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Cold Atmospheric Plasma in Cancer Therapy: Molecular Insights, Novel Concepts, and Future Opportunities

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Abstract

Over the past 2 decades, cold atmospheric plasma (CAP) has been playing increasingly pivotal roles in cutting edge biomedical applications and has developed into an innovative field of research of growing importance. One promising new medical application of CAP is cancer treatment. Being able to richly induce both reactive oxygen and nitrogen species, CAP has been shown to effectively control events critical to cancer initiation/progression; selectively inducing Deoxyribonucleic acid (DNA) damage and apoptotic cell deaths, reducing tumor volume and vasculature, halting metastasis and conferring anti-tumor effects by taking advantage of e.g., synergies among and between chemotherapeutic drugs and nanoparticles. This paper discusses molecular and immunological mechanisms of CAP treatment as a potential tool against cancers, with a focus on the novel mechanisms by which CAP interacts with chemotherapeutic drugs and nanotechnology. We then attempt to draw parallels between what is already known about the mechanisms of CAP activity, some novel concepts and attempts of CAP synergy, and the knowledge we would need to develop CAP as a potential therapeutic strategy in oncotherapy.

Keywords: Cancer treatment, Clinical trials, Cold atmospheric plasma, Reactive oxygen and nitrogen species

Introduction

CAP is a near-room temperature ionized gas medium composed of electrons, ions, UV photons, free radicals, and reactive molecules like reactive oxygen and Reactive Nitrogen Species (RNS) [1,2]. These highly reactive and energetic species impact a variety of bacterial, plant, and mammalian cells in many ways [3,4]. Plasma is considered the 4th state of matter and makes up most of our visible universe [5-7]. In practice, plasma is generated when a gas is heated to completely knock off electrons from the respective nucleus component of atoms [8]. Modern advancement in plasma generation technology has laid the foundation for the generation of plasma within 40 °C, making it ideal for interaction with living tissues and exploring its medical and therapeutic implications [9-12]. During the past decade, CAP interaction with biological systems (cells, tissues, and wounds) has been extensively investigated for its promising application in addressing medical issues in wound healing [13-17], dermatology [18-20], dentistry [21-23], surgery [24,25], and infectious and inflammatory diseases [26-30]. More recently, the potential use of CAP science technology in cancer treatment has been getting increasing attention [31-36] and has been expected to cause a paradigm shift

in cancer therapy [37,38]. There have been increasing reports highlighting CAP selectivity and efficacy against a wide range of cancers in both *in vitro* and *in vivo* studies [39,40]. Indeed, CAP has shown a significant anti-cancer capacity against a wide range of cancer cell lines including and not limited to melanomas [41-44], carcinomas [45,46], neural malignancies [47-50], and hematopoietic malignancies [51-53]. In addition, CAP does exhibit noticeable anticancer effects and resists tumor growth over several engrafted tumor mice models [54-60]. So far, we know that the anti-tumor capacity conferred by CAP happens in 2 subsequent steps. First, there is always an increment in the intracellular Reactive Oxygen Species (ROS) levels in cancer cells upon CAP treatment which, on 1 hand, causes damage to antioxidant homeostasis of cells and, on other hand, causes DNA double-strand breaks (DSB) to a fatal degree. Second, serious DNA damage and other CAP effects on cancer cells result in cell cycle arrest, apoptosis, and necrosis. Most importantly, untransformed and normal cells are always more resistant to CAP application compared to cancer cells [61].

The major role of reactive oxygen and nitrogen species containing in plasma discharge which effects in controlling core cancer state transitions, such as tumor initiation/progression, metastasis, antigenic switch, and drug sensitivity spectrum. This review aims to stimulate discussion on the studies that take advantage of the unique features of CAP therapy for effective tumor management. To achieve this goal, first, we discuss what is known regarding the potential of CAP to control and modulate the critical molecular/immunological events driving cancer initiation and progression. And then we introduce some novel insights into CAP application in cancer management and their future potential. To date, various cellular responses including selective increment of ROS/RNS, DNA and mitochondrial damage, cell cycle arrest and apoptosis, tumor specificity, and even immunologic cell death have already been attributed following CAP treatment [62]. The presented review related to our research work which is also carried out from our laboratory has evaluated the inactivation efficiency of CAP against microorganisms and suggested that CAP has strong antimicrobial properties. The main aim is to demonstrate the effect of CAP-irradiated media as a promising anticancer tool. CAP has been generated even using a high voltage power supply of frequency in kHz instead of RF power supply, making treatment cost-effective. Treated DMEM media by CAP using argon as process gas was transferred to cancer cells and its viability was observed using MTT assay. So, we suggested that CAP gives a lot of potential for biomedical applications [63,64]. CAP treatment selectively killed cancer cells without affecting normal cells *in vitro*. Kaushik *et al.* [65] have reported that non-thermal atmospheric plasma has been suggested as a promising tool for various biological and medical applications. Adhikari *et al*. [66] reported that the survival or death-promoting way is still debatable in different types of cancer cells.

