Part I. Development of a Model System for Studying Nitric Oxide in Tumors: High Nitric Oxide-Adapted Head and Neck Squamous Cell Carcinoma Cell Lines

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Running title: Nitric Oxide-Adapted HNSCC Cell Lines

Abstract

The free radical nitric oxide (NO) is over-expressed in many tumors, including head and neck squamous cell carcinomas (HNSCC). However, the role NO plays in tumor pathophysiology is still not well understood. We herein report the development of an *in vitro* model system which can be used to probe the role of NO in the carcinogenesis of HNSCC. Five HNSCC cell lines were adapted to a high NO (HNO) environment by gradually introducing increasing concentrations of DETA-NONOate, a nitrogen-based NO donor, to cell media. The adaptation process was carried out until a sufficiently high enough donor concentration was reached which enabled the HNO cells to survive and grow, but which was lethal to the original, unadapted ("parent") cells. The adapted HNO cells exhibited analogous morphology to the parent cells, but grew better than their corresponding parent cells in normal media, on soft agar, and in the presence of hydrogen peroxide, an oxygen-based free radical donor. These results indicate the HNO cell lines are unique and possess biologically different properties than the parent cell lines from which they originated. The HNO/parent cell lines developed herein may be used as a model system to better understand the role NO plays in HNSCC carcinogenesis.

Keywords

Nitric oxide, nitrogen reactive species, squamous cell carcinoma, oral cancer, nitric oxide synthase (NOS), free radical exposure, head and neck cancer

Introduction

According to American Cancer Society estimates, the United States reported 48,010 new cases of head and neck cancer in 2009, and an estimated 11,260 deaths from this disease. Tumors arising from the larynx, tongue, mouth, and pharynx account for over 96% of these cases [1], and greater than 90% of these cancers are squamous cell carcinomas [2].

Cigarette smoking—the leading risk factor in head and neck cancers—has been shown to produce free radicals such as reactive oxygen species and nitrogen-based free radicals, including nitric oxide (NO) [3-5]. These molecules may play an important role in tumor development and propagation, as increased concentrations of these free radicals can cause DNA damage such as point mutations and chromosomal breakage. This DNA damage results in mutations to proto-oncogenes and tumor-suppressor genes, ultimately leading to malignant transformation and cancer development [6-8].

NO is a nitrogen-based free radical that is synthesized by a family of enzymes known as nitric oxide synthase (NOS). It plays an essential role in many normal physiological processes such as vasodilation, central and peripheral neurotransmission, hormone secretion, inhibition of platelet aggregation, and mediation of immune response. It has also been implicated in a number of pathophysiological processes such as inflammation, septic shock, hypertension, atherosclerosis, and carcinogenesis [9-11].

While NO is known to be present in normal tissue, and necessary for proper cellular function, its role in tumor development and progression is less clear [12]. Increased serum levels of NO and/or increased expression of NOS has been found in a variety of tumors from different sites [13-18], including head and neck squamous cell

carcinomas (HNSCC) [19-22]. Further studies have suggested chronic overproduction of NO leads to a variety of pathologic disorders, including carcinogenesis [23-25].

Despite the recent advancements in understanding the role of NO in cancer, several clinically relevant questions still remain unanswered. For example, it is still unknown how the NO microenvironment of a tumor affects its progression, and how NO expression is influenced by the treatment of tumors. Similarly, further research needs to be conducted to determine if the expression levels of NO should dictate the use of a particular therapeutic approach. Given the difficulty in attempting to answer these questions directly in humans, it would be extremely valuable if an *in vitro* cell line model system could be established that mimicked the varying degrees of nitrosative stress observed in clinically presenting tumors. Such a system would allow a spectrum of NO expression to be studied—from very low to very high levels of expression—in order to better understand the role NO plays in tumor progression.

To this end, we have recently developed such a cell line system in human and mouse lung adenocarcinoma cell lines [26], as well as in human breast adenocarcinoma cell lines [27]. Over the period of several months, these tumor cells were adapted to increasing concentrations of a nitric oxide donor, ultimately reaching extremely high nitric oxide (HNO) levels. The HNO-adapted cells were able to grow and eventually thrive in a comparatively harsh free radical environment—an environment which was found to be lethal to the original, unadapted ("parent") cell lines. Herein we extend the universality of the cell line model to study five HNSCC cell lines.

