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UNIVERSITY OF CALIFORNIA

Monothematic conference

Therapeutic Applications of Nitric Oxide in Cancer and Inflammatory-related Disorders

Accademia dei Fisiocritici (Siena)
October 4-5, 2018

Co-Organizers:
Lucia Morbidelli, Ph.D
Benjamin Bonavida, Ph.D

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PROGRAM

Therapeutic Applications of Nitric Oxide in Cancer and Inflammatory-related Disorders

Accademia dei Fisiocritici (Siena),
October 4th-5th, 2018

Day 1: Thursday, October 4th

10-10:30am Greetings from the Authorities
Francesco Frati, Rector of University of Siena
Cosima Baldari, Director of Dept. Life Sciences
Alessandro Mugelli, SIF President
Carlo Riccardi, Salvatore Cuzzocrea, Gruppo di lavoro SIF Infiammazione
Opening address: Lucia Morbidelli, University of Siena
General introduction of the meeting: Benjamin Bonavida, University of California at Los Angeles

I. Nitric Oxide and Cancer

Session 1

Nitric Oxide roles in tumor progression and regression

Chairs: Jordi Muntane, Marina Ziche

10:30-11:00am
John Wallace – University of Calgary
Plenary lecture: **Potential for Chemoprevention with Nitric Oxide and Hydrogen Sulfide-Releasing Drugs**
11:00-11:15am
Salvatore Rizza – Danish Cancer Society Research Center
The Roles of the Denitrosylase GSNOR in Tumor Progression
11:15-11:30am
Khosrow Kashfi – University of New York School of Medicine
Nitric oxide and cancer: To inhibit or to induce iNOS – That is the Question?
11:30-11:45am
Karim Zuhra – Sapienza University of Rome
H_2S Metabolism in Colon Cancer Cells Exposed to Hypoxia

11:45-12:15pm ***Coffee Break***

Session 2

Nitric Oxide and Cancer Cell Death

Chairs: Sharon Glynn, Cristiana Perrotta

12:15-12:30pm
Moran Benhar – Technion-Israel Institute of Technology
Characterization of Nitroso-Redox Stress-Induced Cancer Cell Death

12:30-12:45pm

Arnold Stern – New York University School of Medicine

Pro-Apoptotic Effects of S-Nitrosothiols O-Chloro and M-Chloro S-Nitroso-Aryl-Amides in Human Breast Cancer Cell Lines

12:45-1:00pm

Valerio Ciccone – University of Siena

Antitumor Effect of a Metal-Nonoate Through Angiogenesis Impairment

1:00-1:15pm

Lorenzo Cinci – University of Florence

New NO-Releasing Doxorubicins as Targeted Therapy Against Chemoresistance in Castration-Resistant Prostate Cancer: In Vitro and in Vivo Evaluations

1:15 -1:45pm **General discussion (Chairs: Jordi Muntane, Marina Ziche, Sharon Glynn, Cristiana Perrotta)**

1:30-2:30pm ***Lunch***

Session 3

Nitric Oxide and therapy (I)

Chairs: Carlo Riccardi, Stefan Chlopicki

2:30-2:45pm

Greta Varchi – University of Bologna

Light Guided Production of Nitric Oxide and Singlet Oxygen for the Multi-modal treatment of cancer

2:45-3:00pm

Claudiu T Supuran – University of Florence

Carbonic Anhydrase Inhibitor – NO Donor Hybrids and Their Pharmacologic Applications

3:00-3:15pm

Massimo Venturelli - University of Verona

The Role of Nitric Oxide on Vascular Dysfunction During Aging and Alzheimer's Disease

3:15-3:45pm ***Coffee break***

Session 4

Nitric Oxide and Therapy (II)

Chairs: Valentina Rapozzi, Giuseppe Cirino

3:45-4:00pm

Stavroula Baritaki – University of Crete

Nitric Oxide (NO): A Multifaceted Target for Reversal of Cancer Cell Pleiotropic Properties by NO-modulating Therapies

4:00-4:15pm

Cristiana Perrotta - Università degli Studi di Milano

Nitric Oxide in Cancer Resistance to Cisplatin: Tumor Associated Macrophages as Key Players

4:15-4:30pm

Joanna Kopecka – University of Torino

Nitric Oxide Re-Instates Doxorubicin Cytotoxic and Pro-Immunogenic Effects in Refractory Breast Cancer

4:30-4:45pm

Alma Martelli – University of Pisa

Anti-Cancer Activities of Erucin a H_2S-donor Isothiocyanate from Eruca Sativa Mill.: Is H_2S the Real Player?