Methods and discussion

In fact, in this review paper, we have explained CAP treatment to cancer cells besides other several processes such as surgery, chemotherapy, immune therapy, hormone therapy and stem cell transplant. It had been concluded that the CAP has a novel promising anticancer efficacy. Non-thermal(cold) plasma treatment includes DNA damage in cancer cell which can lead apoptosis (programed cell death). The advantage of plasma treatment is that it mainly targets cancer cells whereas minimizing destroy to surrounding healthy tissues which generates reactive oxygen and nitrogen species that affects cancer cells. However, plasma treatment has the risk of affecting normal cells, enhancing unnecessary side effects and difficulties of controlling the extent of DNA damage. In this review article, the following methods were explained briefly.

DNA damage and apoptosis

There is a growing mass of evidence conferring the notion that the anti-tumor activity of plasma is due to the result of DNA damage and apoptosis induction mediated by ROS/RNS (**Figure 1**). In CAP-treated colorectal cancer cells, the NADPH Oxidase -2 (NOX 2) has been significantly up-regulated in both messenger ribonucleic acid (mRNA) and protein levels to produce ROS. The same study has also reported that plasma-induced cell death was due to the generation of ROS and activation of Apoptosis signal-regulating kinase 1 (ASK1) and caspase 3/7 activity [67]. In CAP-treated head and neck cancer cells, there was increased phosphorylation of c-JUN N terminal kinase (JNK) and p38. CAP was able to induce apoptosis through Mitogen-activated protein kinase (MAPK)-dependent mitochondrial ROS [68]. Tumor necrosis factor receptor (TNFR) based apoptosis pathway has been induced by the increase in intracellular ROS [69]. There has been well-established evidence of ASK1 activation through ROS treatment which further activates MAP kinases such as JNK and p38. This ultimately induces apoptosis through caspase activation [70].

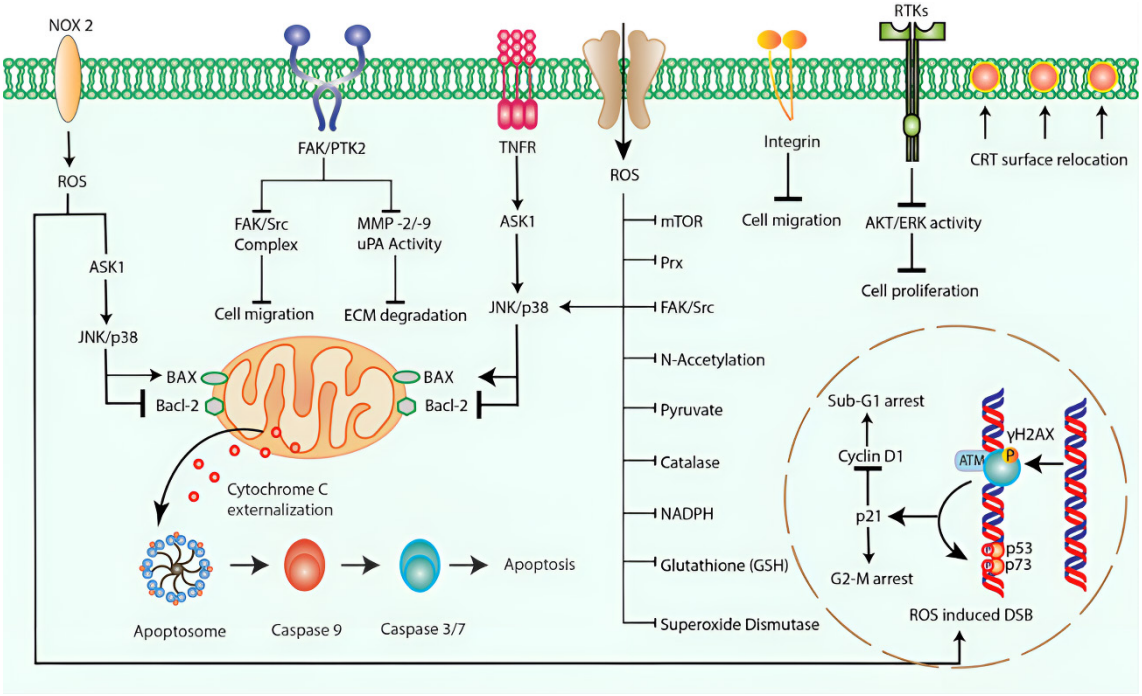

Figure 1 Schematic diagram showing the role of CAP in selective cancer killing via DNA damage, apoptosis, cell migration inhibition and induction of immunogenic cell death (ICD).