Materials and Methodology

Cell Culture and Cell Lines

All media and supplements were purchased from Invitrogen Corporation (Carlsbad, CA, USA) except where noted. Five human head and neck squamous cell carcinoma (HNSCC) cell lines (three originating from the tongue: SCC016, SCC040, SCC056; one from the floor of mouth: SCC114; and one from the alveolar ridge: SCC116) [28] were grown in MEM media. Cell lines were generously obtained from Dr. Susanne Gollin (University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA). All media was supplemented with 10% fetal calf serum (FCS) inactivated at 56ºC for 30 minutes, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 2.5 μg/mL Amphotericin B solution. The MEM media was additionally supplemented with 100 mM MEM nonessential amino acids and 1 mM sodium pyruvate (Mediatech, Inc., Manassas, VA, USA). Cell lines were grown in a humidified incubator at 37 ºC and 5% CO_2.

The nitric oxide donor (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) (Sigma-Aldrich Corp., St. Louis, MO, USA) was utilized in this study. DETA-NONOate stock solutions were prepared in sterilized water and sterile filtered (0.22 μm). Solutions were aliquoted and stored at -20 °C until ready for use.

Determination of Adaptation End-Point

Before the adaptation process was initiated (discussed in the next section), the concentration of NO which was lethal to the cells was determined. Cells from each of the

five cell lines were seeded (100 μL) into 96-well microtiter plates and grown for 24 hours, to ~70% confluency. The media was then removed, and the cells were treated with 100 μL media containing varying concentrations (0-600 μM) of DETA-NONOate. Following an additional 72 hour incubation period, cell proliferation/viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media was removed from each well, and 100 μL of 2 mg/mL MTT (Sigma-Aldrich Corp., St. Louis, MO, USA) in phosphate buffer saline (PBS) was added. Plates were then placed in an incubator (37ºC) for 5 hours, during which time purple formazan crystals formed upon the reaction of the MTT with mitochondrial dehydrogenases of viable cells. Following the incubation period, the MTT solution was removed, and 100 μL of Dimethyl Sulfoxide (DMSO) was added. The absorbance of the resulting purple solution in each well was read (540 nm) using a SpectraMax® Plus 384 spectrophotometer (Molecular Devices, Inc., Sunnyvale, CA, USA). Each experiment was independently conducted a minimum of three times, and a minimum of four replicate wells were tested for each cell line at each concentration. Data was normalized to the mean optical density of the untreated control cells.

Cell Adaptation Process

The five HNSCC cell lines were "adapted" to the nitric oxide donor DETA-NONOate. The adaptation process was initiated by passaging cells with trypsin-EDTA and transferring the cells to a new flask containing media supplemented with 50 μM DETA-NONOate. The DETA-NONOate solution was prepared immediately prior to the addition of cells into the growth media. Cells were then incubated at 37 ºC and 5% CO_2

until they reached ~90% confluency. At this time, the cells were again passaged, and the resulting cultures were grown in media treated with 75 μM DETA-NONOate. The NO donor concentration was increased at 25 μM increments in this fashion until a final concentration of 600 μM DETA-NONOate was reached. Aliquots of the adapted cells were periodically removed for cryostorage during the adaptation process.

In addition to the adapted cells, separate "parent" cells were maintained as controls. No nitric oxide donor was added to the parent cells, and these cells were grown under standard conditions.

The cells were replenished with media containing DETA-NONOate every 2-3 days, both during the adaptation process and the subsequent maintenance of the fully adapted cells. Media was also replenished every 2-3 days for the parent cells.

Cells were imaged at 100x magnification using an InfinityX-32C camera (Lumenera Corporation, Ottawa, Ontario, Canada) affixed to an American Optics BioStar microscope (Reichert, Inc., Depew, NY, USA).

