COMPARISON OF THE TOXICITY OF NBQ 234 TOM AND NBQ 345 TOM ON COLO 205 CANCER CELLS AND ITS FLUORESCENT EMISSION UNDER HYPOXIC VERSUS AEROBIC ENVIRONMENTS

Luis M. Ortiz-Jordán, BS, MSEM[1], Christian Vélez-Gerena, MSEM[2], Noemi Soto-Nieves, Ph.D.[3], Osvaldo Cox-Rosario[4] & Beatriz Zayas-Rivera Ph.D.[4]

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Abstract: Hypoxic solid cancer (low oxygen environment) is associated with difficulty of diagnosis, and poor prognosis, and therefore the need for identification of new markers and indicators with limited secondary effect of such hypoxic tissues. The main purpose of this study was to determine the capacity of two novel compounds, NBQ 234, and NBQ 345 TOM as hypoxic fluorescent markers in colon (COLO 205) tumor cells in comparison with a currently applied marker, which is Pimonidazole. A protocol was developed by exposing COLO-205 human colorectal adenocarcinoma (ATCC CCL-222) under hypoxic and aerobic environments for 24 hours. For cytotoxicity determination, cells were treated at concentrations ranging from 15 to 900 μM for 24 hours. Once the NBQ 234 and NBQ 345 TOM GI50 was determined; cells were then incubated for 24 hours at aerobic or hypoxic conditions to determine fluorescent emission of cells treated with NBQ 234, NBQ 345 TOM or Pimonidazole (control) indicative of the formation of a fluorescent metabolite. After treatment cell hypoxia was first confirmed by measuring through flowcytometry, the expression of HIF1α, a hypoxia inducible factor. Once hypoxia in cells was confirmed, a correlation with the fluorescent emission of treated cells at low oxygen versus aerobic conditions was evaluated. Preliminary results show cell cytotoxicity at 25.29 μM for NBQ 234 TOM and 2.12 mM for NBQ TOM 345 on COLO 205 under hypoxic and aerobic conditions. Fluorescence emission results demonstrated significant ($p < 0.001$) intensity on hypoxic tumor cells in contrast to cells treated under aerobic environment. The correlation between the HIF1α of hypoxic cells and their fluorescence intensity demonstrated the advantages of the experimental compounds over the commercially available marker Pimonidazole. In addition, fluorescence microscopy images of colon cancer cells treated for 24 hours with NBQ'S at hypoxic and aerobic conditions confirmed the stronger fluorescence generation at hypoxic conditions. This research confirms the applicability of NBQ 234 TOM and NBQ 345 TOM as potential markers of metabolically active hypoxic cells.

Keywords: cancer, hypoxia, NBQ's, cytotoxicity, biomarker, public health

Resumen: Los tumores sólidos cancerosos en estado de hipoxia (baja concentración

[1] Scripps Research Institute, Florida. Email. lortiz@scripps.edu
[2] United Stated Department of Agriculture, APHIS, National Veterinary Services Laboratories, Iowa
[3] Natural Sciences Department, Universidad Sagrado Corazón, San Juan, Puerto Rico
[4] Science & Technology Division, Universidad Ana G. Méndez, Cupey campus, Puerto Rico

de oxígeno) se les asocia con dificultad de diagnóstico y una pobre prognosis, por lo cual hay una gran necesidad de identificar nuevos marcadores con limitados efectos secundarios. El objetivo principal de este estudio es determinar la capacidad de dos compuestos noveles, NBQ 234 y NBQ 345 TOM como biomarcadores fluorescentes en células de cáncer de colon (COLO 205) en comparación con el marcador Pimonidazole. Se confirmó la expresión del factor de transcripción asociado a hipoxia (HIF1α) en células expuestas como indicador del estado hipóxico. Una vez confirmado, se evaluó la correlación de la emisión de fluorescencia de las células tratadas con NBQ 234 and NBQ 345 TOM en condiciones aeróbicas vs las hipóxicas. La aplicabilidad de los compuestos experimentales como marcadores de hipoxia fue determinada implementando un protocolo donde células de cancer colorrectal humana (COLO-205; ATCC CCL-222) fueron tratadas a condiciones aeróbicas e hipóxicas por 24 horas. Para determinar la citotoxicidad (GI50), las células se trataron a concentraciones de los NBQ's de 15 a 900 μM por 24 horas. Una vez se determinó la citotoxicidad de NBQ 234 and NBQ 345 TOM, las células fueron incubadas por 24 horas en condiciones aeróbicas o hipóxicas para determinar la emisión de la fluorescencia de las células tratadas con los NBQ's y Pimonidazole. La fluorescencia fue medida utilizando un fluorimetro y la expresión de HIF1α se midió mediante citometría de flujo. Los resultados demuestran citotoxicidad a 25.29 μM para NBQ 234 TOM y 2.12 mM para NBQ TOM 345 en COLO 205 bajo condiciones aeróbicas e hipóxicas. Los resultados de emisión de fluorescencia demuestran la formación significativa (p<0.001) de los metabolitos fluorescentes de los NBQ's bajo condiciones hipóxicas en comparación con las condiciones aeróbicas. En comparación con Pimonidazole, los NBQ's demuestran una intensidad de fluorescencia significativamente más alta. Microscopía de fluorescencia confirmó una generación de fluorescencia de mayor intensidad en células bajo condiciones hipóxicas en comparación con las células en condiciones aeróbicas. Estos resultados demuestran las ventajas y aplicabilidad de los NBQ 234 y NBQ 345 sobre Pimonidazole como uso de marcadores de células en hipoxia metabólicamente activas.

