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Review

Focal photodynamic intracellular acidification as a cancer therapeutic

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ABSTRACT

Cancer cells utilize an array of proton transporters to regulate intra- and extracellular pH to thrive in hypoxic conditions, and to increase tumor growth and metastasis. Efforts to target many of the transporters involved in cancer cell pH regulation have yielded promising results, however, many productive attempts to disrupt pH regulation appear to be non-specific to cancer cells, and more effective in some cancer cells than others. Following a review of the status of photodynamic cancer therapy, a novel light-activated process is presented which creates very focal, rapid, and significant decreases in only intracellular pH (pHi), leading to cell death. The light-activation of the H^+ carrier, nitrobenzaldehyde, has been effective at initiating pH-induced apoptosis in non-cancerous and numerous cancerous cell lines *in vitro*, to include breast, prostate, and pancreatic cancers. Also, this intracellular acidification technique caused significant reductions in tumor growth rate and enhanced survival in mice bearing triple negative breast cancer tumors. The efficacy of an NBA-upconverting nanoparticle to kill breast cancer cells *in vitro* is described, as well as a discussion of the potential intracellular mechanisms underlying the pH-induced apoptosis.

Contents

Abbreviations: NBA, nitrobenzaldehyde; PDT, photodynamic therapy.

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1. Introduction

Increased screening and earlier detection of cancers, and advances in surgical and radiation oncology have resulted in an increase in the overall 5-year survival rates for cancers in the United States to 66%, compared just over 50% in 1990 [1]. Targeted therapeutics also play a role in the improved outcomes for cancer patients, as these treatments are developed to exploit a precise characteristic of cancers. Trastuzumab successfully targets the increased expression of human epidermal growth factor receptor 2 (HER-2) in breast and stomach cancer, reducing the growth of these cancers. Additional targeted therapies include signal transduction inhibitors, angiogenesis inhibitors, immunotherapies, gene expression modulators, and apoptosis inducers. The precision of the targeted therapy approach is attractive and has been successful for given cancers in a subset of patients. However, only recently has attention been directed towards therapeutics which address a characteristic common to all cancers, namely the importance of extra- and intracellular pH regulation.

2. pH regulation in cancer

Cancer cells utilize glycolysis for energy, therefore cancer cells produce extraordinary lactate and H^+ levels. In spite of this increased intracellular H^+ production, cancer cells have been reported to have an intracellular pH that is relatively more alkaline (pH 7.12–7.24) compared to non-cancerous cells (pH 6.99–7.05) [2]. In order to maintain an acidic extracellular environment and alkaline intracellular pH (pHi), many cancer cells exhibit an increase in the expression and activation of pH regulatory proteins. It is well known that an acidic extracellular tumor microenvironment facilitates tumor aggressiveness, migration and metastasis [3–7], chemoresistance [8], and vascular endothelial growth factor-mediated angiogenesis [5]. Cancer cells have multiple mechanisms to regulate both intracellular pH (pHi) and extracellular pH (pHe), maintaining a slightly alkaline pHi and acidic pHe with respect to non-cancerous cells [2]. The monocarboxylate transporters (MCT), vacuolar-type H^+-adenosine triphosphatase (V-ATPase), and sodium hydrogen exchangers (NHE), which directly transport H^+ from the intracellular to extracellular space are ideal candidate targets to disrupt pH dynamics in cancer.

3. Manipulation of pH regulatory proteins

Several studies implicate pH regulation by cancer cells in both cancer cell migration and metastases, and have proposed manipulation of pH regulatory proteins as candidate therapeutics for human cancers. Knockdown of the MCT4 transporter *via* siRNA lead to reduced migration of human retinal pigment epithelial cells (ARPE-19) and canine epithelial cells (MDCK) [9]. The knockdown of the MCT4 transporter in human breast cancer cells (MDA-MB-231) [9] or the inhibition of NHE1 in MDCK cells [10] resulted in reduced invasion, and migration, respectively. Decreased invasion and metastases in response to the disruption of pH regulation *via* the V-ATPase has been reported in triple negative breast cancer cells [11] and B16 melanoma cells [12]. A matrix metallopeptidase 9-dependent decrease in pancreatic cancer cell invasion was observed in response to siRNA knockdown of V-ATPase [13].

3.1. Monocarboxylate transporters

Monocarboxylate transporters are part of the 14-member solute carrier family, however only MCT 1–4 facilitate proton-linked monocarboxylate transport. The rapid transport of pyruvate, lactate, and ketone bodies across the plasma membrane is coupled to the proton-linked MCTs 1–4. The net influx or efflux of monocarboxylates is solely determined by the concentration gradients of protons and monocarboxylates across the membrane of the cell. Decreases in pHi, and subsequent disruption of cancer growth have been achieved by manipulation of the intracellular pHi regulators, MCT1-4, NHE1, and V-ATPase. Gerlinger et al., [14] reported that siRNA knockdown of the MCT-4 resulted in an intracellular acidosis of 0.20 and 0.25 pH units, decreased cellular ATP levels, and 42% decrease in cell proliferation in six of eight clear cell renal carcinoma cell lines. While MCT1 is expressed ubiquitously in normal tissue, it is also highly expressed, along with MCT2 and MCT4 in gastrointestinal tumors [15] and colorectal cancer [16].

3.2. NHE1

NHE1 is ubiquitously expressed in normal tissue and is upregulated in response to hypoxia [17] and intracellular superoxide concentrations [18]. In spite of its systemic expression, targeting NHE1 to induce intracellular acidosis has proven useful *in vitro*. The ammonium chloride prepulse technique was used to induce an intracellular acidosis to approximately pH 6.3 in one rat and three human malignant glioma cell lines (C6, U-87, U-118, and U-251); all cell lines were capable of recovering from this intracellular acidosis, however, these same glioma cells exhibited a significant, prolonged intracellular acidification in response to bath application of amiloride, an NHE1 blocker [19]. Li et al., [20] reported intracellular acidification to a pHi of 6.86 in drug-resistant small cell lung cancer cells in response to NHE1 antisense treatment, which was significantly lower than the empty vector control pHi of 7.25. More importantly, these transfected cells exhibited significant decreases in cell proliferation, and a $12.18 \pm 1.86\%$ increases in caspase-3 mediated apoptosis 48 h after transfection, compared to only $2.37 \pm 0.33\%$ apoptosis control cells.