Verification of Adaptation End-Point

Upon completion of the adaptation process, parent and HNO adapted cells were seeded (100 μL) into 96-well microtiter plates. (The adapted cells were seeded in media supplemented with 600 μM DETA-NONONate; parent cells were seeded in media lacking DETA-NONOate.) Cells were grown for 24 hours, to ~70% confluency. The media was then removed and replaced with media containing either 0 or 600 μM DETA-NONOate. Following an additional 72 hour incubation period, cell proliferation/viability was assessed using the MTT assay described above.

Normal Media Growth Assays

Parent and HNO adapted cells were seeded into 96-well microtiter plates (100 μL) such that a confluency of ~30% would be reached 24 hours after seeding. HNO adapted cells were grown in media supplemented with 600 μM DETA-NONOate. Parent cells were grown in media without DETA-NONOate. Cell viability was measured at 24, 48, and 72 hours after seeding using the MTT assay. At each time point, the media was removed, the MTT dye was added, and cell viability readings were obtained as described above. Each experiment was independently conducted a minimum of three times, and a minimum of four replicate wells were tested for each cell line at each time point. Data was normalized to the mean optical density value at the 24 hour time point, and values lying outside of two standard deviations were removed. Data was plotted as the mean normalized absorbance ± standard error.

Soft Agar Growth Assays

A soft agar mixture consisting of 50% melting point agarose (Sigma A-9539, Sigma-Aldrich Corp., St. Louis, MO, USA) and 50% 2x MEM supplemented with 20% fetal calf serum, 200 U/mL penicillin, 200 μg/mL streptomycin, 4 mM L-glutamine, and 5 μg/mL Amphotericin B solution was prepared. This mixture was incubated at 42 ^{o}C for at least 30 minutes, after which time the agarose mixture (100 μL) was loaded into a 96-well microtiter plate (100 μL). The agarose was then allowed to harden overnight at 4 °C, after which time parent and HNO adapted cells (100 μL) were added on top of the hardened agar. (HNO adapted cells were added in standard 1X growth media

supplemented with 600 μM DETA-NONOate; parent cells were added in standard 1X growth media lacking additional DETA-NONOate.) In order to allow the cells to attach to the soft agar, plates were incubated for 12 hours at 37 ^{o}C; after this time, the media above the agar was removed. The plates were incubated for another 24 or 72 hours, at which time MTT proliferation/viability assays were carried out. A 2 mg/mL MTT in PBS solution (100 μL) was added to each well (on top of the agar), and the plates were incubated at 37 ºC for 5 hours. During this time, purple formazan crystals developed. Following the incubation period, the MTT solution was removed, and the remaining crystals were dissolved in DMSO (100 μL), yielding a purple solution. The resulting supernatant in each well was then transferred to a new 96-well microtiter plate. This new plate was used to obtain the absorbance reading for each well as described above. Four replicate wells were tested for each cell line at each time point. Data was normalized to the mean optical density value at the 24 hour time point. Data was plotted as the mean normalized absorbance ± standard deviation.

H_2O_2 Exposure Growth Assays

Cells were exposed to varying concentrations of hydrogen peroxide to determine if the adapted cells were resistant to high free-radical environments generated by oxygen-based free radicals. Parent and adapted cells were seeded (100 μL) into 96-well microtiter plates in the appropriate media (parent cells in media without NO donor; HNO cells in media supplemented with 600 μM DETA-NONOate) and incubated overnight, reaching approximately 70% confluency by the following day. Cells were treated with varying concentrations (0-1.78 mM) of hydrogen peroxide (30% w/w solution, Sigma H1009,

Sigma-Aldrich Corp., St. Louis, MO, USA) and incubated at 37 ^{o}C for an additional 24 hours. MTT cell viability/proliferation assays were then performed, as described above. A minimum of four replicate wells were measured for each cell line at each concentration, and assays were repeated in triplicate. Data was normalized against the mean of the untreated control cells, and values lying outside of two standard deviations were removed. Data was plotted as the mean normalized absorbance ± standard error.

Data Analysis and Statistics

Two-tailed Student's t-tests were run using Microsoft Excel 2007 in order to determine statistical significance between the growth rates of parent and HNO cells treated under identical conditions. $P < 0.05$ was considered statistically significant.