Among others, most of the evidence showing apoptosis as a cause of plasma-induced cell death is based on DNA damage through the mitochondrial pathway. Double-strand DNA break (DBS) is one of the well-known events upon CAP treatment. Upon plasma treatment on glioblastoma U87MG and colorectal carcinoma HCT-116 cells, a large amount of ROS was generated leading to the formation of DNA damage, multiphase cell cycle arrest, and subsequent apoptosis induction [71]. Phosphorylation on serine 139 on H2Ax histone (γ-H2AX) usually follows DBS and this has also been commonly observed after CAP exposure to cells [72-74]. The phosphorylation event at 139 serine residues in γ-H2AX is mediated by ataxia telangiectasia mutated (ATM) recruited on the DSB site with other DNA damage response complexes [75]. The increased expression of ATM has been reported in the oral cavity squamous cell carcinoma cells after CAP treatment by

Chang *et al.* The activated monomeric ATM can phosphorylate p53 and another substrate in the DNA damage response complexes localized at DSB sites. Phosphorylation of p53 also has been observed in oral cavity squamous cell carcinoma cells and melanoma cells [41, 76] following CAP exposure. Phosphorylation of p53 is a key event in the induction of apoptosis following DBS. Phosphorylated p53 (which is more stable) then mediates DNA damage response by stimulating the nuclear release of histone H1. This phosphorylation event is one of the primary post-translational modifications of p53 and is carried out by kinases such as ATM [76]. Also increased expression of p73 in the CAP-treated melanoma (Me1007) cells has been observed along with the reduced anti-cancer capacity of CAP under p73 knockout. Evidence also points out that ATM phosphorylates p73 along with p53 and further activates pro-apoptotic factors. Subsequent activation of p21 by p53 inhibits the function of cyclic D1 and triggers noticeable sub-G1 and G2/M arrest [76,77].

Cell migration

Other possible molecular mechanisms of the anticancer effects of CAP on cancer cells have also been reported. One is related to the decrease in the expression of cell surface proteins like integrin and Focal adhesion kinase (FAK)/Protein tyrosine kinase 2(PTK2). Plasma-treated melanoma cells showed decreased expressions of integrin α2, integrin α4, and FAK [78]. Likewise, reduced expression of integrin's (integrin β1 and integrin α_v) has been observed in primary fibroblasts and mouse epithelial skin cancer cells (PAM) following CAP treatment [79]. FAK/PTK2 has been reported to ultimately promote migration of cells through complex formation with Src followed by phosphorylation of cytoskeleton adaptor molecule paxillin. Other studies have reported the modulation of assembly and disassembly of the actin cytoskeleton through its effects on the Rho subfamily of small ATPase's as other mechanisms of cell migration regulation by FAK. CAP-treated thyroid cancer cells showed decreased FAK/Scr complex and paxillin signaling and subsequent inhibition of cell migration [80]. The same study has also reported a decrease in MMP-2/-9 expression in CAP-treated cancer cells. The MMP/uPA system is directly related to extracellular matrix (EMC) degradation and is crucial for promoting tumor metastasis. In another hand, the relationship between FAK and the MMP-2/-9 pathway has also been studied. FAK inhibition has been shown to reduce MMP-9 secretion [81].

Induction of ICD through CAP treatment

The initiation, pathogenesis, progression, and metastasis of tumors are directly influenced by immune function. This is particularly interesting as the activation of the immune system following plasma treatment has been increasingly highlighted in recent studies. A recent report suggests the increased efficacy of macrophages in ablating tumor cells mechanistically through ICD following plasma treatment [82]. Similarly, another study has found an increase in tumor-associated antigens subsequently leading to dendritic cell maturation and T cell-mediated immune response against tumors, complementing the results of the previous finding and supporting the role of plasma in eliciting ICD [83]. Another recent study has further supported the notion showing that reactive oxygen and nitrogen species (e.g., hydroxyl radicals, atomic oxygen, and nitric oxide) produced by plasma are the main effectors that induce ICD in melanoma cells [84]. A schematic explaining the mechanism by which ICD is induced in tumor cells following CAP treatment has been summarized in (**Figure 2**).