Palabras claves: cáncer, hipoxia, NBQ's, citotoxicidad, biomarcadores, salud pública

Introduction

Cancer is the name given to a group of diseases characterize by abnormal cell growth and their capacity to invade other tissues. In terms of its incidence, it was estimated that by 2020 around 1,806,590 cancer cases were diagnosed and over 606,520 Americans died of cancer. Making cancer the second most common cause of death in the United States (Siegel, Miller, & Jemal, 2020). Cancer forms solid tumors and non-solid tumors, which are classified by the types of cells that compose them, the most common solid tumors being sarcomas and carcinomas. Three of the most common types of cancer for men are: prostate (191,930 cases), lung (116,300 cases) and colon (78,300 cases); while the three most common cases for women are: breast (276,480 cases), lung (112,520 cases) and colon (69,650 cases (Siegel, Miller, & Jemal, 2020).

The first study done related to hypoxia was performed by Thomlinson & Gray (1955). The researchers performed various incisions made in tumors that demonstrated a lack of capillaries. It was also observed that these cells made a type of encapsulated tumors, where the cells did not demonstrate any proliferative activity. Once these cells were exposed to hypoxic conditions, they transformed into cells that were able to proliferate, which meant that cells could defend themselves from lack of nutrients by remaining under a dormant state for a sometime without loss of proliferation (Thomlinson & Gray 1955). It was also observed that these cells under a dormant state were resistant to radiotherapy. Tumor cells under in vitro and in vivo conditions have showed a need of oxygen for successful radiotherapy. Mice were inoculated with Ehrlich ascites carcinoma and exposed to pure oxygen and irradiated with x-rays. The mice with high amounts of oxygen in their biological systems favored the therapy; hence, the tumors were destroyed, which demonstrates the importance of high oxygen levels in destroying cancerous cells (Gray et al.,1953).

Hypoxia can cause various changes in tumor microenvironments at physiological, chemical and genetic levels. When nutrient levels fall (such as oxygen) due to the distance from the tumor and the vessels, the cell cycle can arrest and cells go under a dormant state where little or no proliferation exists (Ljungkvist et al., 2002). This can pose a problem because most therapeutically drugs in the market are more effective against cells that are under proliferation than dormant cells. In consequence, these dormant or slow proliferating cells can become resistant to therapy (Hirst & Denekamp, 1979; Tannock, 1968; Tannock & Rotin, 1989).

Characteristics of the tumor's microenvironment can also affect the response to treatment. A drastic change in a tumor's pH can affect the effectivity of anticancer drugs. Most drugs are under uncharged states, which help them diffuse passively into a cell's membrane. Certain drugs, especially those with disassociation constants between 7.5 to 9.5 can be protonated, which in consequence will affect the cellular uptake (Gerweck, 2006; Tannock & Rotin, 1989). When cells enter a state of hypoxia, this can cause a chain activation of genes. These activated genes can help a tumor to produce new vasculature (angiogenesis) and lead to cell survival. The culprit that mediates the activation of these genes is Hypoxia Inducible Factor 1-alpha (HIF-1A), which is a transcription factor. This gene can help mediate the regulation of angiogenesis, oxygen homeostasis and the mTOR pathway. All the latter pathways when combined boost the cells' ability to survive, become resistant

to treatments, and to invade other healthy tissues (Gray, 2005; Pouysségur, Dayan, & Mazure, 2006).

With so many pathways occurring in cells, different genes and proteins are activated, which in turn can be used to identify certain characteristics of cancer cells. A biomarker is a measurable indicator that relates to a state or condition of a biological system. In the case of cancer, a biomarker can include a substance that is produced by cancer cells and can help identify a type of cancer, under what state it is, measure the progression of the condition and measure cancer's reaction to clinical treatments. Many types of biomarkers exist, some are unique to normal cells, others are produced by abnormal cells, and some are produced by both normal and abnormal cells. Many biomarkers are produced in high concentrations by cancer cells (Jemal et al., 2017). These markers can be found in blood, stool, tumor tissue and urine. There are drawbacks to the use of biomarkers in cancer, one of these limitations is due to low levels of the biomarkers in early stages of cancer and the level of expression of these markers can highly vary depending on the type of cancer that is involved (Goossens, Sun, & Hoshida, 2015).