3.3. V-ATPase

The movement of protons across the membrane of intracellular organelles, such as endosomes, Gogli complex, lysosomes, and the plasma membrane due to the actions of the V-ATPase enzyme, result in organelle and extracellular acidity, respectively. The V-ATPase couple's proton transport to ATP hydrolysis, and is found in many eukaryotic cell types, plants, and fungi [21]. Because V-ATPases play a significant role in acidification and degradation of the extra-tumoral space, facilitating the invasion of malignant cells, the V-ATPase is an attractive target to disrupt pH dynamics in cancer. In addition, this multi-subunit protein is upregulated in pancreatic cancer [22], and is upregulated on the plasma membrane of the highly aggressive triple negative breast cancer cell line MDA-MB-231, while it is not located on the cell surface of the less aggressive MCF-7 breast cancer cell. Small interfering RNAs specific to each the of four isoforms of the subunit of the V-ATPase were used to quantify the role of these isoforms on cancer invasiveness and cytosolic and endosomal pH on MDA-MB-231 and MCF-7 breast cancer cell lines [11]. Only the knockdown of the a3 isoform lead to decreased cytosolic pH, whereas siRNAs to a3 and a4 significantly inhibited invasiveness in MDA-MB-231 cells [11]. Overexpression of the V-ATPase 16 kDa subunit enhanced invasiveness and matrix metalloproteinases, responsible for degrading extracellular matrix proteins, in mouse fibroblasts, whereas transfection of these mouse fibroblast with antisense oligonucleotides of the 16 kDa subunit of V-ATPase decreased both invasiveness and production of matrix metalloproteinases [23].

The ability to alter or disrupt the pH dynamics, alkaline pHi and acidic pHe, *via* MCTs, NHE1, or V-ATPase provides researchers and clinicians with the potential to reduce cancer growth, invasiveness and metastases, resistance to basic chemotherapeutics, and angio-

genesis while increasing the rate of apoptosis. While the advances described above provide insight into the potential benefits of developing treatments that can disrupt pHi regulation in cancer, other pH regulatory processes, such as carbonic anhydrases, chloride-bicarbonate transporters, and voltage-gated proton channels may also offer therapeutic potential. Although the disruption of pHi regulation *via* the MCTs, NHE1, or V-ATPase appear to be somewhat cancer specific, there does not appear to be a viable approach to altering pHi regulation that can decrease pHi and produce cancer cell death across virtually any cancer. Light-activated intracellular acidosis is a viable candidate technique to produce pH-induced cell death in any cancerous tissue.

4. Photodynamic therapy (PDT) as a cancer therapeutic

The current approach of PDT in cancer therapy has principally utilized the release of high levels of intracellular reactive oxygen species (ROS) to inhibit tumor growth. The use of ROS PDT is often palliative [24] or applicable in limited indications in cancer therapy, such as bile duct cancer, precancerous Barrett's esophagus, non-small cell lung cancer, and bladder cancer. Cancer cells express elevated ROS, but these cancer cells also have increased expression of antioxidant proteins to prevent these higher ROS levels from damaging the cancer cell. Unfortunately, porphyrins may also enter nearby healthy tissue, creating unintended ROS damage as an undesired side effect. In addition to damaging healthy cells, increasing ROS outside of the tumor is potentially tumorigenic and may promote tumorigenesis in nearby, non-cancerous cells. While high levels of ROS can cause apoptosis, low levels of ROS facilitate cancer cell survival [25], therefore using ROS as an anti-cancer therapy requires a delicate balance of ROS levels. Other limitations of ROS PDT include skin and eye sensitivity lasting up to 6 weeks after treatment, burns, swelling, pain, and scarring of nearby tissue [26]. More serious concerns regarding the use of ROS as an anti-cancer therapy treatment involve the harmful effects of low doses of ROS in promoting cell proliferation [27], metastasis [28], and angiogenesis [29] at the tumor site. ROS-regulated Vascular Endothelial Growth Factor (VEGF) was required for inducing angiogenesis in ovarian and prostate cancer cells [30], and VEGF expression is increased during cancer cell nutrient deprivation and hypoxia [31], both which increase intracellular levels of ROS.

Administration of exogenous 5- aminolaevulinic acid (ALA) leads to an increase of protoporphyrin IX (PpIX) [32], a ROS photosensitizer, and was used as part of a fluorescence-guided resection of brain tumors. ALA-induced PpIX was used to perform a fluorescence-guided resection, allowing 63% of patients to receive complete resection through this method compared to 36% of patients that received white light resection. The trial was terminated when analysis revealed that while ALA patient progression-free survival was significantly higher than white light patients at 6 months, both treatments groups drop to less than 10% survival at the 15-month mark [24].

The relatively infrequent and effective use of ROS PDT may stem from the fact that many chemotherapeutic strategies are designed to increase intracellular ROS levels to induce irreparable damage that results in tumor cell apoptosis, thereby eliminating the need for ROS PDT. In addition, many solid tumors are severely hypoxic, thereby rendering ROS therapy relatively limited in its efficacy. One significant difference between our light-activated intracellular acidosis method and traditional ROS PDT is that our technique induces an oxygen-independent intracellular acidosis, exploiting the effects of an acute drop in pHi rather than the generation of ROS. Unlike ROS-mediated chemotherapeutics, there are few chemotherapeutic agents that can selectively cause decreases in pHi in a safe manner. The amiloride-based drugs alter pHi by blocking the sodium/hydrogen exchanger 1 (NHE1). This blockage leads to the accumulation of intracellular H^+ and subsequent pH-induced apoptosis. Unfortunately, the NHE1 is found virtually on every cell in the body and its blockage by amiloride is the basis for many of the unwanted side effects of amiloride-based chemotherapy. While light-activated acidosis appears to initiate an intrinsic apoptotic pathway, it appears to do so independent of cell cycle progression. As noted in Leon et al. [33] "the development of small molecules that act independently of cell cycle progression to engage non-apoptotic cell death mechanisms offers a particularly attractive approach to thwart tumor progression".