Results

Cell Adaptation

Five head and neck squamous cell carcinoma cell lines (SCC016, SCC040, SCC056, SCC114, and SCC116) were selected for this study. Three of the cell lines—SCC016, SCC040, and SCC056—originated from the tongue. SCC114 originated from the floor of mouth and SCC116 from the alveolar ridge. As with the previous studies carried out in our laboratory involving the adaptation of human lung and breast cancer cell lines [26,27], each of the five HNSCC cell lines was adapted to the nitric oxide donor DETA-NONOate. This donor was chosen based on its long half-life (approximately 24 hours at 37 °C and pH 7.4), free radical mode of delivery, and rate of delivery (donation of two moles of NO per one mole of donor) [26,29].

Prior to initiating the adaptation process, the five cell lines were exposed to varying doses (0-600 μM) of DETA-NONOate for 72 hours to determine the minimum concentration of DETA-NONOate that was lethal to the cells. Each of the five cell lines exhibited similar results: cell death increased with increasing concentrations of NO donor [data not shown], and 600 μM was the minimum concentration studied that was found to be completely lethal to the cells. Therefore, 600 μM was chosen as the adaptation endpoint for each of the five HNSCC cell lines.

(We note that all concentrations referred to herein are the concentration of the NO donor *added* to the cells, and not the actual concentration of NO *produced by* the donor to which the cells are exposed. The latter will fluctuate based on the half-life of the donor, the age of the stock solution, and the incubation time used in the cell culture experiments. As such, expressing the concentration in terms of donor added enables for a more consistent measure in which to report the amount of NO applied to the cells.)

The adaptation process was carried out by gradually increasing the amount of DETA-NONOate added to the cell lines. Initially, cells were passaged with trypsin-EDTA and introduced into media containing 50 μM DETA-NONOate. Upon the next passaging event, an additional 25 μM DETA-NONOate was added (for a total of 75 μM DETA-NONOate). This process was continued for each cell line until the cells had been adapted to 600 μM DETA-NONOate. A separate set of the original (i.e., "parent") cells, in which NO donor was not added, were maintained throughout the entire adaptation process.

Exposure to new levels of NO donor (i.e., each 25 μM increase) typically resulted in an initially slower growth rate; however, all five of the cell lines studied herein

recovered and eventually grew robustly at all concentrations studied. Figure 1 shows the adaptation curves for four of the HNSCC cell lines adapted herein. (SCC114 had an identical growth curve to SCC040 and therefore is not shown in the figure.) Each data point on the growth curve represents the addition of fresh DETA-NONOate solution to the growth media, either as a passaging event or as replenishment of the donor. Little difference was observed in the adaptation times for the five cell lines: SCC016 reached 600 μM DETA-NONOate in approximately 85 days, SCC056 and SCC116 in 90 days, SCC040 and SCC114 in 95 days.

As mentioned above, initial reductions in cell growth were typically observed after the concentration of DETA-NONOate was increased. Extreme cell toxicity was observed only once—the initial attempt at introducing SCC116 to a concentration of 425 μM DETA-NONOate. In this case, the concentration of donor was reduced to 400 μM; within two days the cells had recovered and exhibited a normal growth rate. At this time the concentration was increased again to 425 μM, and the cells responded favorably to the increased concentration. The remainder of the adaptation proceeded as expected following the re-introduction of 425 μM donor. Reductions in donor concentration were not needed at any point during the adaptation of the other four cell lines studied.

Each of the HNSCC HNO cell lines exhibited similar morphology to the untreated parent cells throughout the entire duration of the adaptation. Figure 2 compares the morphology of the parent cells versus the HNO cells adapted to 600 μM DETA-NONOate.

Verification of Adaptation End-Point

Upon adapting the HNO cells to 600 μM DETA-NONOate, parent and HNO cells from each cell line were exposed to media containing either 0 or 600 μM DETA-NONOate concentrations for 72 hours [data not shown]. In all five cell lines, the parent cells exhibited consistent growth in media lacking the DETA-NONOate, but were completely killed in media containing 600 μM DETA-NONOate. In contrast, the HNO cells were able to grow robustly in media with or without the NO donor added.

Normal Media Growth Curves

Figure 3 shows the results of cell viability/proliferation of the parent cells in standard media and HNO cells in standard media supplemented with 600 μM DETA-NONOate. Each of the HNO cell lines was found to grow faster than the corresponding parent cell line.

