomposition of nitrosoperoxycarboxylate (11) and the reaction between resultant carbonate radicals with H_2O_2 (12) should allow for generation of singlet oxygen through (13)–(16). (B) Role of nitrite and NO_2. Nitrite (NO_2^-) in PAM or CAP is essential for generation of peroxynitrite (1), whereas the concentration of NO_2 is rate limiting for generation of peroxynitric acid (5). In concert, these reactions lead to formation of singlet oxygen (1)–(8). Even if peroxynitrate (O_2NOO^-) fails to contribute to generation of singlet oxygen due to (7), NO_2 generated through this abortive reaction has a chance to contribute to a new round of peroxynitric acid generation through (9). Even more important for the supply with NO_2 is the oxidation of abundant nitrite by catalase (10).

(10), the overall generation of singlet oxygen are not necessarily reduced, because (11) and (12) lead to generation of hydroperoxyl radicals that are essential for the formation of peroxynitric acid and subsequent generation of singlet oxygen through spontaneous decomposition of peroxynitrate. Thus, the variable reaction potential of peroxynitrite may ensure that the final outcome of singlet oxygen generation is not altered by variable reaction profiles. However, the biological effect of singlet oxygen generated through either pathway is only ensured if singlet oxygen does reach its target structure.

Even if peroxynitrate decomposes into superoxide anions and nitrogen dioxide [(7) in Fig. 7(B)] rather than into singlet oxygen and nitrite [(8) in Fig. 7(B)], this may not necessarily reduce overall singlet oxygen generation. NO_2 derived from (7) may contribute to generation of peroxynitric acid through (9), thus producing a second chance to enhance singlet oxygen generation.

In light of the relative abundance of nitrite in PAM and the potential of catalase to oxidate nitrite to nitrogen dioxide [(10) in Fig. 7(B)], (11) can substantially enhance formation of peroxynitric acid and singlet oxygen. Thus, nitrite in PAM does not seem to be solely necessary for initial generation of peroxynitrite (1) but might also enhance singlet oxygen generation after its oxidation by catalase. The enhancing effects of nitrite and NO_2 most likely contribute to the high efficiency of CAP and PAM. Especially, oxidation of CAP- or PAM-derived nitrite to NO_2 will also contribute to efficient secondary singlet oxygen generation because the otherwise rate-limited NO_2 is then available in relative abundance.

Established reactions between nitrite and catalase are shown in Fig. 8.[139] Figure 8(A) summarizes three essential functions of catalase (decomposition of H_2O_2 and peroxynitrite, oxidation of NO) and points to the role of compound I in these reactions. Figure 8(B) shows that nitrite can compete with NO for interactions with compounds I and II. As a result, the local concentration of NO would be enhanced and lead to formation of additional peroxynitrite. Due to the presence of PAM- or CAP-derived H_2O_2 and competition with peroxynitrite for native catalase, the chances for free peroxynitrite survival may thus increase. This may then lead to generation of secondary singlet oxygen through (9)–(14) in Fig. 8.

Alternatively, if the competition between nitrite and NO leads to a sufficiently high NO generation to result in local catalase inhibition [Fig. 9(A)], the resultant free H_2O_2 and peroxynitrite may establish generation of singlet oxygen and thus establish autoamplification of singlet oxygen generation and catalase inactivation.

If CAP- or PAM-derived H_2O_2 transforms compound II (formed through the reaction between nitrite and compound I of catalase) into the terminally inactive compound III, free H_2O_2 and peroxynitrite might likewise generate singlet oxygen and trigger autoamplification [Fig. 9(B)]. This pathway is much more likely to occur after nitrite/catalase interaction than after NO/catalase interaction, because the concentration of nitrite is several orders of magnitude higher than that of NO.