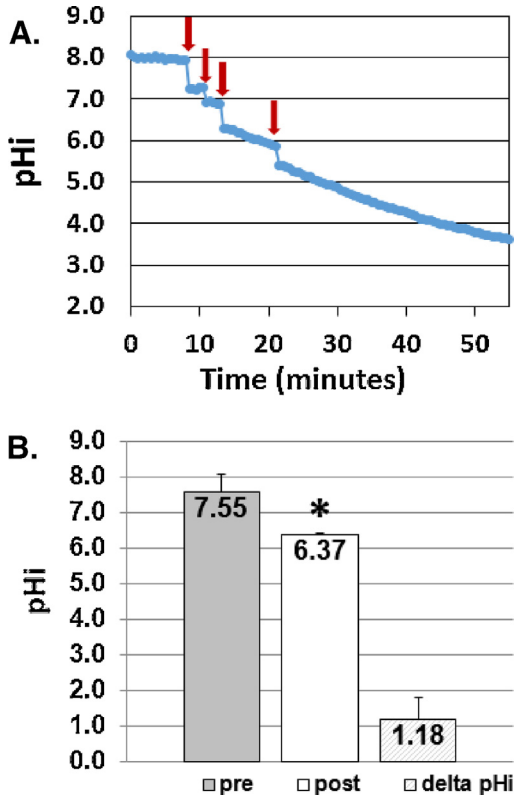

Fig. 1. (A) Optical recording of the ratio of DCFDA fluorescence converted to pHi from an individual PC12 cell loaded with NBA in response to 4 consecutive exposures to UV light (350 nm, 30 s). (B) UV photo activation of NBA (350 nm, 2.5 min) induced a significant reduction in pHi (delta pHi) in PC12 cells (n = 421) loaded with NBA before (pre) and after (post) UV light activation.

5. Light-activated intracellular acidification

We recently developed a technique to use photo activation of a caged H^+ carrier, o-nitrobenzaldehyde (NBA), to cause significant reductions in only pHi. NBA passively diffuses through the cellular membrane and becomes trapped due to the slight positive charge on the molecule. Once inside the cell, NBA is capable of remaining inactive and intact until excited by UV light (200 nm to 410 nm) [34]. NBA is a photo activated molecule capable of immediately releasing a proton upon exposure to ultraviolet (UV) to near-visible light (400–410 nm), leading to decreases only in pHi when extracellular NBA is removed (Fig. 1A). Due to the ease of entry into the cell, the tendency of the molecule to remain inside of the cell, the nontoxic nature of the molecule, and the instant acidification upon exposure to UV light, light-activation of NBA is an ideal mechanism for disruption of pHi . The fast, focal disruption of pHi regulation in cancer cells is an untapped method of cancer treatment that holds great potential as an alternative to chemotherapy and radiation. In addition, cell death *via* photo activation of NBA does not appear to

be "cancer-specific" and can be used in treating a myriad of cancers, including multi-drug resistant (MDR) cancers.

The ability to employ photo activation of NBA to cause significant decreases in pHi (Fig. 1 B) has profound implications in cancer research. Cardone et al., [35] stated that our understanding of how increased pHi and lower extracellular pH facilitates tumor proliferation and invasiveness may lead to new targets for cancer therapy, and that "compounds that selectively decrease pHi would also be of great interest, although it may be difficult to achieve this effect only in cancer cells". Our research approach overcomes this barrier to selectively acidify the intracellular space of cancer cells which only receive NBA and light, and offers the hope of transforming both basic research and clinical practices involving cancer therapeutics.

Effective concentrations of intracellular NBA required to induce significant intracellular acidosis and subsequent cell death are not cytotoxic and do not disrupt cellular function. Kohse et al. [36] photo activated the enzyme acid phosphatase from pH 8.0–6.0 by the activation of a pH jump using flash photolysis of NBA and did not report any degradation of enzyme function as result of exposure to NBA. Flash photolysis of NBA to reduce pHi of rat ventricular myocytes [37] did not alter the H^+ buffer capacity of these cells. More importantly, in the absence of UV exposure, NBA (1 mM) did not alter diastolic Ca^{2+}, cellular contraction, or "the mechanisms of spatial pHi regulation" [37]. We recently loaded the entire *in vitro* tadpole brainstem with 10 μM NBA and observed no disruption in the spontaneous, fictive respiratory motor output or central respiratory chemoreceptor responses, indicating that at this concentration, NBA does not exert cytotoxic effects on cells which compose the respiratory neural circuits [38].

6. Fluorescence ratio recordings of changes in pHi

Our desire to pursue this compartmentalized pH reduction technique as a viable cancer therapeutic began with validation of the effectiveness of pH-induced apoptosis on non-cancerous rat pheochromocytoma (PC12) cells. In order to quantify changes in pHi induced by photolysis of NBA, Jordan et al., [39] performed optical recordings of pHi using the ratiometric, pH-sensitive fluorophore 2′,7′–dichlorofluorescin diacetate (DCFDA), as this technique permits the quantification of dynamic, real-time changes in pHi. A calibration curve was first generated, which permitted the conversion of DCFDA emitted fluorescence ratio to pHi. PC12 cells were loaded with the pH-sensitive dye Carboxy-DCFDA (10 μM in rat aCSF). Nigericin was titrated to a pH of 2.0, 4.0, 5.0, 6.0, 7.0 or 8.0. The emitted fluorescence of DCFDA was recorded from cells at each of the titrated pH solutions. The ratiometric emitted fluorescence intensity was observed at an excitation of 495 nm/440 nm on 30 s intervals until emitted fluorescence (504/530 nm) reach a steady state. Each ratio from individual cells (n = 421) was recorded for each pH. A curve of best fit was created (R^2 = 93.02) and used for the conversion of fluorescence to pHi. Calibration curves for both PC12 cells and MCF-7 breast cancer cells were generated using this nigericin technique.