To verify that the enhanced growth of the HNO cell line was inherent in the cell line created, and not attributed merely to the DETA-NONOate serving as a growth stimulant, the HNO cell lines were grown in media in which the NO donor had been removed. No significant difference was observed between the growth curves of HNO cells grown in media with DETA-NONOate versus HNO cells grown in media without donor [data not shown] after 72 hours, confirming the HNO cells grow more aggressively than their corresponding parent cells.

Soft Agar Growth Assays

The ability of tumors to grow in low nutrient growth media, such as growth on soft agar, is used to measure a tumor's aggressiveness. Figure 4 shows growth curves on soft agar. Four of the five HNO cell lines were found to grow faster than their corresponding parent cell lines on soft agar; only SCC114 exhibited similar growth rates between parent and HNO cells.

H_2O_2 Growth Assays

Parent and HNO cells were grown in the presence of hydrogen peroxide to test if the cells could survive a high free radical environment generated by an oxygen-based donor. Comparatively high concentrations of H_2O_2 (above 55 μM) killed both parent and HNO cells upon 24 hour exposure [data not shown]. However, at lower concentrations (0.4-14 μM), each of the five HNO cell lines exhibited a statistically significant ($P < 0.05$) higher cell viability than their corresponding parent cell line at one or more of the concentrations studied (Figure 5).

Discussion

The five parent cell lines chosen for the adaptation process are all squamous cell carcinomas in origin, and represent tumors arising from three different head and neck locations (tongue, floor of mouth, and alveolar ridge). All five parent cell lines were successfully adapted to high levels of the nitric oxide donor DETA-NONOate (Figure 1). As previously discussed in the adaptation of the human lung tumor [26] and breast tumor [26,27] cell lines, DETA-NONOate was chosen as the NO donor because of its high level

of free radical donation (two moles of NO per mole of DETA-NONOate) and relatively long half-life (approximately 24 hrs at 37°C and pH 7.4). In general, the head and neck tumor cells were able to easily withstand the increased 25 μM DETA-NONOate increments during the adaptation process. It was necessary to reduce the concentration due to significant toxicity in only one case (SCC116 at 425 μM), and in this case, the cells were able to recover quickly when the concentration was subsequently reduced.

As previously found for the adaptation of the lung and breast tumor cell lines [26,27], 600 μM was a sufficient adaptation endpoint for each of the five head and neck cell lines. The parent cells were unable to survive at this concentration, but the HNO cells grew robustly, without a change in morphology (Figure 2).

The differences in the behavior of tumors growing in low and high free radical environments are clearly evident in this study. The HNO cells were all found to grow more aggressively than the parent cells in standard media (Figure 3) and on soft agar (Figure 4). These findings are consistent with the previously observed growth curves for the adapted lung and breast tumor cell lines [26,27]. Furthermore, the ability of the HNO cell lines to grow in media lacking DETA-NONOate demonstrates that short-term growth (up to 72 hours) of the HNO cells is not affected when the NO source is removed. Collectively these results indicate significant biological changes took place during the adaptation process, even though the parent and HNO cells are morphologically similar.

While the nitrogen-based DETA-NONOate was chosen as the donor for the adaptation process, the HNO cells are also able to survive high levels of hydrogen peroxide (Figure 5), an oxygen-based free radical donor. This suggests the cells have protected themselves against free radicals in general, and not only against the nitrogen

free radicals produced by DETA-NONOate or simply the inherent toxicity of the DETA-NONOate donor.

Our previously reported adaptation of the lung and breast tumor cell lines suggested the adaptation process was universal across adenocarcinomas [26,27], regardless of the origin of the tumor. Herein we have successfully utilized the same adaptation process for a series of squamous cell lines, suggesting the universality of this adaptation extends not only across like tumors arising from different sites, but also different tumor *types*. Furthermore, our adaptation process exposes the cells to periodic introduction of NO donor and subsequent decay. Given that free radicals are generated during radiation treatment, we believe our adaptation process mimics the long-term, cyclical free radical exposure many cancer patients receive during current radiation treatment protocols and suggests the need for hyperfractionation beyond what is currently being used.