Hypoxia Inducible Factor 1-alpha (HIF-1A) is a potential biomarker that can be used to identify hypoxia in cancerous tumors. As mentioned before, an ideal biomarker should indicate cancer at an early, intermediate, or advanced stage. In the case of HIF-1A, it is not selective to identify a stage or type of cancer, which impedes its use as a biomarker via anatomical characterization. To help solve this problem, it is important to use endogenous or exogenous biomarkers. Endogenous biomarkers for hypoxia are genes or proteins that are expressed when a cell enters a state of hypoxia. Exogenous markers are compounds that are formed as a by-product when hypoxia is present. An example of an endogenous compound is 2-nitroimidazole also known as Pimonidazole. The compound can form adducts with macromolecules such as DNA in hypoxic conditions, while it is not permitted in tissues with high levels of oxygen. The compound can form adducts at pO2 levels below 10 mmHg and becomes extremely sensitive at extreme low levels of pO2 (Kizaka-Kondoh & Konse-Nagasawa, 2009; Le & Courter, 2008).

A new focus in research has turned to Hypoxic Activated Pro-Drugs (HAPs). These drugs have the capacity to activate and carry out their effects under hypoxic conditions. AQ4N is one of the most studied HAPs (Patterson et al., 2000). It was

tested under aerobic conditions against the National Cancer Institute panel of 60 cancer cell lines. Preliminary results demonstrated cytotoxicity at low concentration (EC_{50} < 100 μM) of AQ4N in various cell lines. Other experiments were done under hypoxic conditions, which showed promising results. The compound was highly cytotoxic, with concentrations observed in the nM range, it was also observed that this compound is not affected by low levels of pH (Patterson et al., 2000). Another highly studied compound is TH-302, in a research study this compound was used in combination with seven chemotherapeutic drugs (docetaxel, cisplatin, pemetrexed, irinotecan, doxorubicin, gemcitabine, and temozolomide) in six types of cancer cells (no small cell lung cancer (NSCLC), colon cancer, prostate cancer, fibrosarcoma, melanoma, and pancreatic cancer). Results showed an increase in anti-tumoral activity when TH-302 is used in combination with chemotherapeutics (Liu et al., 2012).

Tirapazamine is a highly studied anti-cancer drug. Its use as a possible anti-cancer agent in hypoxia, was first described by Zeman et al. (1986), which resulted in the compound being highly studied during the 90's. This compound passed Phase I and Phase II of clinical trials, and since 2000, has been undergoing Phase III clinical trials. But during the past years the compound has had its drawbacks in various studies that demonstrated the compound's ability to activate in aerobic conditions (Marcu & Olver, 2006). Many other drugs have the same situation, which highlights the necessity to design new compounds that can have the ability to selectively activate via a reduction in hypoxic conditions and generate metabolites useful for the detection of hypoxia in cancer tumors (Reddy & Williamson, 2009).

In regard to other members of the NBQ family, Cox et al. (1982) synthesized NBQ 2 which demonstrated potential antitumor activity. In another study made by Colón et al. (2008), the team tested NBQ 95 in cancer cells. The objective was to compare the capacity of the compound to bind itself to DNA in the presence of NBQ 38 under hypoxic conditions. Results showed a major formation of adducts under hypoxic conditions which showed NBQ 38 potential with therapeutic application.

Cox et al. (2014) more recently created two compounds: NBQ 48 and ABQ 48. The goal was to measure both compounds' cytotoxicity on various cancer lines. The result was that ABQ 48 had a higher cytotoxicity level compared to NBQ

48. ABQ resulted to be more toxic for colon KM12, melanoma SK-MEL 5 and nervous system cancers.

In a recent study done by Zayas et al. (2020), the research team measured the fluorescent activity of NBQ 48 under hypoxic conditions in COLO-205 tumor cells. NBQ 48 demonstrated bio reductive activity via the formation of a fluorescent metabolite in COLO-205 tumor cells and demonstrating the capacity as hypoxic marker on 2&3D cultures. Here we report the activity as potential hypoxic markers of two novel NBQ compounds: NBQ 234 TOM and NBQ 345 TOM. The presented results report their cytotoxicity in cancer cells and their ability to create fluorescent metabolites under hypoxic conditions demonstrating their potential as hypoxia biomarkers.

Materials and methods

Experimental Compounds: Purified and characterized NBQ 234 TOM and NBQ 345 TOM was synthesized in house. Stock solutions were prepared at a concentration of 3 mM in sterile dH2O the day before the treatment. The stock solution was stored in a refrigerator at 2.5 °C until used for cell treatment.

Cell cultures: The colorectal adenocarcinoma COLO-205 cell line (ATCC CCL-222) from a 70-year-old male Caucasian was implemented. COLO-205 cancer cells were sub-cultured and kept at a density of 5.0×10^5 per 3.5 mL of RPMI-1640 cell media with 10% Fetal Bovine Serum (FBS). All these products were obtained from the American Type Culture Collection. Cell viability prior and during NBQ's treatment was determined with an automated Countess Cell Counter (Invitrogen) using the trypan blue exclusion method.