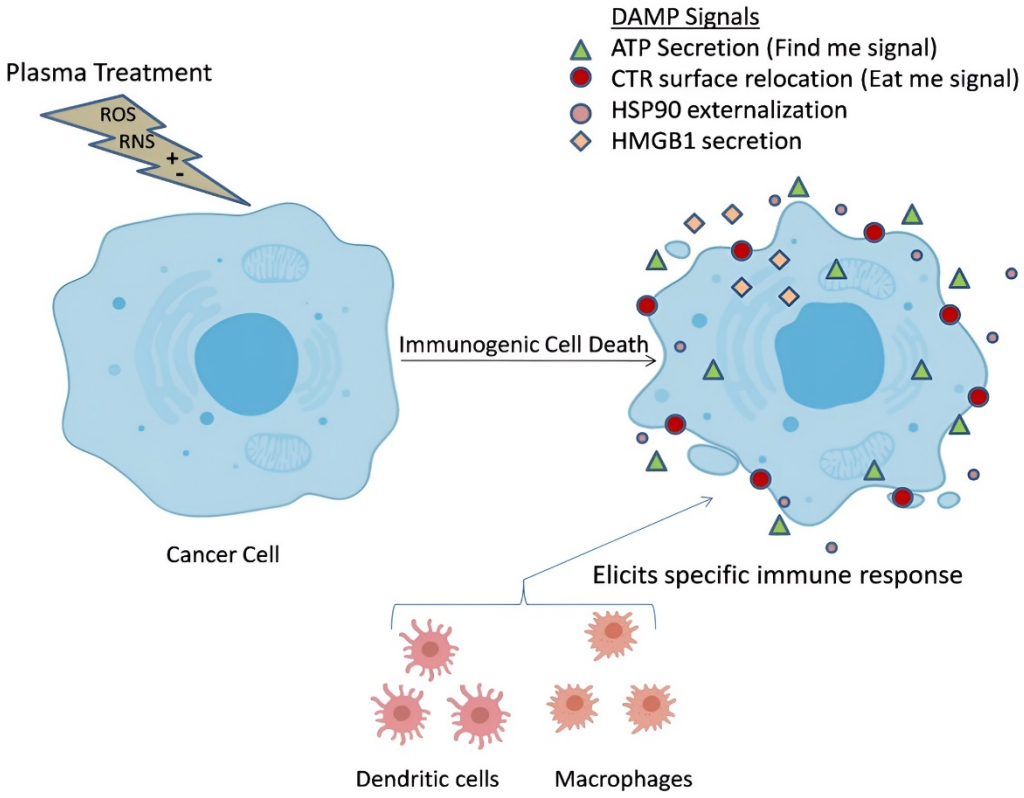

Figure 2 Schematic diagram showing the induction of ICD in cancer cells following CAP treatment. Post treatment, cells undergo cell swelling/bursting and the dying cells secrets damage-associated molecular pattern (DAMPs). These signals initiate the cascade of specific immune response through cytokine secretion, antigen presentation, phagocytosis and finally destruction of target by immune cells.

At specific regimes of plasma exposure, various damage-associated molecular patterns (DAMPs) are produced by the tumor cells in the tumor microenvironment (TME), which acts as a plea for help from immune cells in the vicinity [85]. The 2 most prominent events that follow through are adenosine triphosphate (ATP) secretion and intracellular calreticulin (CRT)/disulfide isomerize ERp57 mobilization into the outer cell surface by the tumor cells. A comprehensive review published by Kroemer *et al*. [86] highlights how the then-produced ATP acts as a 'FIND ME' signal and surface-delocalized CRT acts as an 'EAT ME' signal for immune cells. ATP when secreted into an extracellular matrix, alerts antigen-presenting cells like macrophages and dendritic cell precursors about the potential threat in the surroundings and activates them. Meanwhile, CRT, a 46 kDa Ca^{2+}-binding protein along with disulfide isomerize ERp57 is transported from the lumen of the Endoplasmic Reticulum (ER) to the cellular periphery. CRT then functions as an engulf signal for the ATP-activated DCs [87,88]. One of the strong evidences of CAP as the inducer of ICD comes from a 2018 study by Lin *et al*. [89]. They have reported the echo-CRT and 70-fold increase in ATP on CT26 cancer cells following CAP treatment. Mice immunized with pre-treated CT26 cells with plasma (at ICD-inducing regimes) showed marked slow tumor growth when injected with live CT26 cancer cells compared to the controls. They also showed that plasma could amplify specific T-cell responses in mice, strongly supporting the role of plasma in inducing ICD [89]. Other significant changes that aid the entire process include the secretion of High Mobility Group Box 1 (HMGB1) and HSP70/90 externalization. HMG1

displays immune-stimulatory properties and helps in dendritic cell maturation. Similarly, HSP70/90 attracts monocytes and neutrophils along with elicitation of T-cell-based antitumor immunity. Taken together all these events help initiate tumor antigen presentation and ultimately destruction of the target by immune cells following the plasma treatment.