4:45-5:00pm

Valentina Citi – University of Pisa

Anticancer Effect of a Novel H2S-Hybrid Molecule on Human Breast Adenocarcinoma (MFC-7) and Human Breast Epithelial (MCF-10A) Cell Lines

5:00-5:30pm **General discussion (Chairs: Carlo Riccardi, Stefan Chlopicki, Valentina Rapozzi, Giuseppe Cirino)**

8:00pm ***Dinner in Contrada***

Day 2: Friday, October 5th

II. Nitric Oxide and Inflammatory Related Disorders

Session 5

Nitric oxide and cardiovascular diseases

Chairs: Maria Foti, Andreas Papapetropoulos

9:15-9:30am

Perwez Hussain – National Cancer Institute, Bethesda, Maryland

Nitric oxide (NO•)/kynurenine/AHR signaling enhances disease aggressiveness in pancreatic cancer

9:30-9:45am

Miriam Durante – University of Siena

***In vitro* Assessment of NitDox Toxicity Towards Vasculature**

9:45-10:00am

Asghar Ghasemi - Shahid Beheshti University of Medical Sciences

Effects of long-term nitrite supplementation on gene expressions of GLUT2, GLUT4 and glucokinase in male obese type 2 diabetic rats

10:00-10:15am

Zahra Bahadoran - Shahid Beheshti University of Medical Sciences

Serum Nitric Oxide Metabolites and The Incidence of Metabolic Syndrome and Its Phenotypes: A Population-Based Prospective Study

10:15-10:45am ***Coffee break***

Session 6

Nitric oxide and Non-Cardiovascular Diseases

Chairs: Lucia Morbidelli, Vincenzo Calderone

10:45-11:00am

Andreas Papapetropoulos - National and Kapodistrian University of Athens

Hydrogen Sulfide as a Signaling Molecule in The Cardiovascular System: Actions and Interactions with NO.

11:00-11:15am

Josef Dulak - Jagiellonian University

Heme Oxygenase-1 In Duchenne Muscular Dystrophy: Interaction with Nitric Oxide Synthase Pathway in Satellite Cells Differentiation

11:15-11:30am

Stefan Chlopicki - Jagiellonian University

Pharmacology of Hepatoselective Nitric Oxide - Donors in NAFLD in Mice

11:30-11:45am
Giuseppe Filomeni - Danish Cancer Society Research Center
The Regulation of GSNOR Expression Discloses the Role of *S*-nitrosylation in Aging and Disease

12:00-12:30pm ***Coffee break & snacks***

Session 7
Nitric Oxide and Therapeutic Applications
Chairs: Benjamin Bonavida, Salvatore Cuzzocrea

12:30-12:45pm
Chiara Platania – University of Catania
Therapeutic Potential of Nitric Oxide Modulation in Ocular Diseases: A Focus on Novel NO-Releasing Molecules
12:45-1:00pm
Rosana Pinto – University of Lisboa
Emerging Nitric Oxide-releasing Porous Materials for Therapeutic Applications
1:00-1:15pm
Jan Scicinski - Avicenna Therapeutics, Inc
Cancer and Beyond: Discovery and Development of NO-releasing Therapeutics

1:15-1:45pm **General discussion and concluding remarks (all chairs of sessions 1-7)**

ABSTRACTS

Serum Nitric Oxide Metabolites and The Incidence of Metabolic Syndrome and Its Phenotypes: A Population-Based Prospective Study.

Zahra Bahadoran[1], Parvin Mirmiran[1], Fereidoun Azizi[2], Asghar Ghasemi[3]

[1]Nutrition and Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [2]Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [3]Endocrine Physiology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Background and aim: Although nitric oxide (NO) is emerging as a central regulator of metabolism, its endogenous over-production, especially originated by inducible NO synthase, is now considered as a marker and potential mediator of metabolic diseases. Current knowledge regarding the prognostic relevance of circulating NO is limited and inconsistent. This study was conducted to investigate whether serum NO metabolites (NOx) could predict the occurrence of a metabolic syndrome (MetS) and its phenotypes, in a general population.

Methods: We measured serum NOx concentrations in the Tehran Lipid and Glucose Study participants (aged ≥ 19 years) at baseline examination (2006-2008) and followed them for a median 7.7 years for MetS and its phenotypes. The receiver operator characteristic (ROC) curve analysis was used to determine the optimal cut-off points of serum NOx for predicting MetS. Multivariate Cox proportional hazard models were used to estimate the hazard ratios (HRs) with 95% confidence intervals (95% CIs) of MetS in response to serum NOx values. The incidence (%) of MetS phenotypes across optimal cut-off points of serum NOx was compared using the Chi-square test.

Results: The median (inter-quartile range; IQR) follow-up of MetS was 7.7 (7.3-8.0) years and the incidence rate (95% CI) was 5.18 (4.77-5.62) per/1000 person years. The optimal cut-off points of serum NOx levels for predicting MetS was 26.5 μmol/L. The area under the curve (AUC) of serum NOx for MetS was 0.56 (P 0.001). A significant positive association was observed between Ln-transformed serum NOx levels and the incidence of MetS (HR=1.20, 95% CI=1.03-1.39). After adjustment of potential confounding variables, participants with serum NOx levels ≥ 26.5 μmol/L had a significant increased risk of MetS (HR=1.19, 95% CI=1.01-1.40). The incidence of MetS phenotypes above and below the cut-off point of serum NOx are illustrated in Figure 1.

Conclusion: Our findings imply that increased serum NOx, most probably due to endogenous overproduction of NO, may be considered as an independent factor contributing to the development of MetS and its phenotypes. With respect to these findings, serum NOx may be suggested as a novel predictor of future risk of metabolic disorders.