Hypoxia cell culture validation: Hypoxia chamber (Biospherix) exterior and interior were disinfected before and after every use. Chamber interior was equipped with a Dry Anaerobic Indicator Strip and a petri dish with 10 mL of distilled water. Live cell cultures were incubated at 37 °C for 24 hours. The chamber was connected to a nitrogen tank via plastic tubing and the 99.9% purity nitrogen tank was opened at 25 L/min of nitrogen flowed in the interior of the tank until oxygen levels reached 11%.

GI_{50} determination: COLO 205 tumor cells were seeded in 96 well plates at 50,000 cells/well (100 µL total volume) and treated with NBQ 234 TOM and NBQ 345 TOM for 24 hours in experimental doses ranging from 15 to 100 µM for NBQ 234 TOM and 80 to 900 µM for NBQ 345 TOM. Cell viability

and GI_{50} determination implemented by the presto blue (PB) reagent according to the manufacturer's protocol (Invitrogen). Cell were analyzed using fluorostar Optima (BMG) fluorescence reader. Changes in cell viability were detected with fluorescence determination at excitation 570 nm; and emission 610 nm. A dose–response curve was generated and plotted using a non-linear regression analysis for the GI_{50} determination of treated cells.

Fluorescence intensity: Fluorescence Intensity was determined with the use of a fluorimeter and fluorescence microscopy. All cell lines were cultured at a density of 5 x 10^5 cells in 12.5 cm2 flasks and exposed to 25 μL of NBQ 234 TOM, NBQ 345 TOM or Pimonidazole (positive control) for 24 hours. Two groups of cells were incubated: Group A under aerobic conditions and group B under hypoxic conditions. Flasks were incubated for 24 hours in a cell incubator at 37°C with 5% CO_2 in the case of cells exposed to aerobic condition; cells exposed to hypoxic conditions were incubated for 24 hours in a hypoxia chamber at 37 °C. After incubation, cells were detached from the flask and washed with PBS. Cells were segregated in a 96-well plate and each well completed with media to 100 μL with phosphate saline buffer. Fluorescence reading was done with OPTIMA BMG fluorimeter. This equipment was programmed with an excitation wave range of 355-612 nm.

Fluorescence imaging: Ten microliters of each condition from Fluorescence intensity experiment were added to a microscope slide to obtain fluorescence images using an Olympus FSX 100 Bio Imaging System of cells that were treated with compounds under aerobic and hypoxic conditions.

Expression of HIF α: Anti-HIF α with PBS (less than 20μL on PBS) was prepared. Five microliters of Zenon Alexa Flour 647 rabbit labeling agent were mixed with anti-HIF α solution and incubated for 5 minutes at room temperature. Once the solution was prepared, 1.0x 10^6 cells from each condition of the fluorescence intensity experiment was fixed in a four percent formaldehyde / PBS solution and incubated for 15 minutes at room temperature. Once incubation was complete, cells were centrifuged at 2500 RPM and supernatant is discarded. Cells are permeabilized with one percent of PBS-Tween or PBS-Triton 0.1 % for five minutes at room temperature. Then cells are centrifuged at 2500 PBS and washed with PBS. Cells were centrifuged again at 2500 RPM for two minutes; the supernatant was discarded, and eleven microliters of anti HIF α solution was added. The mixture was incubated for 30 minutes at room temperature and protected from light. Once the last incubation was complete, cells were centrifuged at 2500 RPM for a minute, supernatant is discarded, and cells are re-suspended in PBS. Analysis performed by Attune Flow Cytometer.

Statistical analysis: Statistical analyses were performed with Graphpad statistical software. Data was expressed as mean ± standard deviation. In order to measure the significant difference in fluorescence between the two environments, a one-way ANOVA with Dunnett's multiple comparison test was performed to prove significance, where $p<0.05$ is considered significant.

Results

GI_{50} determination. Determination of the NBQ 234 TOM and NBQ 345 TOM GI_{50} concentration on cells treated at aerobic environment at 15 to 100 μM and 80 to 900 μM respectively, demonstrated that under aerobic conditions both compounds have cytotoxicity effects at low concentrations on COLO 205 cells after 24 hours treatment. NBQ 234 TOM induced GI_{50} in COLO 205 cells at a concentration of 25.26 μM and NBQ 345 TOM at 2.12 mM. In terms of cytotoxicity, NBQ TOM 345 is less toxic then NBQ TOM 234.