Plasma effects on TME

The tumor not only consists of a mass bulk of cancer cells but also consists of and closely interacts with its vicinity area comprising other non-transformed cells, vasculature, signaling molecules, immune cells, and extracellular matrix, collectively known as the TME. The progression and metastasis fate of a particular tumor is in fact directly dependent upon its interaction with its surroundings. This interaction occurs via soluble factors like cytokines, chemokine, growth factors, antigenic factors, and various enzymes. Such communication is a prerequisite for the formation of new blood vessels, recruitment of cancer-associated effector immune cells, stromal remodeling, and the overall metastatic process. Although a lot of immune effector cells are also infiltrated into the tumor site, their anti-tumor efficacies are largely attenuated in response to various signaling factors generated by the tumor cells. This ultimately aids tumors to evade the host immune surveillance and acquire some of the hallmark functions requisite to tumor growth, progression, and metastasis [90].

All kinds of components present in the tumor vicinity are in fact susceptible to plasma-derived reactive oxygen and nitrogen species (ROS/RNS). It has been proposed that long exposure to plasma inhibits as well as induces necrotic cell death in cancer-associated fibroblasts (CAFs) [91,92]. CAFs are generally recognized as critical players in cancer progression as they communicate with cancer cells mediated by cytokines, and exosomes and exert resistance to chemotherapy and radiotherapy [93]. Thus, the notion of plasma-treated elimination/modulation of CAFs activity might provide a further impetus to assist tumor control. Another important activity that inevitably happens in TME is angiogenesis i.e. formation of new blood vessels. This is particularly significant as tumors require a constant supply of oxygen and nutrients for their progression. A recent study has shown that direct treatment of CAPs on Human endothelial cell line HDMEC was able to reduce migration and decrease capillary tube formation abilities, clearly indicating some preliminary support along the notion of angiogenesis inhibition by CAP [94]. It is now a well-established notion that tumors adjust the surrounding environment in such a way that non-transformed cells in and around often confer a tumor-promoting function via immunosuppression of adaptive immune response. This corner of the immune defense recognizes molecules via specific receptors to distinguish between self and non-self/foreign/mutated/oncogenic structures and gets rid of the latter [95]. The major population of cells associated with this process is T cells and B cells. Plasma exposure has been shown to increase the infiltration, priming, and activation of T cells in murine pancreatic tumors. The same study has also shown that plasma-induced ROS unregulated Major Histo-Compatibility Complex-I (MHC-I), a vital entity for the pre-activation of T cells [96]. Similarly, B cells play a major role in modulating tumor response as they secrete antibodies against tumor antigens and promote antibody-dependent cytotoxicity. Unlike in T cells, however, plasma effects on B cells are yet to be studied in detail.

CAP meets nanotechnology where synergy counts

The notion of nanotechnology on biological and biomedical applications is yet another recent, fairly unexplored but potentially beneficent field that has gathered critically acclaimed interest within the research community. The notion goes well beyond synthesizing

materials at Nanoscale (1 - 100 nm) and focuses on designing as well as manipulating their physical, chemical and biological properties with a goal of integrating them well into the biological systems. These small-sized particles confer substantial variation in their optical, structural, electronic, magnetic, and biological properties well distinct from their own bulk material and can therefore be exploited for various medical applications. While the monumental scale of these opportunities tends researchers to focus on either of the one technology alone, there may be even substantial benefits in their synergy [97].

In one study, Fluorouracil (5-FU) encapsulated electro-sprayed core-shell nanoparticles in conjunction with CAP were studied against MDA-MB-231 breast cancer cell lines. Gene expression profiles revealed a significant down regulation of metastatic genes (VEGF, MMP9, MMP2, and MTDH) along with increased anti-proliferative effects. One interesting mechanism they reported was enhanced cellular internalization of carrier nanoparticles following CAP treatment, thus demonstrating the effectiveness of drug-loaded nanoparticles and CAP for potential synergistic anti-tumor effect compared to each treatment separately [98]. A similar study to analyze the synergy between CAP and iron nanoparticles (FeNPs) was carried out against the MCF-7 breast cancer cell line by Jalili *et al*. [99]. Their finding also revealed enhanced synergistic anti-cancer effect along with induction of apoptosis through significant down regulation of anti-apoptotic gene BCL2 [99]. A recent study looked at integrating CAP with novel paclitaxel (PTX)-loaded magnetic nanoparticles for targeting A549 cells. They found that PTX-loaded nanoparticles and CAP synergistically inhibited the growth of A549 cells more effectively than when each was used individually in an *in vitro* experiment setup. The study also concluded that the combination resulted in a marked increase in PTX retention *in vivo*. CAP was also able to induce an effective concentration of intracellular drug-loaded nanoparticles and enhanced tumor cell-killing effects [100]. The newest report in this arena comes from a 2020 study by Manaloto *et al.* [101]. They have reported > 100-fold cytotoxic effects in U373MG cells when treated with both CAP and PVA-coated silver nanoparticles (AgNPs) compared to either therapy alone. The same study also concluded that CAP was able to induce enhanced cellular uptake, intracellular aggregation, and increased toxicity, all supporting and confirming previous findings [101].