D. Apoptosis-Inducing Signaling after CAP and PAM Action on Tumor Cells

Following catalase inactivation by secondary singlet oxygen, NO/peroxynitrite and/or HOCl signaling can be established [Fig. 10(A)]. This occurs analogous to the situation

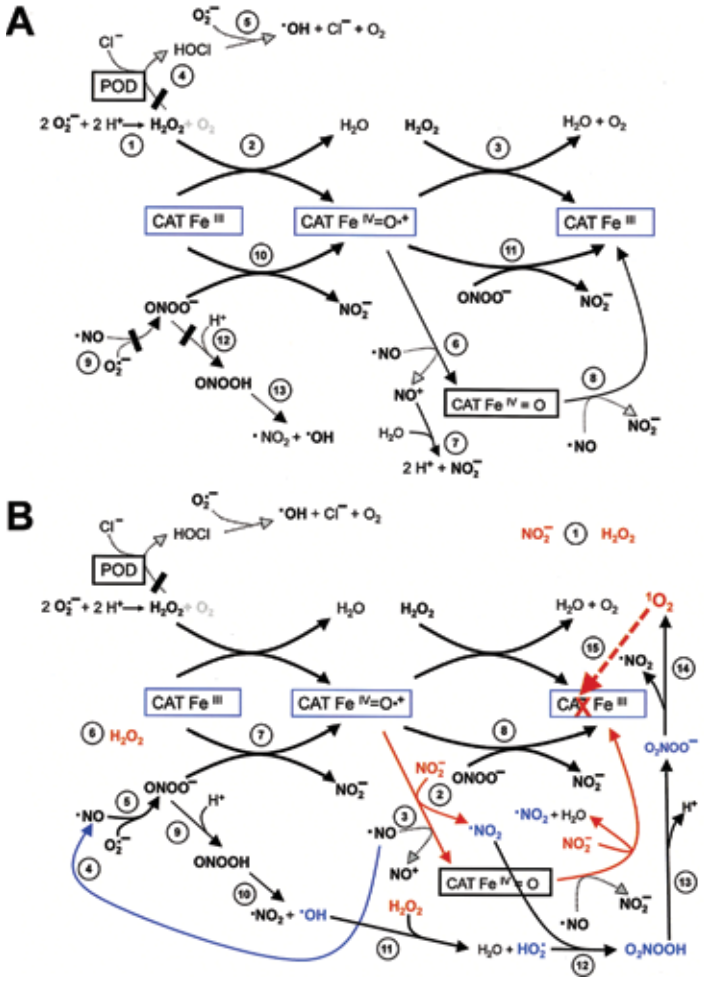

FIG. 8: Reaction between catalase and nitrite. (A) Basic reactions of catalase. Catalase decomposes H_2O_2 in a two-step reaction (1)–(3). This prevents apoptotic induction of tumor cells through the HOCl signaling pathway (4),(5). Compound I of catalase ($CATFe^{IV} = O^{\bullet+}$), the active intermediate obtained after catalase interaction with one molecule of H_2O_2, oxidates NO in a two-step reaction, with compound II ($CATFe^{IV} = O$) acting as intermediate (6)–(8). Eventually, formed peroxynitrite (9) is decomposed by catalase in a two-step reaction (10),(11), thus preventing apoptosis-inducing NO/peroxynitrite signaling (12),(13). **(**B) Interaction between nitrite and catalase. Nitrite contained in PAM or CAP (1) can compete with NO in the interaction with compound I of catalase (2),(3). Due to the abundance of nitrite, this process can be expected to be dominant. Free NO (4) then has a high chance of reacting with superoxide anions to form peroxynitrite (5). Relatively abundant H_2O_2 (6) from PAM or CAP competes with peroxynitrite for interaction with catalase and thus reduces or prevents (7) and (8). This then allows for singlet oxygen generation through (9)–(14), followed by inactivation of additional catalase molecules (15). In this scenario, peroxynitrite formation through interaction between nitrite and H_2O_2 is not required. A detailed description of catalase function and regulation is available elsewhere.[114]