7. NBA photolysis decreases pHi

Jordan et al., [39] reported the mean optical recordings of pHi from PC12 cells (n = 421) loaded with 10 μM DCFDA and 1 mM NBA before (7.55 pHi) and after (6.37 pHi) flash photolysis. We were able to induce a significant acidosis ($P < 0.01$) in response to flash photolysis of NBA (350 nm light for 2.5 min) [39]. The mean change in pHi (Δ pHi) was 1.18 pH units. These data support our abilities to (1) optically record valid pHi measurements, and (2) quantify the pHi decreases associated with the uncaging of H^+ from NBA. Flash photolysis of NBA produced similar reductions in DCFDA ratios in the breast cancer cell lines MCF-7 and MDA-MB-231, prostate cancer cell lines 22RV-1 and LNCap, and the pancreatic cancer cell line BxPC3. The ability to utilize photolysis of NBA to induce significant, rapid, and sustained decreases in pHi which these cancers cannot mount a pH regulatory response to, reinforces the potential to further develop NBA photodynamic therapy as a non-cancer specific modality to treat virtually any cancer.

8. Quantification of cell death

After exposing NBA-containing cells to UV light, the presence of cellular blebs was observed and recordings of the pH in the intrablebular inflationary space (pH_b) were acquired using the emitted ratio of DCFDA fluorescence. The blebs visualized with DCFDA fluorescence showed continuous expansion which never reduced in size. We quantified the unretracted blebs as cellular death based on previously published findings which described all unretracted blebbing was an indication of an irreversible death process [40]. In addition, one hour after the UV flash paradigm to induce intracellular acidosis, the cells were lightly washed and the media was replaced with the phosphatidylserine indicator Annexin V Alexa Fluor 568 (488 nm). Cell death was quantified as either the presence of an unretracted bleb and the expression of Annexin V fluorescence.

9. MCF-7 cell death in response to light-activated intracellular acidosis

After focal acidification was achieved in MCF-7 cells, Annexin V Alexa Fluor 568 was bath applied to mark for phosphatidylserine membrane inversion resulting from and indicative of apoptosis. The cells were monitored for at least 1 h after flash photolysis in the presence of NBA for fluorescence which would indicate apoptosis. MCF-7 cells were also observed to exhibit cellular blebbing (Fig. 2) upon focal intracellular acidification and did not exhibit fluorescence in the presence of Annexin V Alexa Fluor 568 until the bleb completely separated from the cell or after several hours (1–6 h) post treatment. As previously discussed, research indicated that unretracted blebbing was indicative of apoptosis [40]. The number of blebs was counted upon formation as a function of time as another indication of cell death. While previous research indicates that upon activation of the sodium-calcium exchanger (NCX1) there is often a reduction in the size of the bleb [41], we did not observe any reductions in the occurrence or size of blebs over time in response to our NBA-UV treatment. Jordan et al., [39] reported significant apoptosis in both PC12 and MCF-7 cells, as indicated by cellular blebbing or apoptosis *via* fluorescence of Annexin V, in response to intracellular acidification *via* photolysis of NBA. Apoptosis was observed in 85% of PC12 (n = 362; R^2 = 0.98) within two hours after intracellular acidification. MCF-7 cell apoptosis ranged from 94.9 to 100.0% (n = 262; R^2 = 0.92) with an average apoptosis over a two-hour time period of 98.3 ± 0.3% (Fig. 2C; [39]). Linear regression analysis revealed a significant decrease ($P < 0.01$) the percentage of apoptosis for MCF-7 cells exposed to only UV light (7.1%) over 2 hours. The percentage of apoptosis observed in MCF-7 cells exposed only to NBA (2.3%, n = 236, R2 = 0.87) was significantly less ($P < 0.0001$) than the percentage of apoptosis in MCF-7 cell treated with NBA and UV light. Our control experiment to evaluate the percentage of apoptosis in response to time alone (n = 20) revealed no apoptosis over 2 hours. As our results produced no cell death what so ever, linear regression was not performed.