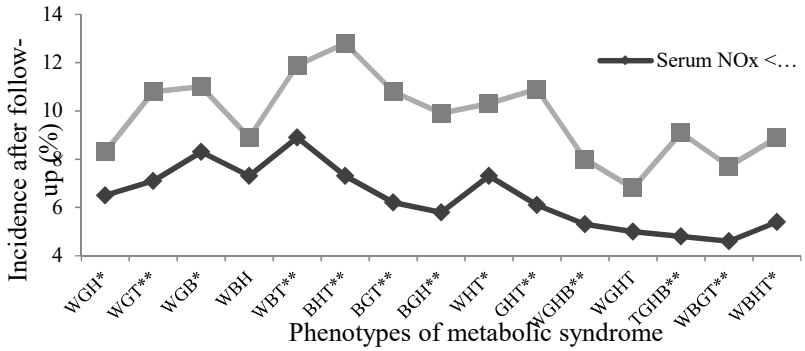

Figure 1. The incidence of metabolic syndrome phenotypes below and above the defined optimal cut-off points for serum NOx (≥26.5 μmol/L). W: elevated waist circumference, G: elevated blood glucose, T: elevated triglyceride levels, B: elevated blood pressure, H: low-HDL-C.* P<0.01, ** P<0.001

Nitric Oxide (NO): A Multifaceted Target for Reversal of Cancer Cell Pleiotropic Properties by NO-modulating Therapies.

Stavroula Baritaki[1] *and Benjamin Bonavida*[2]

[1]Division of Surgical Oncology, School of Medicine, University of Crete, Heraklion, 71110, Crete, Greece. [2]Department of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine, UCLA, Los Angeles, CA, 90095.

During the last two decades, Nitric Oxide (NO) has emerged as a molecule of interest in cancer cell biology. There are several lines of experimental evidence showing that NO, produced by cancer cells, or released by surrounding cells in the tumor milieu, contributes critically to various functions in cancer cells as well as the tumor microenvironment. These functions, among others, include carcinogenesis, tumor cell viability, growth and invasiveness, maintenance of cancer stem cell properties, angiogenesis, immunoediting of immune signals and tumor resistance to conventional chemo/immuno-therapeutics. NO may affect, either positively or negatively, the above processes in various cancer types, thus creating a considerable controversy and confusion in the understanding of the role of NO in cancer biology. In the context of the contrasting roles of NO in cancer, recent findings clarified this controversy, namely, that NO exerts pro- or anti-neoplastic properties in the cancer milieu depending on the NO concentration and the location and timing of its release.

Several studies have shown that NO, at high doses, acts as an anti-neoplastic agent, when used either as a single agent or in combination with other anti-neoplastic compounds. These findings demonstrate the importance of NO modulation in the response to cancer therapeutics. We and others have reported that compounds able to release high concentrations of NO, such as NO donors, are able, among others, to overcome tumor cell resistance to immune-mediated cytotoxicity and conventional chemotherapy, as well as to inhibit the initial metastatic steps of tumor cells, known as the "oncogenic epithelial to mesenchymal transition (EMT)." NO mediates the above anti-neoplastic properties via modulation of signaling pathways and feedback loops comprised of multiple interactions among gene products involved in the regulation of cellular apoptosis and tumor invasive and migratory properties. We will present findings on 1) the range of anti-neoplastic activities of NO in cancer cells and the tumor microenvironment 2) the various molecular mechanisms by which NO mediates its anti-neoplastic activities and 3) NO's novel potential applications in cancer treatment and prevention.

Characterization of Nitroso-Redox Stress-Induced Cancer Cell Death.

Moran Benhar

Faculty of Medicine, Technion-Israel Institute of Technology. Haifa, Israel.

Inhibiting cellular antioxidants and promoting oxidative stress are an anticancer strategy that is the focus of much recent research. In particular, the two major cellular antioxidant systems, the glutathione (GSH) and the thioredoxin (Trx) systems, are increasingly recognized to play important and interrelated roles in tumor progression and resistance to therapy, providing a strong rationale for targeting these antioxidant systems in tumor cells. There is also an ongoing interest in developing nitric oxide (NO) donors as anticancer agents. Yet, so far, the progress in this area has been limited and there are still many unanswered questions regarding mechanisms that determine tumor cell resistance versus sensitivity to NO-mediated stress (nitrosative stress). We have recently found that treatment of HeLa cancer cells with BSO (a GSH depleting agent) results in significant sensitization to cell death-induced by NO or S-nitrosothiol (SNO) donors. Mechanistically, combined BSO/SNO treatment leads to profound inhibition of both the GSH and Trx systems, thereby amplifying the NO/redox stress and committing the cells to death. Further findings suggested that cells die via a nonapoptotic mechanism, likely through a regulated necrosis. Using a redox proteomics approach, we identified hundreds of proteins that are oxidized in BSO/SNO-treated cells, including key regulators of cell redox, mitochondrial function and cytoskeletal dynamics. Consistently, the early steps in BSO/SNO-induced cell death involve rapid induction of thiol stress and collapse of the actin cytoskeleton. Similar to the observations in HeLa cells, we found that the BSO/SNO combination therapy was effective against various lung cancer cell lines. Our findings provide new insights into the mechanism of nitroso-redox stress-induced cancer cell death and suggest a possible avenue for improving NO-based anticancer therapies.

Pharmacology of Hepatoselective Nitric Oxide - Donors in NAFLD in Mice.