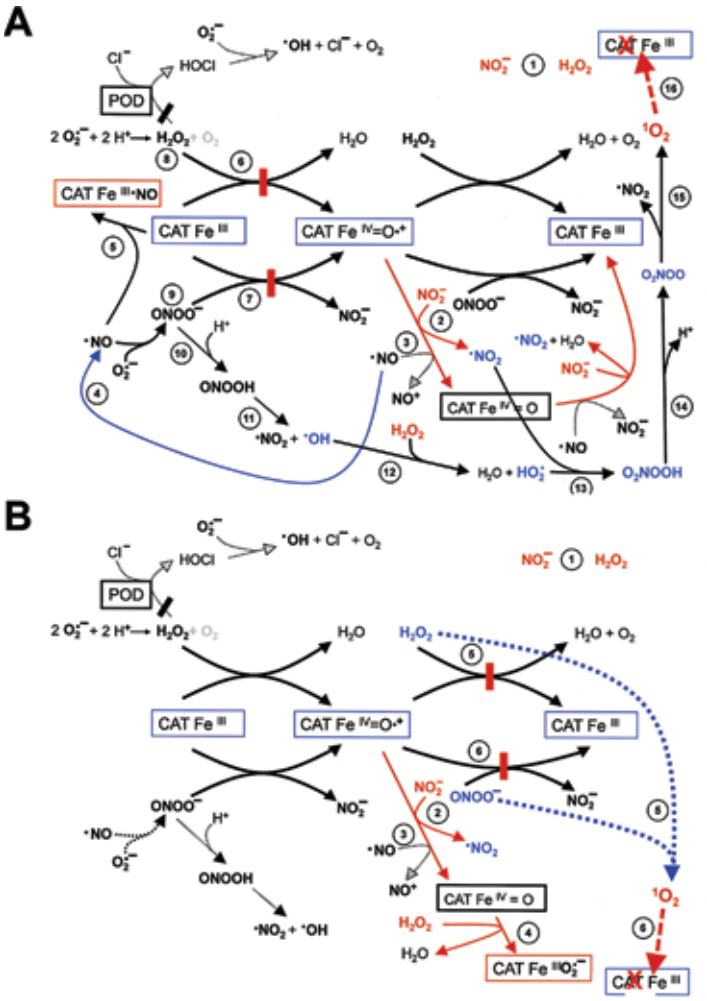

FIG. 9: Alternative modes of nitrite action. (A) NO-mediated inhibition of catalase. Nitrite from CAP or PAM (1) efficiently competes with NO for compound I and oxidates to NO_2 (2),(3). If free NO reaches local concentrations > 0.18 μM, an inactive NO–catalase complex is formed (5).[74] As a result, H_2O_2 and peroxynitrite are not decomposed at the site of inhibited catalase and generate singlet oxygen through (9)–(15). Singlet oxygen then inactivates further catalase molecules (16). (B) Formation of inactive compound III after nitrite action. Efficient competition of nitrite with NO as well as H_2O_2 for compound I might lead to a transiently high concentration of compound II ($CATFe^{IV} = O$) that is converted to the terminally inactive compound III ($CAT-Fe^{III}O_2^{\bullet -}$). As a result, free H_2O_2 and peroxynitrite generate singlet oxygen according to (9)–(15), as described in (A).

found for other modes of catalase inhibition or inactivation.[75,108,111–113] In the continuous presence of CAP- or PAM-derived H_2O_2, HOCl signaling is favored (Bauer, manuscript in preparation). HOCl and NO/peroxynitrite signaling pathways are finalized by genera-