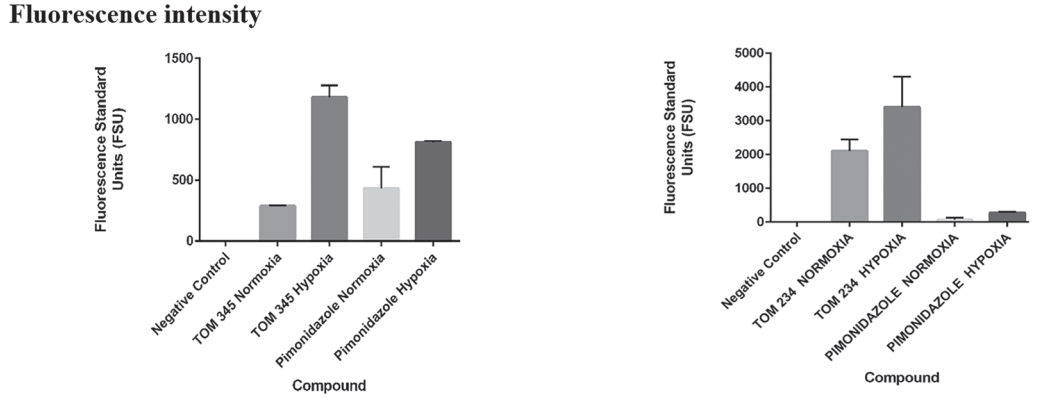
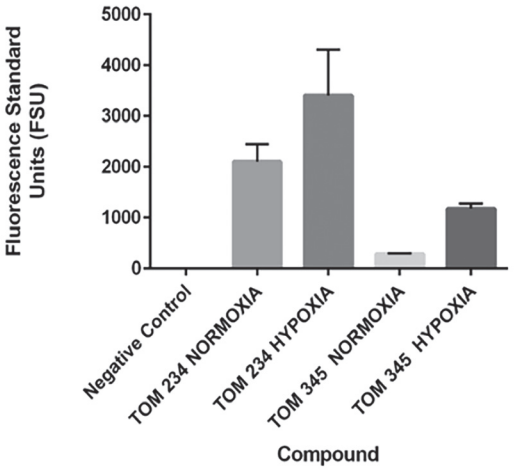


Figure 1 - Fluorescence emissions of COLO 205 cells exposed to (A) NBQ 234 TOM versus Pimonidazole and (B) NBQ 345 TOM versus Pimonidazole for 24 hours under normoxia (20% Oxygen in the air) versus hypoxia: A one-way ANOVA with Dunnett's multiple comparison test was performed to determine significance. Significant differences were observed in the fluorescence emission (adjusted p-value < 0.001) of cell cultures treated with NBQ 234 TOM and NBQ 345 TOM under normoxia versus hypoxia cell cultures.

Quantitative fluorescence intensity results were obtained for COLO 205 cells exposed to 25 μM of NBQ 234 TOM, NBQ 345 TOM and Pimonidazole under aerobic and hypoxic conditions with the use of an OPTIMA fluorimeter. Statistical analysis performed with one-way ANOVA and Dunnett's multiple comparisons test, where the standard is of $p<0.05$, but results indicated < 0.0001, which was significant. Due to cells having a natural level of fluorescence, all data was normalized to account for baseline fluorescence. After 24 hours, results demonstrated that both

NBQ's had a higher mean fluorescent intensity under hypoxic conditions Figure 1A and Figure 1B. NBQ 234 TOM had a mean of 8,526.7 fluorescence standard unit (FSU) and 12,522 for aerobic and hypoxic conditions respectively with a significant mean difference of 3995.3 FSU (Figure 1A). Thus, there is a significant difference between the mean FSU of NBQ 234 and NBQ 345 TOM. The mean difference in FSU between normoxic and hypoxic conditions is important to determine if the NBQ's are: truly going through a reduction in hypoxic conditions; and for future use of the compounds as biomarkers for hypoxia detection with equipment that use fluorescence as a method to identify hypoxia. NBQ 345 TOM presented a mean of 5,458.1 FSU of in aerobic conditions and 6,429.3 under hypoxia with a mean difference of 971.2 FSU which is significantly different Figure 1B. It is noteworthy to mention that when comparing mean intensities of both compounds under aerobic and hypoxic conditions, NBQ 234 TOM has a higher mean FSU in both conditions and a greater difference of these mean intensities compared to those of NBQ 345 TOM Figure 2.

Figure 2 - Fluorescence emissions of COLO 205 cells exposed to NBQ 234 TOM



versus NBQ 345 TOM for 24 hours under normoxia versus hypoxia: A one-way ANOVA with Dunnett's multiple comparison test was performed to determine significance. Significant mean fluorescence difference was observed in the fluorescence emission (adjusted p-value < 0.001) of cell cultures treated with NBQ 234 TOM when compared to NBQ 345 TOM under normoxia versus hypoxia cell cultures.