Stefan Chlopicki

Jagiellonian Centre for Experimental Therapeutics (JCET), Jagiellonian University, Krakow, Poland.

The impairment of the function of liver sinusoidal endothelial cells (LSECs) associated with the impairment of NO production by LSECs play an important role in the pathogenesis of NAFLD [1]. We provided evidence that treatment of mice fed high fat diet (HFD) with a liver selective NO-donor such as V-PYRRO/NO were protected against the development of liver steatosis and attenuated insulin resistance [2]. The effects of V-PYRRO/NO were stronger as compared with metformin and the protection was mediated by a different mechanism as that afforded by metformin [2]. V-PYRRO/NO inhibited synthesis of *de novo* fatty acid and increased insulin resistance, while metformin increased mitochondrial biogenesis. Distinct mechanisms of action of V-PYRRO/NO and metformin suggest a possible additive value of the combined treatment based on the hepatoselective NO delivery and metformin. Interestingly, V-PROLI/NO, despite its similar structure to V-PYRRO/NO, displayed a completely different pharmacokinetic profile, including weak NO release by a cytochrome P450 –mediated metabolism in the major part of the kidney metabolism and subsequently was not effective in the prevention of liver steatosis and insulin resistance in mice fed HFD. [3, 4]. In summary, our studies [1-4] were complemented recently by the demonstration of the beneficial effects of V-PYRRO/NO –based treatment on the liver microcirculation perfusion in NAFLD [5]. These suggested that liver-targeted delivery of NO represents an effective approach to normalize metabolic disturbances and impairment of liver microcirculation of the blood flow occurring in NAFLD. Our ongoing studies are aimed to better understand the regulatory role of LSECs-derived NO in the progression of NAFLD.

Acknowledgements

This work was supported by the Grant from the resources of the European Regional Development Fund under the Innovative Economy Programme (grant coordinated by JCET-UJ, No POIG.01.01.02 00 069/09) and by National Science Centre grant Symfonia no. DEC 2015/16/W/NZ4/00070

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Antitumor Effect of a Metal-Nonoate Through Angiogenesis Impairment.

Valerio Ciccone[1] *and Lucia Morbidelli*[1,2]

[1]Department of Life Sciences, University of Siena, Siena, Italy and [2]Noxamet Ltd, Milan, Italy.

Nitric oxide (NO) has been reported to induce different effects on tumor biology depending on its concentration and availability. We studied the antitumor activity of Ni(SalPipNONO), a new NO donor belonging to the metal-nonoate family. The antitumor activity of nonoate was characterized on A549 cells (human lung carcinoma).

Ni(SalPipNONO) reduced tumor cells survival, clonogenicity and invasiveness, and promoting apoptosis. The effect was due, in part, to the classic NO/cGMP pathway and, in part, to reactive oxygen species (ROS) production. ROS promoted ERK1/2 activation, which in its turn induced p53 upregulation. Finally, we assessed the anti-angiogenic activity of the metal-nonoate. Ni(SalPipNONO) treatment reduced the angiogenic factor expressions of tumor cells (HIF-1α and VEGF) that were associated with the impairment of the endothelial cell function that is related to angiogenesis.

Overall, Ni(SalPipNONO) exerts antitumor activity through different mechanisms, involving an anti-angiogenic effect [1]. Further studies should be done to develop this compound alone or in combination with conventional therapy for the treatment of cancer.

Acknowledgements

We tank Prof. Luigi Casella, University of Pavia, for providing the metal-nonate.

Reference

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New NO-Releasing Doxorubicins as Targeted Therapy Against Chemoresistance in Castration-Resistant Prostate Cancer: In Vitro and in Vivo Evaluations.

Elisabetta Bigagli[1],Cristina Luceri[1], Maria De Angioletti[2], Konstantin Chegaev[3], Mario D'ambrosio[1], Chiara Riganti[4], Elena Gazzano[4], Simona Saponara[5], Mariangela Longini[6], Francesca Luceri[7] and Lorenzo Cinci[1]

[1]Department NEUROFARBA, Section of Pharmacology and Toxicology University of Florence, Florence, Italy. [2]Cancer Genetics and Gene Transfer Laboratory, Core Research Laboratory, Istituto Toscano Tumori, Florence, Italy; and Institute of Chemistry of Organometallic Compounds, CNR, Sesto Fiorentino, Florence, Italy. [3]Department of Drug Science and Technology, University of Turin, Turin, Italy. [4]Department of Oncology, University of Turin, Turin, Italy. [5]Department of Life Sciences, University of Siena, Siena, Italy. [6]Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy. [7]General Laboratory Unit, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy.

Chemotherapy for castration-resistant prostate cancer (CRPC) is only temporarily effective due to the onset of chemoresistance. We investigated the efficacy of NO-releasing doxorubicins (NitDox) in overcoming drug resistance and evaluated their safety.

New and innovative NO-releasing doxorubicins (NitDox) showed a good intracellular accumulation and high cytotoxic activity in vitro in an androgen-independent and doxorubicin-resistant DU-145 prostate cancer cell line. Nude mice were subcutaneously injected with $4x10^6$ DU-145 cells and treated once a week for three weeks with 5 mg/kg doxorubicin, NitDox, or vehicle, i.p. Animal weight, tumor volume, intra-tumoral drug accumulation, apoptosis and the presence of nitrotyrosine groups within the tumor were evaluated. Cardiotoxicity was assessed by measuring troponin plasma levels and the left ventricular wall thickness.