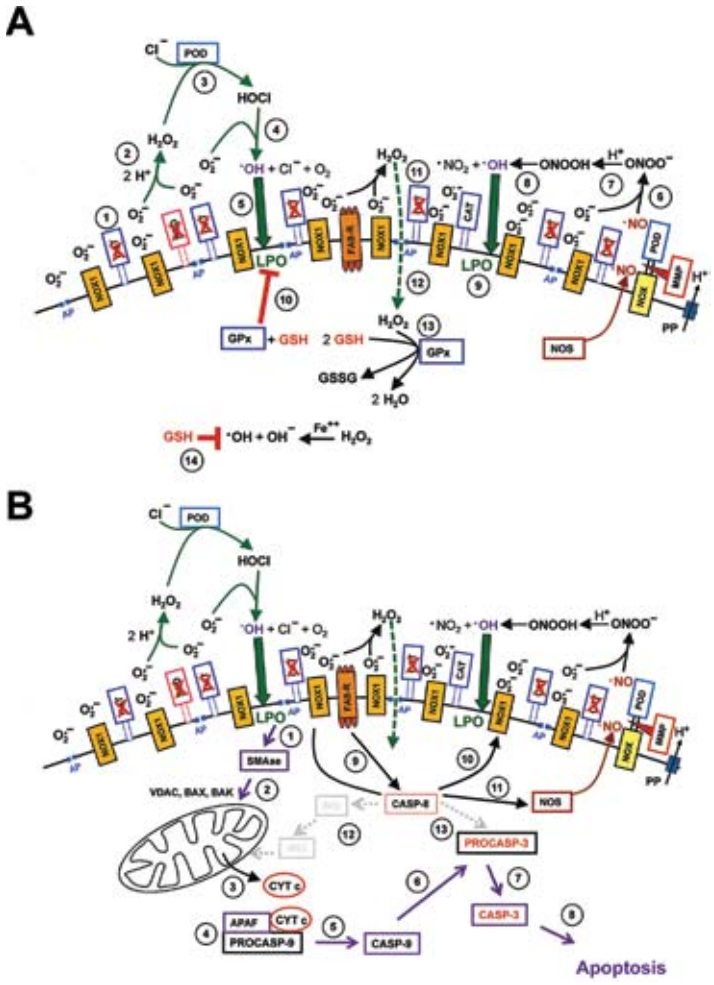

FIG. 10: Intercellular ROS signaling after singlet oxygen–mediated inactivation of tumor cell catalase. (A) Balance between extracellular ROS signaling and intracellular glutathione-mediated protection. After sufficient singlet oxygen–mediated catalase inactivation on the outer surface of tumor cells, the HOCl signaling pathway (1)–(5) and potentially the NO/peroxynitrite signaling pathway (6)–(9) are established. Both pathways are finalized by hydroxyl radical–mediated lipid peroxidation. Initially, glutathione peroxidase and glutathione counteract apoptosis induction by lipid peroxides.[140] However, the lack of protection by inactivated catalase allows strong influx of cell-derived H_2O_2 into cells, leading to efficient depletion of glutathione. This renders the cells vulnerable to apoptosis induction by lipid peroxidation and intracellular hydroxyl radicals. (B) Mitochondrial pathway of apoptosis. Lipid peroxides (1) activate sphingomyelinase (2) and trigger the mitochondrial pathway of apoptosis, mediated by voltage-dependent anion channel (VDAC) and proteins BAK and BAX and characterized by release of cytochrome c (3), formation of apoptosome (4), processing of inactive caspase-9 to active caspase-9 (5), and followed by activation of executing caspase-3 (6)–(8).[141] The role of caspase-8 in this scenario is restricted to (9)–(11), as long as FAS receptor is typically expressed at low levels.

tion of hydroxyl radicals that trigger lipid peroxidation. Lipid peroxides can be removed by intracellular glutathione peroxidase and glutathione.[140] This repair reaction initially interferes with hydroxyl radical–mediated apoptosis induction. However, local inactivation of catalase by singlet oxygen allows aquaporin-dependent influx of extracellular H_2O_2 (either derived from CAP or PAM or generated through dismutation of NOX1-generated superoxide anions). Subsequent glutathione peroxidase/glutathione–mediated decomposition of H_2O_2 leads to decreased intracellular glutathione concentration. Therefore, interference of glutathione peroxidase-4/glutathione with lipid peroxidation-dependent triggering of the mitochondrial pathway of apoptosis is gradually abrogated. This central control step explains the findings by Yan et al., who have convincingly shown that aquaporins play a central part during CAP- and PAM-dependent apoptosis induction in malignant cells.[118,119] Recent experimental evidence supports the concept that these findings extend to other ROS/RNS-mediated apoptosis-inducing principles in general.