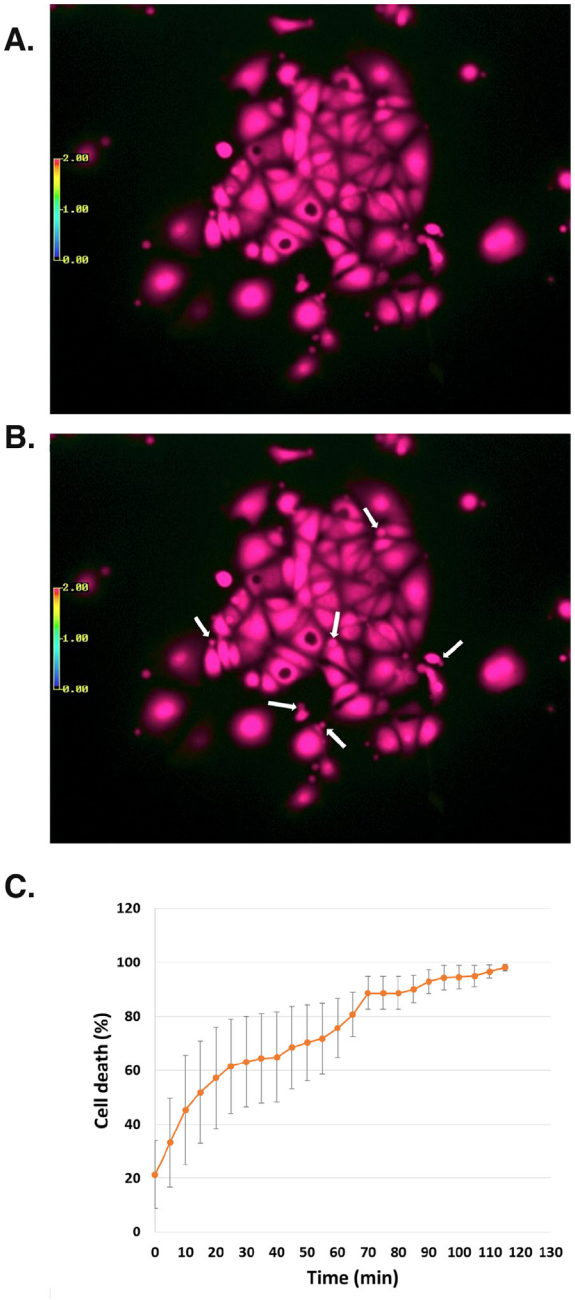

Fig. 2. DCFDA emitted fluorescence from MCF-7 breast cancer cells before (A) and after (B) NBA photodynamic treatment. Note the presence of cellular blebs following intracellular acidification (C) Mean ± SD MCF-7 cell death following 2.5 min of NBA PDT (time 0; from Jordan et al., [40]).

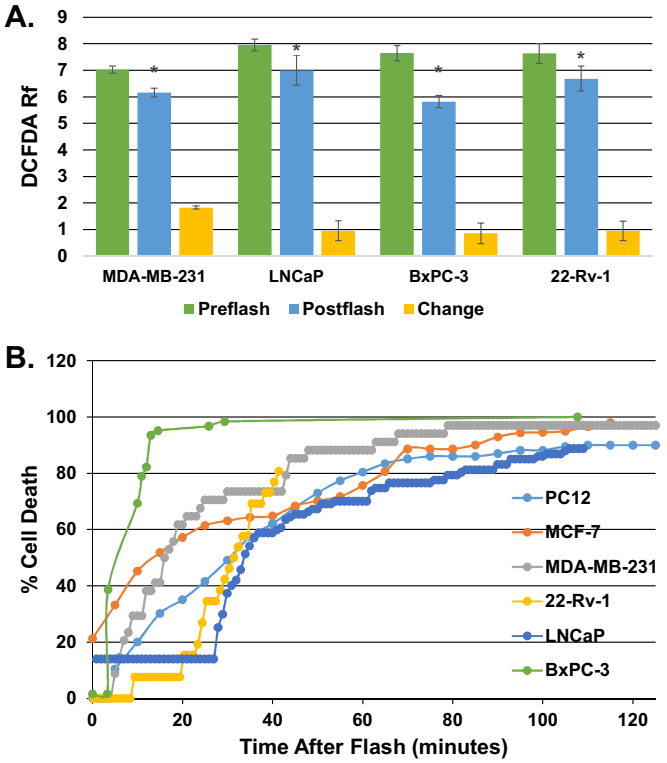

Fig. 3. (A) The pre-flash, post-flash, and change in ratio of the DCFDA emitted fluorescence four cancer cell lines. These values were not substantially different from similar DCFDA ratios recorded in PC12 and MCF-7 cells. *Indicates significantly different from the pre-flash value. (B) percent cell death over time in response to NBA light-activated intracellular acidosis in PC12, MCF-7, MDA-MB-231, 22Rv1, LNCaP, and BxPC3 cell lines.

10. Light-activated NBA cell death is not cancer specific

To test the hypothesis that light-activated NBA represents a non-cancer cell specific treatment, several types of cancer cell lines and the non-cancerous PC12 cell line were incubated with DCFDA and NBA. Exposure to the cell resulted in a decrease in DCFDA ratio which was consistent with a rapid decrease in pHi. Using the PC12 calibration curve, we calculated a decrease in pHi in PC12 cells of 1.18 pH units (Fig. 1B). Based upon the emitted DCFDA fluorescence ratio (Rfl), we observed in 4 cancer cell lines, changes in ratio before and after NBA flash photolysis which were not substantially different from the decreases in ratio observed in PC12 cells (Fig. 3A). Based upon the PC12 calibration formula, we observed decreases in pHi in response to photo activation of NBA in MDA-MB-231 of 0.67 pH units (n = 34). LNCaP cells decreased pHi by 0.80 pH units (n = 107). BxPC3 cells decreased by 1.46 pH units (n = 62), and 22Rv1 cells decreased pHi by 0.77 pH units (n = 22). Preliminary data reveal that the decreases in pHi associated with photolysis of NBA were immediately followed by increases in unretracted cellular blebs and rupture of the cell membrane (Fig. 3B). Cell death was rapid in all 4 cancer cell lines, with the greatest rate of cell death in the BxPC-3 cancer cell line, which experienced the greatest intracellular acidification. These data support that the photolysis of NBA within cells produces a focal and significant intracellular acidification, followed by a reliable pattern of cell death. The ability to produce a significant, rapid reduction in pHi appears to induce apoptosis, regardless of whether the target cell is a non-cancerous PC12 cell or a cancerous cell line as reported above.