Among others, gold nanoparticles (AuNPs) confer unique properties including their readily controlled chemistry, biocompatibility, lower cytotoxicity and their capacity to be used as tumor-specific imaging and drug carrier agents. One of the 1st studies in this area came in 2008, where Kim *et al*. [102] utilized antibody-conjugated 30 nm AuNPs and showed a fivefold increase in G361 human melanoma skin cancer cell death compared to plasma treatment alone. Similarly, another study showed up to a 30 % overall increase in U87 glioblastoma human brain cancer cell death with CAP treatment along with AuNPs compared to the control group with plasma treatment alone [103]. Kaushik *et al.* were able to demonstrate a significant decrease in the proliferation of glioblastoma multiform T98G and lung adenocarcinoma A549 cancer cells through co-treatment of 100 nm PEG-coated gold nanoparticle and cold plasma (for 150 s). The study also highlighted their potential synergy to abrogate the PI3K/AKT signaling pathway, and the reversal of epithelial-mesenchyme transition (EMT) as confirmed by both *in vitro* as well as *in vivo* studies on tumor cells [104]. The synergetic potential offered by integrating AuNPs and CAP thus provides a promising therapeutic dimension for newer paradigms in cancer therapeutic strategies.

This synergy can be exploited to reduce the minimum plasma dose/Nano drugs against cancer to further reduce toxicity (**Figure 3**). Researchers are also using herbal-based Nano-formulation in combination

with CAP treatment. Adhikari *et al.* studied the combined effect of CAP and Silymarin Nano emulsion on the G-361 melanoma cell line. Silymarin is a hepatoprotective and antioxidant agent obtained from the herbal plant *Silybum marianum* L. (milk thistle). Generation of both ROS (< 3 times) and Reactive Nitrogen Species (< 2.5 times) was significantly higher in co-treatment compared to control. DNA damage studies were evaluated by estimation Levels of γH2AX (1.8-fold), PD1 (> 2-fold), and DNMT which showed damage in G361 cells. They observed an increase in Caspase 8, 9, 7/3 level (> 1, 5 times), PARP level (2 times), apoptotic gene level, and eventually blocking off the HGF/c-MET pathway. They also observed a decrease in EMT markers (E-cadherin, YKL-40, N-cadherin, and SNAI1). Markers for melanoma cells (BRAF, NAMPT) and stem cells (CD133, ABCB5) are simultaneously reduced. Similar results were observed with a reduction in tumor weight and size in the tumor engraft mice model. In another study by Adhikari *et al.* they assessed the effect of CAP and SN on autophagy induction in G-361 human melanoma cell lines. It was found that autophagy is induced in the G261 cell line by activating PI3K/mTOR and EGFR pathways, expressing autophagy-related transcription factors and genes.