Following lipid peroxidation and glutathione depletion, the mitochondrial pathway of apoptosis is activated,[141] summarized in Fig. 10(B). Essential elements are sphingomyelinase; mitochondria; the apoptosome consisting of apoptotic protease-inhibiting factor, cytochrome c, and procaspase-9; and active caspase-9 and -3. Mitochondria involvement in the apoptosis pathway includes release of cytochrome c and decoupling of the mitochondrial respiratory chain. As a result of decoupling, mitochondria involved in apoptosis induction generate high concentrations of superoxide anions that are dismutated to H_2O_2 by mitochondrial SOD [Fig. 11(A)]. H_2O_2 may then reach neighboring intact mitochondria and cause ROS-dependent ROS release through oxidation of the permeability transition pore, followed by uncoupling of the mitochondrial respiratory chain.[142] In total, this scenario explains the massive increase in ROS (i.e., primarily H_2O_2) in cells that undergo the mitochondrial pathway of apoptosis. ROS increase may promote oxidative effects in the cell population, as suggested by Pletjushkina et al.[143]

Cytochrome c that is released from mitochondria has recently been found to use H_2O_2 to generate HOCl in a reaction analogous to myeloperoxidase or the perioxidase domain of dual oxidase (Bauer, unpublished result). HOCl that is generated inside cells through this pathway may (1) oxidate tumor cell antigens, thus making them attractive to dendritic cells,[144–149] (2) interact with superoxide anions and generate damaging hydroxyl radicals,[124,129] or (3) react with either H_2O_2 or lipid peroxides and generate singlet oxygen.[150–152] The biological relevance of these massive secondary reactions has not yet been fully investigated. Detection of mitochondrial singlet oxygen by Bekeschus et al.[84] may be due to these reactions following the execution of the mitochondrial pathway of apoptosis, although these authors may have missed detecting primary and secondary singlet oxygen on the membrane due to the scavenging function of membrane-associated catalase.

The role of HOCl does not seem to be restricted to apoptosis induction through the HOCl signaling pathway. Due to its high reactivity, HOCl can also very efficiently modify tumor cell antigens and enhance T-cell responses directed to tumor cells.[144–149] It is conceivable that HOCl-dependent antigenic modification may act in concert with