In vivo, NitDox accumulated inside the tumors, significantly reduced tumor volumes by 60%, increased the percentage of apoptotic cells in both the inner and the outer parts of the tumors and showed the presence of nitrotyrosine. Doxorubicin treatment was associated with reduced body weight and cardiotoxicity. On the contrary, NitDox was well tolerated and had a better safety profile.

Combining the efficacy with reduced cardiovascular side effects, NitDox is a promising novel therapeutic agent for reversing chemoresistance in CRCP.

Anticancer Effect of a Novel H2S-Hybrid Molecule on Human Breast Adenocarcinoma (MFC-7) and Human Breast Epithelial (MCF-10A) Cell Lines.

Citi V, Martelli A, Piragine E, Barresi E, Taliani S, Testai L, Da Settimo F, Calderone V.

Department of Pharmacy, University of Pisa, via Bonanno n.6, 56125 – PI, Italy.

Hydrogen sulfide (H2S) is a gasotransmitter produced endogenously in the mammalian system. Many in vitro studies described the role of H2S in regulating cell growth and survival, resulting in apoptosis of cancer cells mediated by the activation of the caspase pathway. Recently, our group described some synthetic and natural isothiocyanates (ITCs) as H2S-donor agents. Noteworthy, there is an overlap between the anticancer effects widely attributed to many ITCs and those exhibited by the treatment with some known H2S-donors, such as the inhibition of human cancer cell viability and the arrest of the cell cycle in the G2/M phase [1,2]. The development of H2S-hybrid drugs, obtained through the conjugation of H2S-releasing moieties to conventional drugs, is aimed to reduce possible adverse effects of the old drugs and/or improve their therapeutic impact. In this work, an innovative molecule (named "hybrid") has been obtained from the combination of a well-known drug (the "native" drug) exerting anticancer effect, and the H2S-releasing moiety ITC. Firstly, the characterization of the H2S-generating properties of the hybrid and the native drug has been carried out in a cell-free model by an amperometric approach. Then, the antiproliferative effect of the two molecules at different concentration (5mM; 1mM; 500μM; 100μM; 50μM; 10μM; 5μM; 1μM; 500nM and 100nM) after 72h-treatment has been assessed in the MFC-7 cell line (human breast adenocarcinoma cells) and the MCF-10A cell line (human breast epithelial cells). Furthermore, the intracellular H2S-release of the two compounds (1mM, 100μM and 10μM) has been recorded, using a cell-based spectrofluorometric method. Finally, in order to investigate the anticancer mechanism of action, the flow cytometry analyses of the cell cycle, annexin V (early apoptosis) and caspase 3/7 (mild apoptosis) activities have been performed on the MCF7 cells after 72h treatment at the concentration of 20μM.

As concerns of the amperometric results, the incubation of the hybrid 1 mM in the presence of L-cysteine 4 mM led to a slow increase of the H2S concentration (about 8 μM); in contrast, after the incubation of the hybrid 1 mM in the absence of L-cysteine, no H2S release has been recorded. The incubation of the native drug did not lead to the formation of H2S either in the presence or in the absence of L-cysteine. The cell treatment with the hybrid for 72h showed a concentration-dependent antiproliferative effect on MCF7 cells, with a maximum efficacy value (100% inhibition of cell viability) at the concentration of 500μM and a parameter of potency of 20 μM (IC50, concentration evoking the inhibition of the 50% of cell viability). In contrast, the active drug showed a dramatic decrease in the antiproliferative effect on MFC7, showing an inhibition of cell viability of about 50% at the highest concentration (5mM). Furthermore, the hybrid was able to release H2S into MCF7 cells: in fact, at the concentration of 1mM the amount of H2S released by the hybrid was similar to that evoked by the reference H2S-donor diallyl disulfide (DADS) at 100μM. The antiproliferative effect of the two molecules has been also assessed in the non-tumorigenic MCF10A: while the native drug showed an anti-proliferative behaviour similar to that exhibited in MCF7, the hybrid showed a lower parameter of potency (about 170μM) and a maximum efficacy value only at the concentration of 1mM of about the 90% and, interestingly, did not promote any detectable H2S release when incubated with MCF10A. On MCF7 cell cycle, the hybrid induced a significant reduction of the G0/G1 phase cells and an increase of the G2/M phase; moreover, an increased number of cells in early and mild apoptosis, respectively of about the 10% and 20% was recorded in cells treated with the hybrid compared to vehicle-treated cell. In conclusion, this hybrid molecule showed a significant improvement of the native drug anticancer properties, indicating that ITC can be viewed as a versatile chemical moiety for developing novel hybrid compounds in this field.

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Heme Oxygenase-1 In Duchenne Muscular Dystrophy: Interaction with Nitric Oxide Synthase Pathway in Satellite Cells Differentiation.

Józef Dulak

Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland.