The *in vitro* model system created herein can be used to study two distinct types of human head and neck tumors: those with slower propagation rates, growing in low-NO environments (represented by the parent cell lines) and those with faster propagation rates, which can grow aggressively in, and are resistant to, high NO environments (represented by the HNO cell lines). As evident from the results of the cell lines in this study, long-term exposure to NO can transform slower growing, less resistant tumor cells into faster growing, more resistant cells. However, the mechanism behind this transformation has not yet been determined. Understanding this parent-to-HNO transformation will be instrumental in determining the key factors that result in clinically aggressive tumors.

In summary, this study, along with the previously reported work [26,27], confirms that both adenocarcinoma and squamous cell carcinoma cells can adapt to high concentrations of NO. These adapted cells exhibit biologically different properties from the parent cells, despite the identical morphologies found between the parent and HNO cells. Long-term exposure to increasing levels of NO results in fast-growing, resistant cells that mimic clinically aggressive tumors. While it is currently unknown why morphologically similar tumors vary in their aggressiveness, the model system developed herein could help to understand this clinical phenomenon by clarifying the parent-to-HNO transformation process and identifying key biological differences between the parent and HNO cells. This new model system may also allow us to determine the mechanism which drives the *in vivo* progression from slower growing, NOS-expressing cells to faster growing, more aggressive cells. Furthermore, the parent/HNO model system could be used to predict therapeutic response to various treatment regiments, as well as potentially leading to new treatments designed to target the tumor microenvironment. To this end, the initial molecular and cellular characterization of the three parent/HNO human tongue squamous cell carcinoma cell line pairs developed herein (SCC016, SCC040, SCC056) are discussed in an accompanying manuscript [30].

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Figure Legends

Figure 1. Adaptation curves of the head and neck cancer cell lines (Top: SCC016, SCC040; Bottom: SCC056, SCC116). SCC114 had an identical adaptation curve to SCC040, and is therefore not shown. Each data point represents the addition of fresh DETA-NONOate, either as a passaging event or a replenishment of media

Figure 2. Images depicting cellular morphology of parent (left) and HNO (right) head and neck cell lines (Top-to-bottom: SCC016, SCC040, SCC056, SCC114, SCC116). Images collected at 100x magnification

Figure 3. MTT viability/proliferation curves of parent and HNO head and neck tumor cells in standard growth media. The media of the HNO cells was supplemented with 600 μM DETA-NONOate. Data is shown as the mean normalized absorbance ± standard error ($n = 3$; *P < 0.05 versus parent cells, **P < 0.01 versus parent cells)

Figure 4. MTT viability/proliferation curves of parent and HNO head and neck tumor cells grown on soft agar. The media of the HNO cells was supplemented with 600 μM DETA-NONOate. Data is shown as the mean normalized absorbance ± standard deviation ($n = 1$)

Figure 5. MTT viability/proliferation curves of parent and HNO head and neck tumor cells exposed to varying concentrations of hydrogen peroxide for 24 hours. The media of the HNO cells was supplemented with 600 μM DETA-NONOate. Data is shown as the mean normalized absorbance ± standard error ($n = 3$; *P < 0.05 versus parent cells, **P < 0.01 versus parent cells)