11. Light-activated NBA reduces tumor growth and promotes survival *in vivo*

We have recently conducted preliminary *in vivo* experiments to determine the efficacy of intratumoral acidosis on tumor growth. Four-week-old female nude mice were injected with MDA-MB-231 cells into the mammary fat pad. All animals were sacrificed when humane endpoints were reached, as defined by the Institutional Animal Care and Use Committee at The University of Texas at San Antonio. Tumor growth was monitored daily, and upon the tumor reaching 5 mm in length, we treated animals with either an aCSF injection (Controls, n = 4) or 1 mM NBA followed by photo activation of intratumoral NBA using a 200 μM fiber optic can-

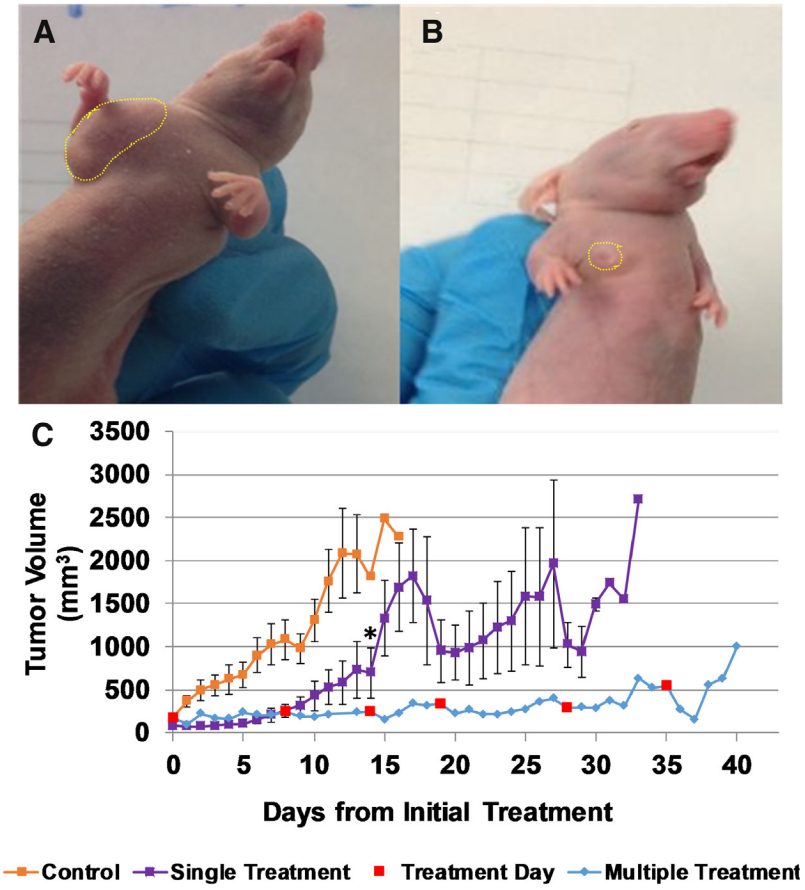

Fig. 4. MDA-MB-231 breast cancer tumors in nude mice (A) five days after control injection of aCSF, or (B) three days after a one-time treatment of NBA and light-activated intracelluar acidosis. The change in tumor volume (mean ± SEM) following treatment (aCSF control, n = 4, Single treatment NBA with photo activation, n = 5, and Multiple treatment) over a 40-day period. The single NBA with photo activation treatment on day 0 resulted in a significant ($P < 0.05$) reduction in tumor volume (*) for 14 days when compared to the control treatment.

nula (405 nm, 85 mW for 2.5 min; n = 5). Control treated animals exhibited rapid and continued tumor growth (Fig. 4A) over time, as illustrated in Fig. 4C. Our single injection of NBA and one-time photo activation resulted in a significant reduction in both tumor volume (mm^3) and the percent change in tumor volume following treatment (Fig. 4B and C). The one-time treatment with photo activated NBA resulted in a significant reduction of tumor growth for 14 days; thereafter tumors continued to grow. We also performed multiple NBA treatments on a single mouse, delivering an NBA injection and photo activation every 4–7 days. The multiple treatments of triple negative breast cancer resulted in a maintained decrease in tumor growth for over 40 days (Fig. 4C). The multiple NBA treated animal was sacrificed when modest tumor growth at day 37 migrated medially, compromising the animal's ability to swallow. Animal survival was significantly improved in the one-time NBA treated mice (37.4 ± 7.0 days) compared to the control animals (16.5 ± 1.6 days). With only one animal in the Multiple Treatment condition, we were unable to perform statistical analysis on the effects of the treatment on growth rate and survival time; however, these preliminary results provide a clear demonstration of the efficacy of photo activating NBA intratumorally to reduce tumor volume in an animal model.

12. Upconverting NBA nanoparticle

We have improved the ability to induce discrete decreases in pHi only to virtually any desirable pHi lower than the intrinsic pHi, and we have demonstrated that this effect is reproducible and highly effective at initiating significant pH-induced apoptosis *in vitro* (Fig. 3) and reductions in TNBC tumors *in vivo* (Fig. 4). We created an additional delivery system to cause pH-induced cancer cell death in the form of a photo-upconverting nanoparticle. Upon entry into the cancer cell, the nanoparticle is exposed to 980 nm near infrared light and absorbs this energy and quickly emits 350–400 nm light around the core of the particle, thereby releasing the H^+ to the intracellular space.

Upconverting nanoparticles (UCNPs) are nanocrystalline mineral-phase particles doped with rare earth (RE^{3+}) lanthanide ions. They use multi-photon upconversion fluorescence to emit visible and ultraviolet (UV) light upon stimulation with longer-wavelength electromagnetic sources, such as near infrared (NIR) lasers. Recent improvements in the upconversion efficiency of tetrafluoride based UCNPs have yielded order-of-magnitude gains in blue-violet and UV luminescence [42–46]. Enhancement of such wavelengths further improves their utility in this application such as light harvesting to photolabile polymers and biomolecules [47]. The use of 980 nm diode sources provides the advantages of low cost, low photo-damage, low auto-fluorescence, high directionality, and deep penetration in biological tissues [47]. Nash and coworkers [48–50] have investigated the spectroscopic characteristics of RE^{3+} species in highly hydrophilic, tissue-like environments and demonstrated useful therapeutic windows for NIR therapies involving blood contact. Similar findings have been applied toward advancing the development of UCNPs for deep-