Muscle damage in Duchenne muscular dystrophy (DMD), caused by the lack of dystrophin, is strongly linked to inflammation. Accordingly, modulation of inflammation can ameliorate the disease progression. Heme oxygenase-1 (HO-1; *Hmox1*) is the crucial anti-inflammatory protein affecting also muscle stem cells differentiation. Surprisingly, the role of HO-1 has not been so far deeply addressed in DMD.

The results of our recent studies [1-2] showed that HO-1 is strongly upregulated in response to cardiotoxin-induced muscle injury and in DMD muscles. Lack of HO-1 augments the injury and, consequently, multiple rounds of degeneration/regeneration in conditions of HO-1 deficiency may lead to exhaustion of satellite cells (SCs) pool. Accordingly, the number of SCs is decreased in old mice lacking HO-1.

Lack of HO-1 affects the severity of DMD in *mdx* mice deficient of dystrophin. Accordingly, double knockout animals (*Hmox1*$^{-/-}$*mdx)* demonstrate strongly impaired exercise capacity. Moreover, *mdx* SCs show disturbed and enhanced differentiation, which are further aggravated by *Hmox1* deficiency. Interestingly, in contrast to the effect observed in *mdx* striated muscles, in which the *Hmox1* is strongly induced in comparison to healthy animals, the expression of *Hmox1* is decreased in dystrophin-deficient SCs. Importantly, SCs of *mdx* mice demonstrate disturbed and enhanced differentiation, which are further intensified by *Hmox1* deficiency. RNA sequencing revealed downregulation of Atf3, MafK, Foxo1 and Klf2 transcription factors, known to activate *Hmox1* expression, as well as attenuation of nitric oxide-mediated cGMP-dependent signaling in *mdx* SCs. Accordingly, treatment with NO-donor induces *Hmox1* expression and inhibits differentiation. Finally, differentiation of *mdx* SCs was normalized by CO, a product of HO-1 activity

In summary, HO-1 can be considered as a therapeutic target to alleviate muscle injury and DMD severity. Our study indicates of the interaction between nitric oxide and heme oxygenase-1 in SC differentiation.

Acknowledgements

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In vitro Assessment of NitDox Toxicity Towards Vasculature.

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Doxorubicin (Dox) is one of the most powerful anticancer drugs, but its therapeutic efficacy is hampered by the development of drug resistance, mainly due to the overexpression of ATP-binding cassette (ABC) efflux transporters [1].

The conjugation of Dox with nitric oxide (NO)-releasing groups gives rise to novel multitarget anthracyclines [2-4], such as nitrooxy-Dox (NitDox), capable to overcome drug resistance by decreasing the activity of ABC transporters *via* nitration of critical tyrosine residues on the pumps [2]. Moreover, NitDox preferentially accumulates in mitochondria and affects its function, thus representing a prototype of novel anthracyclines, which have cellular targets different from, and of greater efficacy against drug-resistant tumor cells than the parent compound [5-7].

The widely described anthracyclines cardiovascular toxicity, however, might limit their clinical use. The aim of this study was to investigate the NitDox-induced vascular effects, as potential hazard, on vascular smooth muscle A7r5 and endothelial EA.hy926 cells viability, on the mechanical activity of freshly and cultured rat aorta rings, and on L-type Ca^{2+} current [$I_{Ca(L)}$] of A7r5 cells [8]. Dox was used as a reference compound.

Both A7r5 and EAhy.926 cells proved to be more sensitive to Dox than to NitDox at concentration of comparable to those accumulating and exerting antitumor activity in drug-resistant cells after 24h exposure. Both compounds, however, promoted similar apoptotic effects in A7r5 cells. Dox was more active than its derivative in rising sub-diploid-, dapi- and annexin V-positive-cell number in endothelial cells. The pro-apoptotic activity of both Dox and NitDox was due to the generation of intracellular ROS and changes in the mitochondrial potential. Finally, NitDox doubled basal NO cell content after 24h of treatment in both A7r5 and EAhy.926 cells.

Dox exhibited a modest contracturing effect in endothelium intact rings, while NitDox induced a significant vasodilation in endothelium denuded rings, that disappeared in presence of ODQ. NitDox and Dox did not significantly affect KCl-induced contraction. In arteries cultured with both drugs for 7 days, NitDox blocked both phenylephrine- and KCl-induced contractions at a concentration 10-fold higher than that of Dox. NitDox exhibited weak Ca^{2+} antagonist properties in single A7r5 cells.

In conclusion, NitDox is an NO-releasing Dox with a more favourable vascular toxicity profile and a greater efficacy against drug-resistant cells than the parent compound, worthy of further investigations in preclinical and clinical settings.

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The Regulation of GSNOR Expression Discloses the Role of *S*-nitrosylation in Aging and Disease.

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S-nitrosylation, the reversible modification of cysteine residues by nitric oxide (NO) represents a prototypic redox-based signal that is frequently dysregulated in disease.

By examining the literature, we were struck by a constellation of changes in mice deficient in the denitrosylase *S*-nitrosoglutathione reductase (GSNOR) that typifies aging.

Here, we go into depth on the molecular mechanisms underlying this phenotype. In particular, we show that GSNOR deficiency – and excessive *S*-nitrosylation resulting from it – drives cell senescence and aging in mammals by affecting mitochondrial dynamics and homeostasis. We report that GSNOR-null cells show an accumulation of fragmented and dysfunctional mitochondria which are not properly recognized by the autophagic machinery. As a result, they are not removed and degraded by mitophagy, with this alteration being a marker of cell aging.