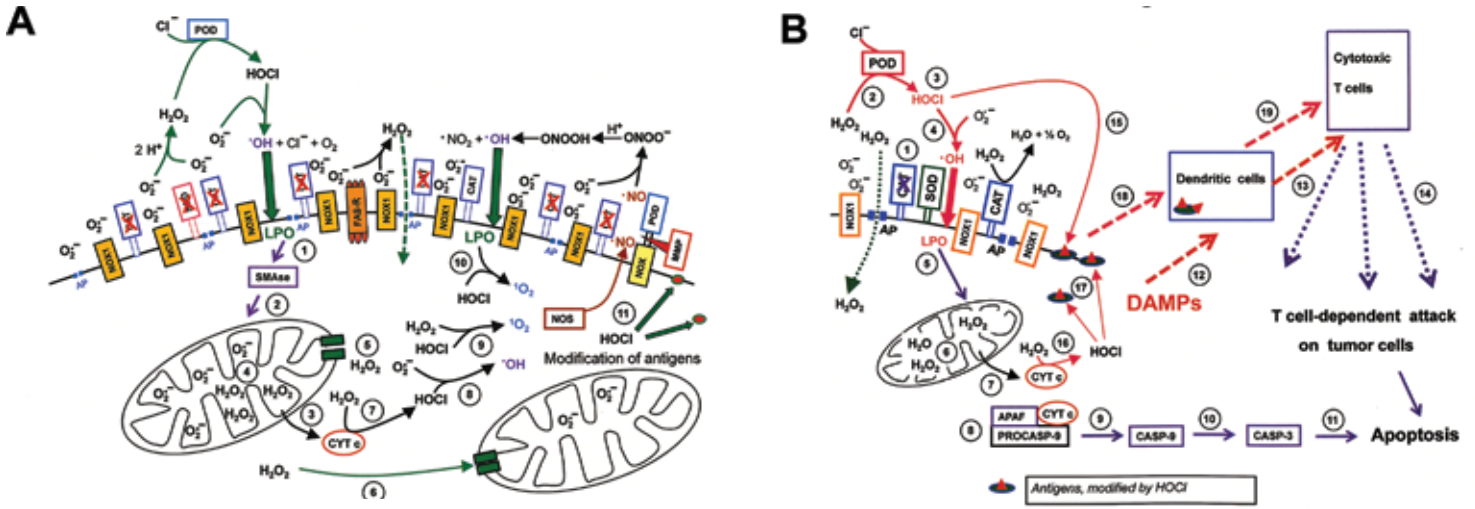

FIG. 11: Further consequences of the mitochondrial pathway of apoptosis. (A) Secondary mitochondria-dependent ROS generation. Following lipid peroxidation, activation of sphingomyelinase, release of cytochrome c from mitochondria (1)–(3), and uncoupling of the mitochondrial respiratory chain cause generation of superoxide anions. These dismutate to H_2O_2 both spontaneously and through activity of mitochondrial SOD (4). H_2O_2 can leak out from mitochondria through aquaporins and potentially through VDAC (5). It may act on the permeability transition pore of neighboring mitochondria and induce ROS-dependent ROS release (6).[142] In total, high concentrations of ROS are generated through these effects. Recently, cytochrome c has been shown to react with H_2O_2 to form a compound I–analogous structure that allows generation of HOCl (7) (Bauer, unpublished result). HOCl may interact with superoxide anions (8), leading to generation of hydroxyl radicals, or with H_2O_2 (9), leading to generation of singlet oxygen.[150] The interaction of HOCl with lipid peroxides may cause additional generation of singlet oxygen (10).[151,152] Finally, HOCl may react with cellular proteins and render them more attractive for dendritic cells, the primary cells of immune responses (11).[144–149] (B) Immunogenic cell death. Inactivation of catalase allows for establishment of the mitochondrial pathway of apoptosis (1)–(11). Death-associated molecular patterns (DAMPs) stimulate dendritic cells to provoke a cytotoxic T-cell response (12),(13) that is directed to tumor cells (14). In an analogous mode, HOCl generated through primary HOCl signaling (1) or through cytochrome c–dependent HOCl synthesis (16) modifies tumor cell antigens (17). This seems to generate a DAMP-analogous signaling pathway, climaxing in a cytotoxic T-cell response (18),(19).[144–149]

canonical death-associated molecular patterns that are involved in classical immunogenic cell death that triggers a T-cell response to tumor cells [Fig. 11(B)]. There is general consent that T-cell responses after initial tumor treatment are of central importance to overall outcome of most if not all antitumor therapies presently under investigation.[77,87–91]

III. CONCLUSIONS

The selective action of CAP and PAM on tumor cells seems to be based on the interaction between singlet oxygen (derived from CAP or generated through the interaction between H_2O_2 and nitrite in CAP or PAM) and the specific redox biology-related enzyme composition on the surface of tumor cells. Of central and determining importance in this context are membrane-associated catalase and SOD, because they interact with reaction products of membrane-associated NOX and intracellular NOS. FAS receptor also has a modulatory role in this context.

This cooperative system of enzymes and FAS receptor represents a tumor cell–specific biochemical switchboard that can be triggered by singlet oxygen. Catalase and SOD can be inactivated by singlet oxygen, whereas FAS receptor is activated by singlet oxygen. Thus, even a very low concentration of singlet oxygen that is derived from CAP or PAM can establish a strong and sustained biochemical and cellular response through this mechanism. The maintained and autoamplificatory nature of this response is due to the

(1) establishment of local concentrations of free H_2O_2 and peroxynitrite at the site of inactivated catalase molecules,
(2) potential of H_2O_2 and peroxynitrite to interact in a complex series of reactions, yielding *de novo* generation of secondary singlet oxygen,
(3) attack of neighboring active catalase and SOD by the secondary singlet oxygen.

After inactivation of a sufficiently high portion of protective catalase, intercellular ROS/RNS-mediated apoptosis-inducing signaling is established and causes elimination of malignant cells. Because this biochemical switchboard is unavailable to nonmalignant cells, selectivity of CAP and PAM action for tumor cells is ensured as long as the dose of CAP and PAM is in a range that does not allow nonselective apoptosis induction in nonmalignant cells by hydrogen peroxide or peroxynitrite.