Figure 1

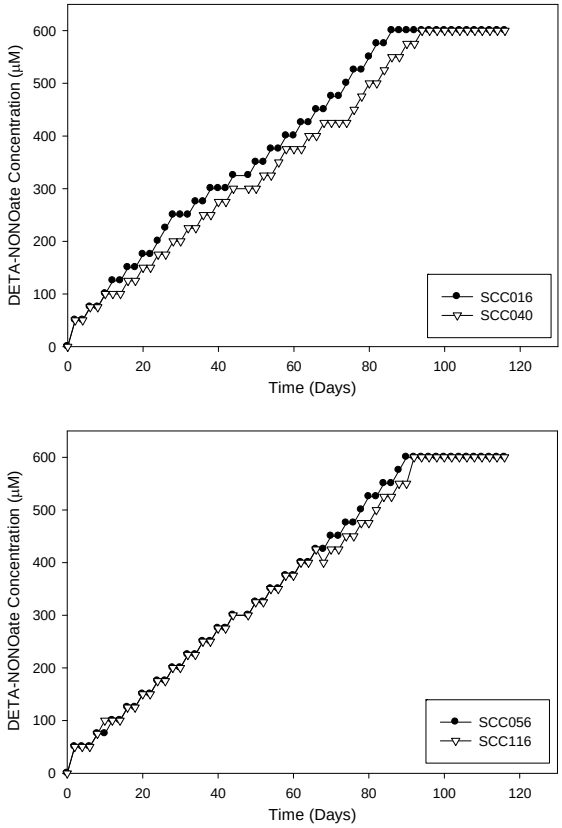

DETA-NONOate Concentration (μM)
Time (Days)
SCC016
SCC040
SCC056
SCC116

Figure 2

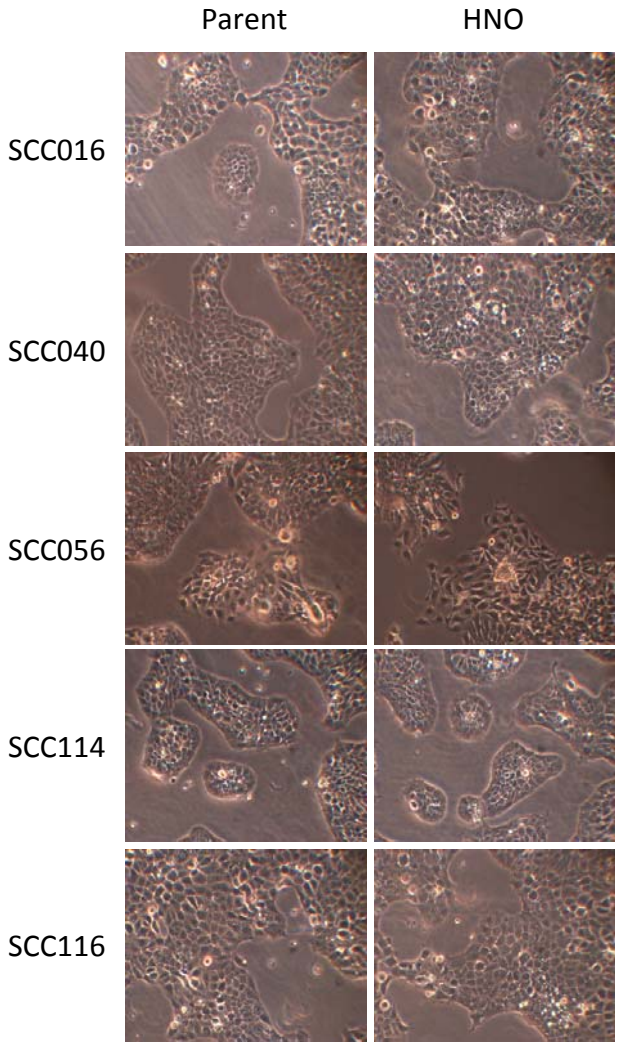


Figure 3

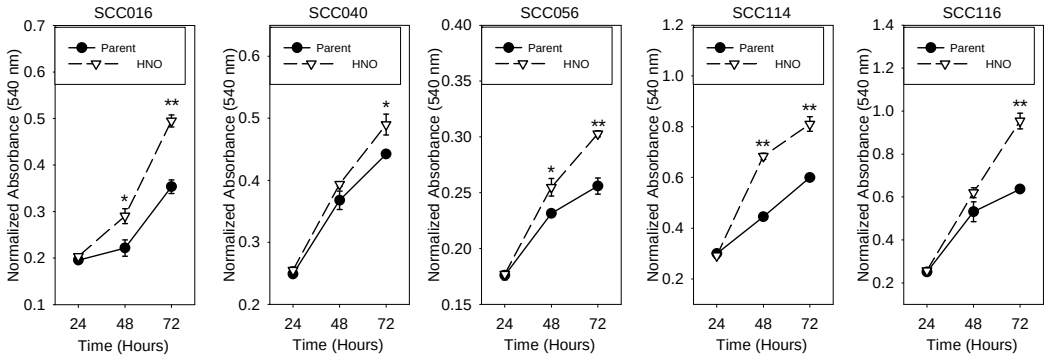

SCC016
SCC040
SCC056
SCC114
SCC116
Parent
HNO
Normalized Absorbance (540 nm)
Time (Hours)
24
48
72
*
**