In order to provide the biological relevance to our results, we demonstrate that GSNOR expression physiologically decreases in senescent cells in mice and humans during their life span owing to an epigenetic regulation of the methylation status of the *GSNOR* promoter. In stark contrast, but in line with the hypothesis that GSNOR represents a new longevity protein, exceptionally long-lived individuals maintain GSNOR levels.

Finally, we also provide preliminary evidence about GSNOR expression being regulated at translational levels by the ataxia telangiectasia mutated (ATM)-induced signaling cascade in response to nitro-oxidative insults. We speculate that this event is crucial to rapidly modulate intracellular glutathione levels, and, in turn, face up oxidative stress, providing new cues for the comprehension of the ataxia-telangiectasia etiopathogenesis.

Effects of long-term nitrite supplementation on gene expressions of GLUT2, GLUT4 and glucokinase in male obese type 2 diabetic rats.

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Purpose: Reduced bioavailability of nitric oxide (NO) is associated with the pathogenesis of type 2 diabetes. Nitrite can act as a substrate for boosting the nitrate-nitrite-NO pathway in diabetes. The aim of this study was to determine the effects of nitrite on gene expressions of GLUT2, GLUT4 and glucokinase in male obese type 2 diabetic rats.

Methods: Male Wistar rats were divided into 4 groups: Control, control+nitrite, diabetes, and diabetes+nitrite. Diabetes was induced using a high-fat diet combined with a low-dose of streptozotocin. Sodium nitrite (50 mg/L in drinking water) was administered for two months. At the end of the study, mRNA expressions of GLUT2 and glucokinase were measured in the pancreas and mRNA expression of GLUT4 was measured in the soleus muscle and epididymal adipose tissue.

Results: Compared to the controls, in diabetic rats, the mRNA expressions of glucokinase (70%, $p<0.001$) and GLUT4 (52% and 40%, in the soleus muscle and epididymal adipose tissue, respectively) were significantly lower while there was no significant change in GLUT2 mRNA expression. Nitrite administration increased GLUT4 mRNA expression in both the soleus muscle (25% and 54%, in control and diabetic rats, respectively) and the epididymal adipose tissue (120% and 70%, respectively) as well as increased glucokinase (231% and 153% in control and diabetic rats, respectively) mRNA expression in the pancreas. Nitrite administration increased mRNA expression of GLUT2 only in control rats (123%, $p<0.001$) but not in diabetic rats.

Conclusion: Chronic supplementation with nitrite in a rat model of obesity and type 2 diabetes increased mRNA expression of GLUT4 in insulin-sensitive tissues and glucokinase in pancreas while had no effect on GLUT2 mRNA expression.

Nitric oxide and cancer: To inhibit or to induce iNOS – That is the Question?

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NO has a dichotomous role in cancer biology. Some reports suggest that NO possesses anti-inflammatory and anti-tumor properties, mainly exerted by NO-activated apoptotic pathways, while others implicate NO in inflammation, tumor promotion and metastasis. For example, iNOS overexpression has been reported in breast, prostate, lung, brain and colon cancer. It appears that low concentrations of NO (<100 nM) prevent certain cell types from apoptosis, favoring tumorogenesis and progression, whereas high concentrations of NO (>500 nM), may be pro-apoptotic, producing cytotoxicity and antitumor activity. NO-dependent cytotoxic effects on tumor cells are derived from macrophages, neutrophils, endothelial cells, hepatocytes, cardiac myocytes and chondrocytes.

In some studies, high iNOS expression has correlated relatively well with poor patient survival. These include colon, breast, gastric, hepatocellular carcinoma, melanoma, ovarian, leukemia, gastric, prostate, esophageal, and cervical cancers. It has thus been suggested that iNOS expression may be used as a biomarker of poor patient prognosis and perhaps survival. By contrast, favorable prognosis has been associated with high iNOS expression in ovarian (10-year prognosis) and non-small cell lung cancers. Treatment of triple-negative breast cancer (TNBC) cell lines, which express high levels of iNOS with various NOS inhibitors, reduced cell proliferation, migration, and mammosphere formation. TNBC xenografts treated with NOS inhibitors, significantly reduced tumor growth. Growth of glioma or melanoma cells in xenografts was significantly reduced when iNOS was silenced in these cells before they were implanted.

L-arginine supplementation can reduce tumor occurrence and development in some tumor models, including chemically induced solid tumors, transplantable solid tumors, and human tumors. In xenograft studies, L-arginine treatment significantly lowered MMP-2, MMP-9 and VEGF receptor levels in tumors whereas it inhibited the progression of colon cancer by increasing NO levels and consequently enhancing spleen natural killer cell activity. High iNOS expression is associated with favorable prognoses in ovarian and lung cancers. Overexpression of iNOS by gene transfer in prostate and colon cancer cells increased the sensitivity of these cells to cisplatin-induced cell death or to radiation therapy. In a mouse model of thyroid cancer, iNOS overexpression inhibited tumorogenesis. iNOS expressing pancreatic cancer cells did not produce tumors or metastases in xenograft mouse models due to NO upregulation and apoptosis. Overexpressing iNOS by other means has also produced anti-cancer effects.