These autoamplificatory processes that are activated by singlet oxygen on tumor cells use, in a sustained mode, the same ROS/RNS that also act in CAP and PAM and show a partial overlap to intercellular ROS/RNS-dependent apoptosis-inducing signaling. Essential compounds for the autoamplificatory mechanism are superoxide anions, nitric oxide, nitrogen dioxide, peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), hydrogen peroxide, hydroxyl radicals, hydroperoxyl radicals, peroxynitrate (O_2NOO^-), peroxynitric acid (O_2NOOH), and singlet oxygen. Due to involvement of enzymes and

proton pumps on the tumor cell membrane, this ROS/RNS-dependent process is localized to the membrane. This ensures high efficiency selectivity of these processes.

Catalase inactivation of secondary singlet oxygen far exceeds inactivation of catalase by primary, CAP-, and PAM-generated singlet oxygen. Catalase inactivation allows an essential aquaporin-mediated influx of tumor cell–derived H_2O_2 into the targeted cells, causing depletion of intracellular glutathione. This results in the abrogation of glutathione peroxidase 4/glutathione inhibitory effects to apoptosis-inducing signaling by lipid peroxides. Hydroxyl radicals induce lipid peroxidation that is generated by intercellular ROS/RNS signaling.

The subsequent induction of the apoptosis mitochondrial pathway results in activation of caspase-9 and -3. In parallel, the release of cytochrome c in apoptotic cells increases concentrations of superoxide anions and H_2O_2, generated by the uncoupled respiratory chain. The potential contribution of these ROS, arising late in the apoptotic process of targeted cells, is considered to contribute to the spread of the apoptotic response in the cell population.[143] So far, its potential biological relevance seems to be underestimated.

These considerations demonstrate the multiple roles of H_2O_2 for the chemical biology of CAP and PAM action, starting from an involvement in singlet oxygen formation, maintenance and modulation of HOCl signaling, to modulation of apoptotic pathways. These findings add to multiple other roles of H_2O_2 that have been pointed out earlier by Sies.[153,154] The strong ROS/RNS-driven autoamplificatory network induced by CAP and PAM has inherent potential for the following:

- synergistic interaction between parallel inactivation of membrane-associated catalase and SOD, because both enzymes are inactivated by singlet oxygen and biochemically interactive,
- enhanced effect of singlet oxygen–mediated activated FAS receptor activation, with an impact on enhancement of superoxide anion and NO production, thus enhancing generation of secondary singlet oxygen,
- oxidation of nitrite to nitrogen dioxide, which is a rate-limiting reaction partner during the generation of secondary singlet oxygen,
- interaction of nitrite with the catalase cycle, potentially slowing enzyme activity
- competition between nitrite and NO for the reaction with compound I of catalase, including potential local increase of NO and subsequent inhibition of catalase.

These potential synergistic interactions between ROS- and RNS-dependent processes of CAP and PAM action are the basis for an additional and essential strong immunological process, termed immunogenic cell death, that is characterized by the release of death-associated molecular patterns (DAMPs). Therefore, the chain of biochemical and cellular events initiated by CAP- or PAM-derived singlet oxygen and amplified by sustained generation of cell-derived secondary singlet oxygen leads to massive finalization of antitumor action through the immune system. HOCl that is primarily involved

in intercellular apoptosis-inducing signaling after the inactivation of catalase may also contribute to immunogenic cell death through modification of tumor cell antigens.

IV. EPILOGUE

We established the novel model of Bauer and Graves[20,21] in a deductive approach, in which established knowledge of the composition and action of CAP and PAM was connected to experimentally obtained results involving the differential action of defined ROS/RNS on malignant and nonmalignant cells.

This model was confirmed in a bottom-up experimental approach using long-lasting compounds of PAM. A recent experimental approach with CAP and PAM generated by a plasma generator confirmed the results obtained by the preceding model experiments.

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