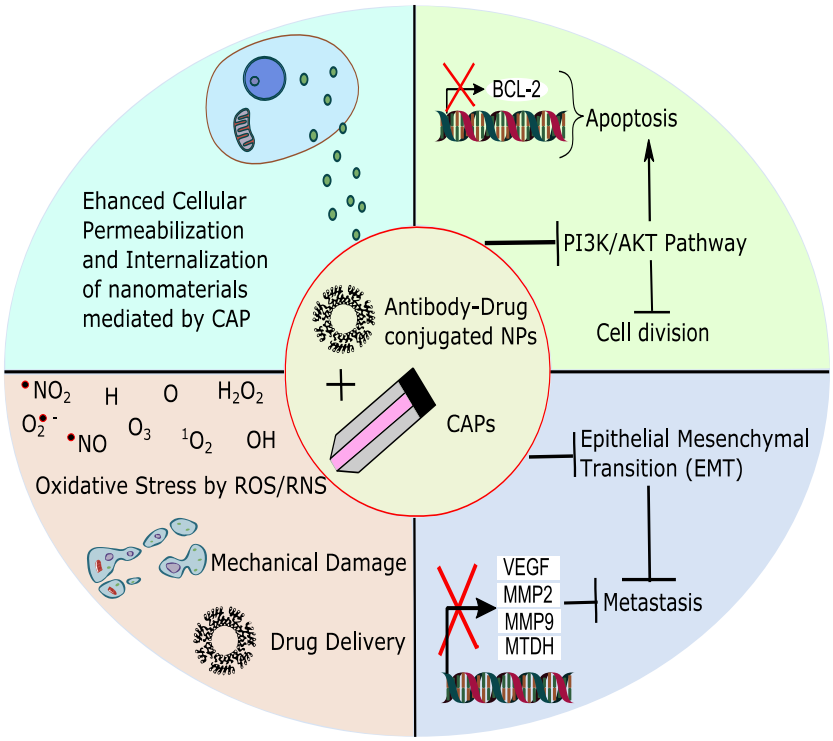

Figure 3 Nanoparticle and CAP synergy capsule. Schematic showing various anti-tumor processes enhanced as the result of NPs and CAP coupled synergy based on multiple studies.

Combination of chemotherapeutics with CAP: A potential new treatment option

Recently, Daeschlein *et al.* [105] reported enhanced anti-cancer efficiency of chemotherapeutic drugs when coupled with CAP exposure in melanoma and glioblastoma cell lines. They used 3 common chemotherapeutics namely bleomycin, paclitaxel, and decarbonize against human melanoma (B16 cells), mouse melanoma (A375 cells), and glioblastoma (A172 cells) in their study. They reported a strong reduction of

proliferation of all the cell lines followed by CAP treatment, which is also consistent with the anti-cancer notion of CAP and previous findings. Most importantly, they reported a maximal reduction in proliferation when drug-CAP combinations were used. This suggests that the combination of chemotherapy with CAP as an indirect treatment may be beneficial in enhancing tumor regression and less drug toxicity of chemotherapeutics [105]. The study is also consistent with previous results reported by Köritzer *et al*. [106]. Along with the anti-cancer potential of CAP, they were also able to report restoration of sensitivity of Temozolomide (TMZ) resistant glioma (LN18) cells. They found that LN18 cells were arrested at G2/M-phase after 1 single treatment with CAP coupled with TMZ while in contrast, repeated treatments of LN18 cells with TMZ were not able to induce comparable cell cycle arrest even in higher dosages (**Figure 3(A)**). Their data clearly showed that a previously applied CAP treatment restores the sensitivity of TMZ-resistant LN18 cells, leading to an induction of cell cycle arrest in the G2/M phase (**Figure 3(B)**). A possible mechanism can be analyzed in a 2012 study by Volotskova *et al*. [40], where they also reported approx. 2-3-fold G2/M increase in 2 different cancer cell lines accompanied by a decrease in the number of cells in the G0/G1 fraction. They reported increased phosphorylation of histone ϒH2A.X at the serine residue 139, which indicated oxidative damage to DNA. This in turn caused the deceleration of cells through the S phase leading to the accumulation of cells in the G2/M checkpoint [107].

Cold plasma: A new frontier in cancer medicine

In the past decade, 2 major approaches have been developed to use CAP in cancer treatment. First, directly deploying plasma jet or dielectric barrier discharge (DBD) plasmas to treat cancer cells seeded in plates (*in vitro*) or tumors grown in mice (*in vivo*). Second, using the plasma-activated solution/liquid cold plasma which includes cell culture media and other solutions to treat cancer cells both *in vitro* and *in vivo* [107]. To overcome the problem of limited penetration of previously used direct plasma, Nguyen *et al.* [108] dissolved the CAP in a liquid (also called liquid cold plasma) and demonstrated that it was able to induce oxidative stress and apoptosis in a heterogeneous population of cancer cells. The mechanisms underlying the liquid-plasma-induced tumor cell death revealed that liquid-plasma treatment generates ROS and RNS and sequentially causes apoptosis aided by DNA fragmentation, and caspase activation. They have shown that the liquid plasma induces apoptotic cell death in cancer cells, regardless of genetic variation through mitochondrial dysfunction mediated by ROS production [108]. Similarly, Plasma treated Phosphate-Buffered Saline (PBS) was able to reduce cancer cell metabolism and proliferation in PDA6606 murine pancreatic cells and COLO 357 human pancreatic cancer cells. They reported that induction of apoptosis was responsible for this antitumor effect identified [109]. Wang *et al*. [110] treated CAP on PBS and Dulbecco's Modified Eagle's Medium (DMEM) to obtain CAP-activated media and have reported that CAP-activated media decreased proliferation and induced apoptosis in A431 cells [110]. In fact, plasma activation can be paired up with any clinical liquid form used in cancer research, making the entire concept of liquid cold plasma a flexible tool for clinical use [111]. CAP has a significant role in cancer treatment and acts as a therapeutic agent to counter melanoma and cause DNA damage [111]. Yan *et al*. [112] reported that the CAP near room temperature ionized gas consists of reactive species especially peroxide which produces thermal radiation, UV radiation, and EM waves that can lead to the death of melanoma cells.