Both NBQ's have a higher FSU values compared to pimonidazole. Pimonidazole was used as a positive control due to it use as an indicator of hypoxia (Kizaka-Kondo

& Konse-Nagasawa, 2009). This study demonstrated the advantage of using NBQ's over pimonidazole to study hypoxia. Results demonstrated higher mean fluorescence intensity under hypoxic conditions among fluorescent intensities between aerobic and hypoxic conditions and a faster activation time then pimonidazole, which needs more than 24 hours to reach peak fluorescent intensity. The probable explanation for this outcome is due the position of a trimethoxy in NBQ 234 TOM which can cause a certain electronic demand that can affect the interaction with a reductase in the cell. The observed shift of fluorescent intensity under hypoxic conditions is mediated by enzymes of COLO 205 that interact with NBQ's and Pimonidazole. It is evidenced that some enzymes from the oxidoreductase family are known to mediate the activation of drugs under hypoxic conditions (Travica et al., 2013). Also, it's been demonstrated that advanced cancer tumors express high quantities of oxidoreductive CYPZW1 enzyme (Stenstedt et al., 2012). It is known that two enzymes involved in the reduction of NBQ's are DT diasphorase and Cytochrome P450 reductase (Velez, 2015, unpublished data).

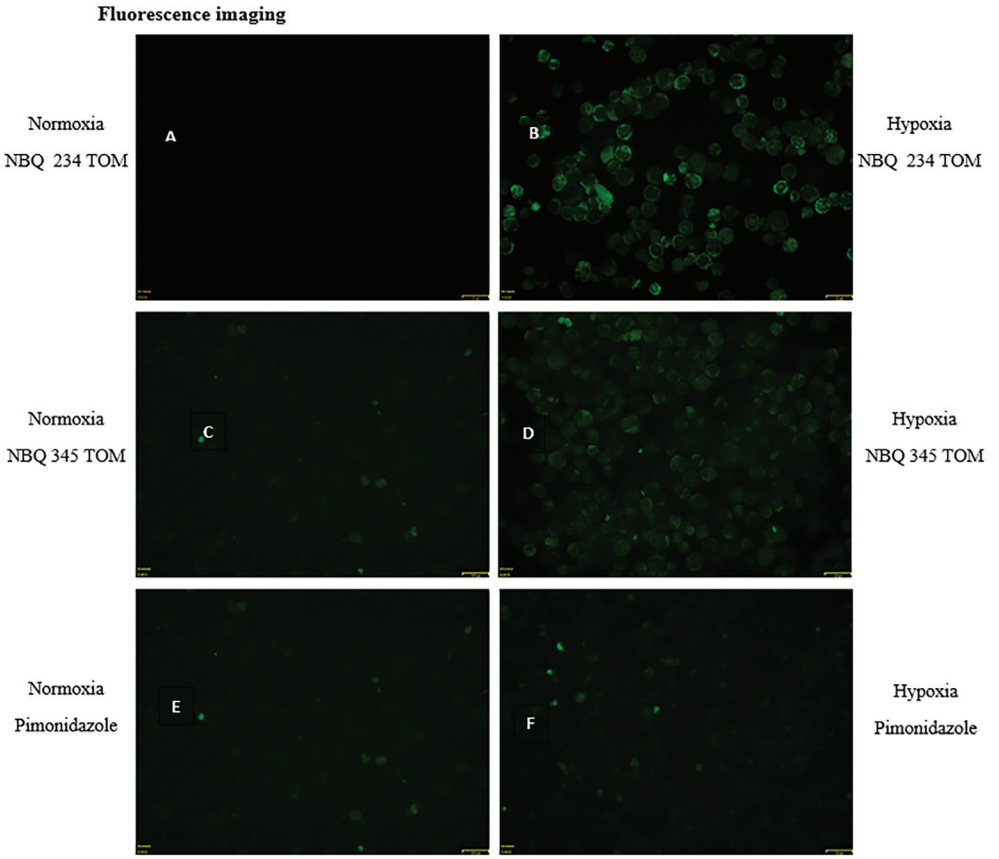


Figure 3 - Fluorescent Microscopy analysis of COLO 205 cancer cells treated with NBQ 234 TOM, NBQ 345 TOM and Pimonidazole after 24 hours emerging from bio-reduction of NBQ's and Pimonidazole into fluorescent metabolites:

(A) Cells with NBQ 234 under normoxia; (B) Cells with NBQ 234 under hypoxia; (C) Cells with NBQ 345 under normoxia; (D) Cells with NBQ 345 under hypoxia; (E) Cells with Pimonidazole under normoxia; (F) Cells with Pimonidazole under normoxia. The green fluorescent cells on B, D and F indicate the hypoxic formed metabolite. Minimum auto fluorescence (background) of COLO 205 cells are observed in images of normoxia samples A, C and E.