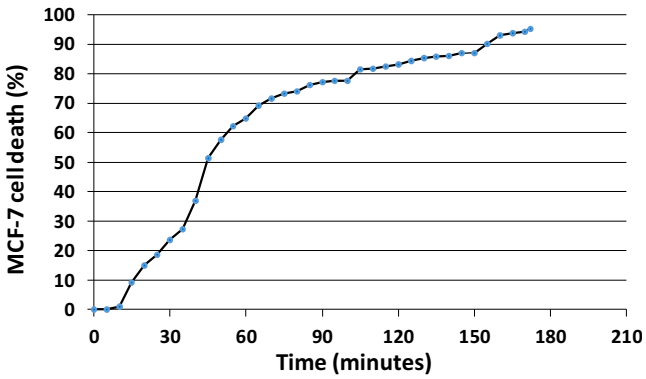

Fig. 5. The percentage of MCF-7 breast cancer cell death over time in response to photo activation (980 nm) of an upconverting NBA nanoparticle. Upconversion of the NBA nanoparticle produced 91.5% breast cancer cell death within 3 hours.

tissue biomedical imaging [51–55] and chemotherapy [56,57]. Our preliminary work employed a NaYF4 nanoparticle with 200 μM polyethylene glycol (PEG) linked to NBA. These small particles (10–30 nm diameter) easily crossed the cell membrane of DCFDA loaded MCF-7 cancer cells. The 980 nm near infrared upconverting light (400 mW; 10 min) produced an intracellular acidosis and concomitant 91.5% cell death pattern (Fig. 5) which was virtually identical to our NBA and UV light paradigm described earlier (Fig. 2C).

This NBA upconverting nanoparticle may also be targeted to a specific receptor unique to cancer, or a receptor that is highly up-regulated in cancer. This nanoparticle delivery system has the potential to address a significant barrier currently facing the treatment of all tumors, specifically the ability to kill the tumor without damaging adjacent healthy tissue. Successfully developing an effective, targeted PDT upconverting nanoparticle that can cause significant, focal pH-induced apoptosis represents an improved method in the emerging field of PDT. The ability to systemically administer the cancer-specific targeted upconverting nanoparticle would permit virtually any cancer to be treated this technique, as the 980 nm light needed to activate the particle penetrates relatively deeply into human tissue.

13. PHi-dependent mechanisms of cell death

Alterations in intracellular pH have been reported to occur both during the *initiation,* as well as the progression of the apoptotic process [58], and intracellular acidification is an important component of the initiation phase of apoptosis [59,60]. The light-activated intracellular acidification caused by photo activation of NBA is the initiating stimulus in our cancer cell death pathway. Studies that report decreases in pHi as potential *initiators* of apoptosis may provide much more insight into the potential cellular mechanisms of apoptosis, rather than studies which report decreases in pHi during the *progression* of apoptosis. The intrinsic intracellular apoptosis pathway involves the translocation of the pro-apoptotic protein Bax to the mitochondrial membrane, and the subsequent oligomerization of Bax to create a mitochondrial pore to release cytochrome c to the intracellular space. Increased intracellular levels of cytochrome c are the hallmark of both the caspase-8-dependent extrinsic and the caspase-9-dependent intrinsic apoptosis, as well as caspase-independent cell death (CICD; for review see Tait and Green [61]). Further understanding of the potential mechanisms of rapid intracellular acidosis *via* the light-activation of NBA on cell death requires a review of studies which describe alterations in intracellular processes which were preceded by changes in pHi.

It is certainly possible that intracellular mechanisms in response to alterations of pHi vary within different cell types. For example, ATP depletion-induced apoptosis in tubular cells was reduced if the pH of the culture media was lowered from 7.0–7.4 to 6.0–6.5, and was associated with the inhibition of caspase activation, indicating that an alkaline pHi was responsible for the ATP deletion-induced apoptosis in renal tubule cells [62]. Importantly, Brooks et al., [62] reported that the decrease in media pH **did not prevent** Bax translocation, oligomerization to the mitochondrial membrane, and cytochrome c release. While their work concluded that the acidic pH blocked caspase-9 activation in the apoptosome, the acidity did not alter Bax-mediate cytochrome c release from the mitochondria. Khaled et al. [63] showed an increase in Bax activation of apoptosis after withdrawal of interleukin-7 in an IL-7 dependent cell line, and that this increase in Bax activation was *preceded by a **rise in pHi***. Kahled et al. [63] argued that the increase in pHi caused a transformational change in Bax which induced translocation to the mitochondria and subsequent cytochrome c release. They proposed a model whereby at a neutral pH, the negative residues of Bax N-terminus could interact with the positive residues on the C-terminus, thereby concealing the hydrophobic transmembrane region, and that at an alkaline pHi would lead to "reduced attraction between the termini, exposing the membrane seeking region". When testing the effects of an acidic pHi on intracellular levels of Bax, Khaled et al. [63] found reduced Bax levels in the cytosol, "likely resulting from the effects of protein aggregation or activation of proteolytic digestion".

Decreases in pHi in many cell lines accelerate the initiation of the intrinsic apoptotic pathway. Silencing the MCT4 transporter in clear cell renal carcinoma resulted in significant decreases in pHi of approximately 0.2–0.25 pH units, and decreases in cell proliferation [14]. Decreases in pHi (magnitude) have been shown to increase caspase activation 2–3 fold. Matsuyama et al. [60] report the induction of apoptosis in approximately 50% Jurkat T-cell leukemia cells within two hours of exposure to the apoptosis inducer staurosporine, ultraviolet light, or anti-Fas antibodies. Staurosporine application leads to an increase in mitochondrial matrix pH and a concomitant decrease in cytosolic pHi, increases in cytosolic cytochrome c, caspase activation, and apoptosis, which was inhibited by application of the anti-apoptotic protein Bcl-2. Overexpression of Bax in the 293T cell line caused apoptosis within 8–10 h of transfection and resulted in a similar mitochondrial matrix alkalinization and cytosolic acidification. The cytochrome c-stimulated caspase activation initiated by staurosporine or transfection of Bax were pH sensitive, with a 3–4-fold increase at pHi 7.1 compared to the control pHi of 7.4. The efficiency of cytochrome c activation of caspases was pH-sensitive, with an optimum pHi of 6.3–6.8 *in vitro* [60]. Matsuyama et al. [60] concluded that Bax-induced changes in mitochondrial and cytosolic pH are a very early event in mitochondria-dependent apoptosis and that the decreases in pHi regulate caspase activation in the mitochondrial pathway of apoptosis.