Clinical aspects of CAP

Despite burgeoning scientific data supporting the anti-tumor potential of CAP in cell cultures (*in vitro*)

and mouse models (*in vivo*), the efficacy has to be ultimately proven in a clinical setting. Metelmann *et al*. used CAP in a cohort of 6 patients with advanced squamous cell carcinoma of the head and neck to evaluate its anti-cancer effects in 2015. With the use of CAP, they reported decreased requests for pain medication, decreased fetid order, decreased microbial load, and partial remission along with wound healing of infected alterations. Similar wound healing capacity as well as a significant decrease in the width of plasma-treated ulcers has been reported elsewhere [113]. Mostly, clinical trials concerning CAP application have been evaluated in the treatment of chronic cutaneous ulcers. This is particularly because there is frequent bacterial colonization in cutaneous ulcers and the development of bacterial resistance is usually unlikely based on the CAP mechanism of action [114]. In a study done by Chuangsuwanich *et al.* [115], low atmospheric pressure plasma was used to treat the ulcer. Patients treated with plasma showed significantly better wound healing compared to the control group. Also, there was a reduction in bacterial load after the plasma treatment [115]. U.S. Medical Innovations LLC (USMI) and the Jerome Canady Research Institute for Advanced Biological and Technological Sciences (JCRI/ABTS) have obtained first-ever approval from the FDA to treat cancer with CAP. They are using high-frequency electrosurgical generators with cold plasma for the selective treatment of cancer [116]. A clinical trial where CAP was used as an oncotherapy was also approved by FDA in mid-2019. The trial aimed to save the life of a 33-year-old patient with a relapsed incurable peritoneal sarcoma patient and prolong the lifespan of a 75-year-old patient carrying late-stage pancreatic cancer for 2 years. A much recent work by Zhou *et al.* has designed a novel CAP injecting source for *in vivo* cancer treatment called *in vivo* Pen and proposes the potential synergy between plasma and conventional drugs as oncotherapy.

Perspectives

In short, CAP emerges rapidly as a potential treatment option for cancer. With many studies showing high selectivity, high potency, and/or promising drug-like properties, the already developed CAP technology shall provide potential alternatives or adjuvants to the current therapeutic arsenal that frequently relies on cytotoxic agents. While the area is in its infancy, we wish to pinpoint several directions that may broaden the application of CAP in oncology.

Conclusions and future directions

Our review underscores the known molecular and immunological actions involved during CAP-cancer treatment and possible clinical applications of CAP in the field of oncology. Its antitumor mechanism is mainly mediated by the production of reactive oxygen and nitrogen species. Among the wide biological effects, plasma being able to induce apoptosis of cancer cells resistant to orthodox therapies and being able to exert synergistic and complementary effects while combining with other treatment options is very exciting. Potential synergies between CAP and orthodox therapies like chemotherapy or with novel nanoparticle-based therapies could be a paradigm shift in cancer management and thus requires more attention. Similarly, research on the use of modalities where an optimal and safe biological dose of CAP is combined with a minimal dose of chemotherapeutic drugs is quite imperative. Our results are mainly co-related to some extent that the results presented by Shakya *et al.* [117]. This is crucial for minimal adversary effects on normal cells while targeting only cancerous cells. In addition, various physical and chemical properties of nanoparticles and drugs can be studied in such a way that plasma can be absorbed, combined, and directed in cancer sites with more efficiency. In addition, substantial research needs to be made to effectively couple nanoparticles and CAP to increase therapeutic value and ultimately establish a completely new era of

plasma oncology. For the time being, it's also highly imperative to study the feasibility of applying CAP for treating human samples. It has discovered a unique alternative known as CAP, which works against cancer like chemotherapy and radiation therapy and involves an ionization process with the production of ions, electrons, radicals, and excited species that can eliminate cancerous cells and contribute to anti-cancer effects. Thus, in the future, more *in vivo* and human studies shall give us many clear results and insights into the probable advantages and disadvantages of CAP's anti-cancer potential. For such studies, in situ injection sources of CAP like plasma jets can be highly useful. Future studies focused on CAP resistance, toxicity, and combinatory treatment strategies would be necessary to further optimize the existing leads into this useful treatment option for clinical trials.

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