Fluorescent microscopy images were obtained for qualitative purposes of fluorescence intensity of NBQ 234 TOM, NBQ 345 TOM and Pimonidazole Figure 3. Each image was normalized. Images were generated using the 20X objective for a total magnification of 200X for an exposure time between one-to-one point two to 1.2 seconds. Very little fluorescence was observed for cells in aerobic conditions exposed to NBQ's and Pimonidazole under aerobic conditions. Cells with compounds under 24-hour hypoxic conditions demonstrated a dramatic shift in fluorescent intensity compared to those under aerobic conditions. This confirms data obtained from the previous fluorescent intensity results. Pimonidazole showed less fluorescent intensity shift compared to NBQ's under aerobic and hypoxic conditions, while NBQ 234 TOM demonstrates to have the highest intensity shift among the three tested compounds. Fluorescent images contribute by giving visual data of the compound's behavior under hypoxic conditions, where not only the color, but the intensity of the fluorescence can be observed. Fluorescent microscopy is a very powerful and useful tool for obtaining images of cancer cells and tumors under hypoxic conditions. In a study done by Cui et al. (2011), reactive fluorescent compounds were synthesized. P-nitro benzyl was introduced in the RHP reactive which was exposed to hypoxic conditions in presence of a nitroreductor (NTR) enzyme, which permitted the liberation of an amino group. A549 cells were exposed to the RHP reactive under aerobic and hypoxic conditions. The research group observed a higher intensity level in cells exposed to hypoxic conditions with RHP. The fluorescence was determined by a color change and intensity from blue to green due to the NTR reductive enzymes. These images were obtained with the use of fluorescent microscopy.

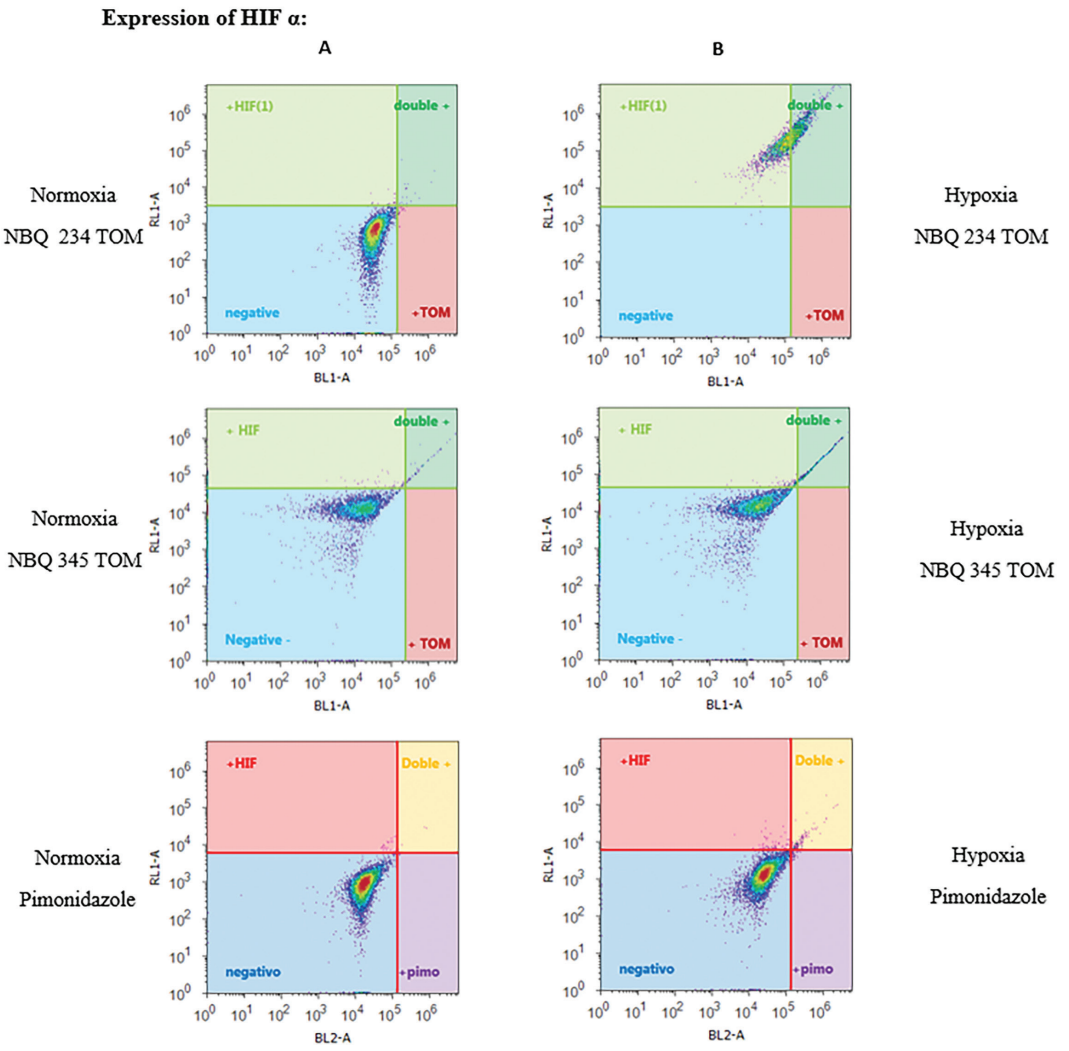


Figure 4 - HIF-1α expression analysis by flow cytometry on COLO 205 cancer cells under normoxia versus hypoxia exposed to NBQ 234 TOM, NBQ 345 TOM and Pimonidazole after 24 hours. Panel A represents cells that are under normoxia, while panel B are cells under hypoxia. The upper left quadrant represents a positive presence of HIF-1-α, the bottom right expresses a positive presence of fluorescent metabolites of NBQ's and Pimonidazole, upper right quadrant represents positive presence of both HIF-1-α, fluorescent metabolites of NBQ's and Pimonidazole. Bottom left quadrant is negative for the presence of HIF-1-α, fluorescent metabolites of NBQ'S and Pimonidazole.