Somatostatin receptor-mediated cytotoxic signaling triggers intracellular acidification and subsequent apoptosis in MCF-7 and T47D breast cancer cell lines and clamping pHi prevents apoptosis [64–66]. Liu et al. [59] reported that decreases in pHi and mitochondrial dysfunction in MCF-7 breast cancer cells exposed to somatostatin-induced apoptosis is preceded by caspase-8. Furthermore, they conclude that intracellular acidification is an early initiator of the apoptotic process and that mitochondrial dysfunction and activation of the effector caspases 3–7 and 11–13 occur only in response to decreases in pHi [59].

Khaled et al. [63] described conformational changes in Bax structure in response to intracellular alkalosis which promoted

translocation, oligomerization, and insertion of Bax into the mitochondrial membrane. Tafani et al. [67] described intracellular acidosis occurring in HeLa cells in response to exposure to tumor necrosis factor (TNF), and that the TNF-induced decrease in pHi produced conformational changes in Bax similar to those conformational changes associated with alkalosis, and that these changes in Bax conformation and subsequent translocation to the mitochondria is directly influenced by pHi. Mutagenesis of Bax revealed that both positively and negatively charged amino acids contribute to the pH dependence of Bax conformation [63]. Thus, a sufficiently acidic intracellular pH also should alter Bax conformation in a manner similar to the conformational changes associated with alkalinization, increasing the insertion of Bax to the mitochondrial membrane, cytochrome c release, and caspase-mediated apoptosis.

Our light-activated intracellular acidosis produces significant cell death across multiple cell types in a similar pattern of death: (1) a very rapid onset of membrane blebbing; (2) Annexin V expression; and (3) a high percentage of cell death. While we have just begun to quantify intracellular mechanisms, each of these characteristics associated with light-activated intracellular acidosis are consistent with Bax-mediated apoptosis. As described above, we propose that the rapid creation of an acidic intracellular space causes a rapid translocation of Bax to the mitochondrial membrane and the subsequent release of cytochrome c. At this point, we cannot exclude the possibility that our light-activated intracellular acidosis technique is also initiating caspase-independent cell death.

14. Conclusion

There is a great, unmet need in the field of cancer treatment for those patients that cannot receive effective treatments or for patients who wish to keep invasive interventions at a minimum. Reducing tumor volume in cancer patients prior to surgical resection, *via* chemotherapeutics and radiation, represents a current standard of care that often results in less scarring and complications, and increases the likelihood of removing the tumor in its entirety, leaving clean margins. For example, reduction of tumor volume is especially important in breast cancer patients that wish to have breast conservation surgery and retention of breast function. Unfortunately, both chemotherapy and radiation present the patient with debilitating systemic side effects, such as hair loss, weakness, anorexia, gastrointestinal discomfort, and dysfunctional lymph drainage. In addition to the drawbacks of chemotherapies and radiation, comorbidity often excludes patients as candidates for chemotherapy and/or radiation prior to surgical resection. While surgical resection of a local tumor often has the potential to be "curative," surgery is often eliminated as a viable treatment due to patient co-morbidity. Furthermore, surgery may not be a viable treatment option if the tumor is located on or near vital structures or structures that if impaired, may have a significant impact on quality of life (*i.e.* colon resection). Like surgery, radiation therapy may offer many patients an effective cancer treatment, however radiation is often discontinued when patients reach the maximum radiation dosage. Radiation therapy may not be successful in all tumor types and has long term side effects such as radiation induced tissue damage and secondary malignancies.

Attempts to reduce intracellular pH within tumor cells has been fraught with disappointing results. Amiloride-based chemotherapeutic drugs block acid extrusion from cells, and while this chemotherapeutic approach has been successful in reducing pH within cancer cells, the target of these drugs are found in virtually every cell in the body, leading to devastating systemic side effects. The innovation of producing intracellular acidosis in cancer cells may be further developed as a valuable tool to reduce tumor volume of any tumor type in a precise manner while reducing the side-effect burden to the patient. Application of light-activated intracellular acidosis technology caused significant cell death in both non-cancerous and cancerous cell lines *in vitro*, and when applied in mice, resulted in significant reductions of triple negative breast cancer tumor growth with *no physical or behavioral side effects* to the animals. Light-activated acidosis produces a rapid and focal intracellular acidosis that cancer cells do not appear to be able to overcome. Evidence exists which supports that the acidosis causes a conformational change in the pro-apoptotic protein Bax, which initiates the intrinsic apoptotic cell death pathway, however, other pathways may be involved in the cell death associated with the decreases in pHi associated with this technology.

Light-activated intracellular acidosis *via* the uncaging of H^+ from NBA produces a rapid, precise, and significant reduction in pHi and concomitant significant cell death. Data indicate that light-activated intracellular acidosis does not appear to be cancer specific, as all cells, including non-cancerous cells, are subject to the profound dysfunction of pHi and cell death associated with this technology. The light-activation of NBA within triple negative breast cancer *in vivo* also demonstrates its potential as a viable therapeutic in humans. Based on the efficacy and proposed mechanism of action on inducing cancer cell death, light-activated intracellular acidosis may also be effective in causing apoptosis in multi-drug resistant cancers. Further development of this intracellular acidification technique may offer therapeutic value to both surgeons and patients, as the acidification treatment can reduce tumor volume without systemic side effects or unwanted damage to surrounding healthy tissue.

Conflict of interest

None.

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