Figure 4

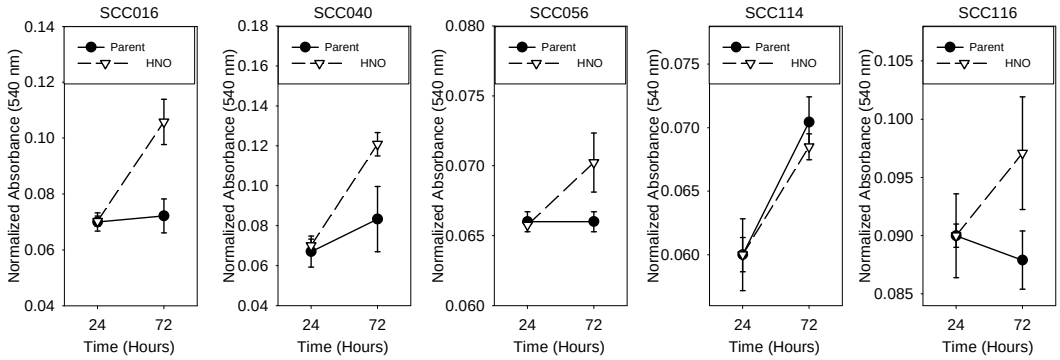
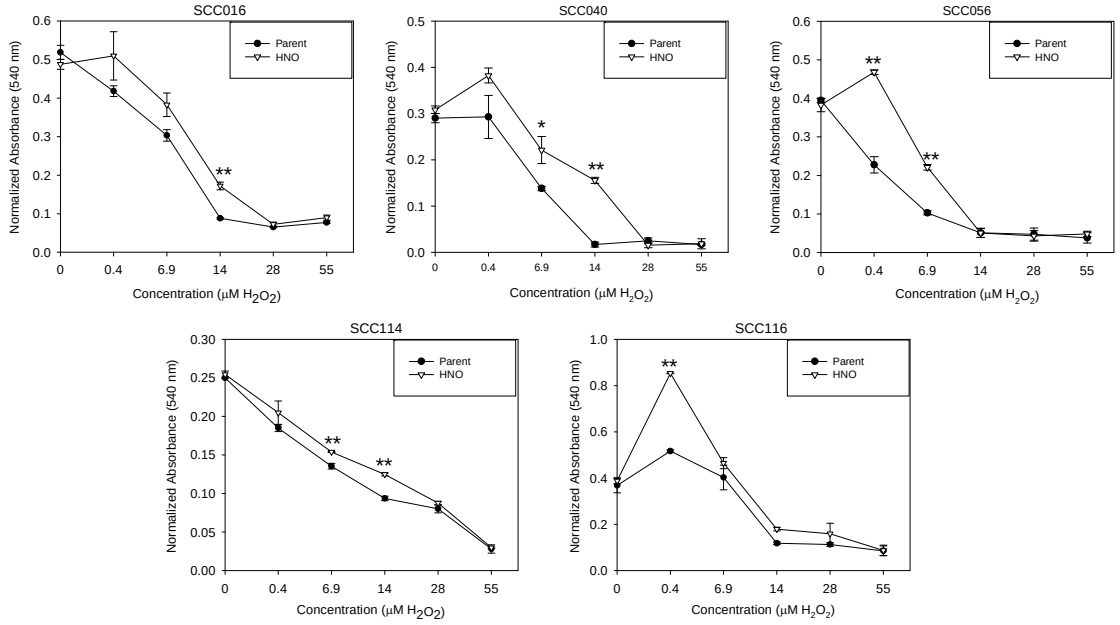

SCC016
SCC040
SCC056
SCC114
SCC116
Parent
HNO
Normalized Absorbance (540 nm)
Time (Hours)
24
72

Figure 5


SCC016
SCC040
SCC056
SCC114
SCC116
Parent
HNO
Normalized Absorbance (540 nm)
Concentration (μM H2O2)
0
0.4
6.9
14
28
55
*
**