Flow cytometry was used to confirm the expression of Hypoxia-inducible factor 1-alpha (HIF-1-α) in COLO 205 cells under hypoxia. The presence of HIF-1-α would confirm that most of the reduction of NBQ's and pimonidazole is caused by reductases in hypoxic conditions and not by other potential cellular processes. COLO 205 cells were marked with anti- HIF-1-α antibodies to confirm HIF expression in cells. Flow cytometry graphs demonstrated a shift in cell population exposed to the compounds from aerobic to hypoxic conditions (Figure 4). It must be mentioned that there will always be a very small number of cells that are expressing HIF under aerobic conditions due to possible cell stress (Figure 4A). Cell populations under aerobic conditions appear in the lower left quadrant of the graphs while cells positive for HIF expression and compound reduction appear on the upper right quadrant. In a study done by Papandreou, Cairns et al. (2006), the team used flow cytometry to quantify the fluorescence intensity of the expression of HIF with the used of antibodies. When HIF is activated, it upregulates gene expression of CYP2C8, CYP2C9 among other genes and in consequence, leads to the larger production of cell reductase in hypoxia (du Souich & Fradette, 2011).

Discussion

Cancer is the second cause of death in the United States (Siegel, Miller, & Jemal, 2020). Hypoxia, which is a lack or low levels of oxygen, is associated to solid tumors and low prognosis of cancer patients. There is a great need for biomarkers that can selectively detect hypoxia in vivo, in an efficient way where the biomarker is stable under diverse conditions and non-toxic. To create a relevant hypoxic condition in cancer cells, a hypoxic environment was implemented to maintain a low level of oxygen to simulate oxygen-deprived cancer cells. The results of this research demonstrated: both compounds have cytotoxicity at low concentrations and selected reduction of NBQ 234 TOM and NBQ 345 TOM, 345 TOM under hypoxic conditions after 24 hours.

Both compounds have a higher mean intensity than pimonidazole under hypoxic conditions; of the two NBQ's tested, NBB 234 TOM is the compound with the highest mean fluorescence intensity. These results are also confirmed with data presented by fluorescent microscopy. Flow cytometry confirms the expression of HIF which assures the reduction of NBQ's and pimonidazole by reductases under hypoxic conditions. Fluorescence occurs as a product of reduction under hypoxic conditions, which is confirmed by studies done by Zayas et al. (2007) which demonstrated the reduction of NBQ's under hypoxic environments.

Pimonidazole was used as a positive control due to it use as an indicator of hypoxia. This study demonstrated the advantage of using NBQ's over pimonidazole

to study hypoxia. In general, results demonstrated a higher mean fluorescence intensity under hypoxic conditions and a statistically difference among fluorescent intensities between aerobic and hypoxic conditions in NBQ_234 TOM. It is also important to mention that there is a faster activation time for NBQ_234 TOM to produce fluorescence than pimonidazole, which needs more than 24 hours to reach peak fluorescent intensity due to activation time and dose dependency, such as described by Durand & Raleigh (1998).

The authors recognized that some areas of opportunity for this study are using include two or more cell lines to compare results, metabolite characterization and exposition to hypoxia to more than 24 hours. Since cell lines can present different metabolism depending on the type of tissue it represents, this could affect the pathways which activate HIF under hypoxic conditions and in consequence can affect the types and amount of reductase that are produced. du Souich & Fradette (2011) explained in their review how HIF pathways can change depending on the metabolism of different cell lines. Metabolite characterization is of extreme importance since results presented by Gisela et al. (2008) demonstrate the formation of DNA adducts when NBQ 98 is reduced. Studying the exact metabolite produced can help explain the compounds toxicity. Activation of hypoxia can vary in time depending of cell metabolism, but usually commences around 24 hours. Data from an unpublished study done by Zayas et al. (2020) demonstrated that peak intensity of NBQ_48 occurred after 48 hours.

Conclusion

This research project demonstrated the applicability of NBQ_234 TOM and NBQ_345 TOM to identify cancer cells under hypoxic conditions. The shift in fluorescent emission levels and fluorescent microscopy images evidence the necessity of hypoxic environments. Cell cytometry evidenced the expression of HIF-1α, which confirms that fluorescent metabolites were formed by reductase enzymes associated to hypoxia. To further confirm the use of NBQ's to detect hypoxia in cancer, more studies must be performed. The studies that can be performed include: NBQ's testing on other cell lines to determine dose/response results for different cell lines, perform future studies on time intervals of more than 24 hours to study the compounds fluorescence intensity behavior, test NBQ's in 3D cell models and determine if the nitro reduction of the NBQ's occur in the cell cytoplasm or nucleus
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