In this conference, we will discuss the yin and yang of NO and in particular the role of iNOS in cancer.

Nitric Oxide Re-Instates Doxorubicin Cytotoxic and Pro-Immunogenic Effects in Refractory Breast Cancer.

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Doxorubicin fails in triple negative breast cancer because of chemoresistance, mainly due to the presence of membrane drug transporters, such as P-glycoprotein (Pgp), which limit intracellular doxorubicin accumulation, prevent cytotoxic and immunogenic cell death (ICD) mediated by this drug. ICD occurs in the condition of endoplasmic reticulum (ER) stress when calreticulin (CRT) is translocated from ER to plasma membrane. Here CRT works as a bait for dendritic cells (DC) which phagocytose tumor cells and activate anti-tumor CD8 T-cells. This type of tumor death promotes long term protection of the host immune system against tumor [1].

We have shown previously that nitric oxide (NO) is a very effective inhibitor of Pgp activity because it nitrates tyrosine in the active center of this transporter. NO in the cells is produced by 3 NO synthases (NOS) which can be stimulated by doxorubicin in sensitive cells, but not in resistant ones [2]. Moreover, NO causes ER stress and induces ICD promoting CRT translocation [3].

Here, we show that the proteasome inhibitor bortezomib and the lysosome inhibitor chloroquine activate NOS I and NOS III, respectively, in human and murine triple negative triple negative breast cancers, expressing Pgp and resistant to doxorubicin. The increased NO levels caused ER stress and the activation of the C/EBP-β LIP transcription factor, that triggers apoptosis via a CHOP/TRB3/caspase 3 pathway, down-regulates Pgp expression and up-regulates CRT transcription, which mediate ICD. In parallel, NO nitrates Pgp and inhibits its catalytic efficacy, increasing the intracellular accumulation of doxorubicin and the drug-mediated cytotoxicity via ER stress-dependent cell death and ICD in resistant cells. The combination of bortezomib, chloroquine and doxorubicin was effective in immunocompetent models of triple negative triple negative breast cancers refractory to doxorubicin, where it reduced tumor growth, induced intratumor ER stress-dependent apoptosis and increased the amount and activity of intratumoral DC and $CD8^+$ T-cells.

In conclusion, the increase of intratumor NO levels by the FDA-approved drugs bortezomib and chloroquine was an excellent adjuvant to doxorubicin chemotherapy in refractory triple negative breast cancer, representing an effective approach to overcome doxorubicin resistance.

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Anti-Cancer Activities of Erucin a H_2S-donor Isothiocyanate from Eruca Sativa Mill.: Is H_2S the Real Player?

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Erucin (4-methylthiobutyl isothiocyanate) is produced by the enzymatic hydrolysis of the glucosinolate glucoerucin present in *Eruca sativa* Mill. seeds (*Brassicaceae or Crucifers*). Erucin has structural analogies with sulforaphane (SFN), an isothiocyanate derived from glucoraphanin, a glucosinolate present in some edible *Crucifers*, and known in the literature for its chemopreventive properties. Indeed, more in general, different isothiocyanates exert anti-cancer properties on many tumor types (liver, breast, bladder, lung and pancreas) [1] and in particular SFN is actually employed in a pilot randomized controlled clinical trial in advanced pancreatic cancer [2]. In this work, we evaluated the anti-cancer properties of erucin on human pancreatic adenocarcinoma cells (AsPc-1) with particular focus on the possible role of its hydrogen sulfide (H_2S)-releasing properties [3]. Indeed, also the gasotransmitter H_2S is recognized as anti-proliferative agent in melanoma and intestinal cancers [4]. **Methods:** Erucin (purified from *Eruca sativa* seeds and available in *Brassica Collection* at CREA CI of Bologna, Italy) anti-proliferative effects at 72h on AsPc-1 viability were evaluated; then, its H_2S-donor properties were also verified both in cell-based and cell-free assays. Moreover, the erucin effects on cell cycle, mitochondrial potential and caspase 3/7 were investigated. Finally, an evaluation of a possible involvement of the mitogen-activated protein kinase (MAPK) pathway, and in particular of ERK1/2, was carried out. **Results:** Erucin inhibited AsPc-1 viability with a potency index (IC_{50}) of about 30μM and its ability to release H_2S was evident both in an *in vitro* assay buffer and on the AsPc-1 cells. Moreover, erucin evoked a reduction of the cell cycle G0/G1 phase, due to an enlargement of both the S and G2/M phases, and increased the number of cells exhibiting depolarization of the mitochondrial potential as a hallmark of early apoptosis. These effects were coupled with an increase of the number of apoptotic cells, recorded by the evaluation of Caspase3/7, as markers of a more advanced stage of apoptosis. Finally, the investigation of a possible mechanism of action highlighted a significant reduction of p-ERK1/2 activation suggesting an involvement of the MAPK pathway in erucin an anti-proliferative effect on AsPc-1. **Conclusion:** Our data demonstrated that erucin induced anti-proliferative effect on AsPc-1 cell altering cell cycle phases, inducing mitochondrial depolarization and increasing caspase 3/7 levels as markers of apoptosis. A significant decrease of p-ERK1/2 activation accounted for an involvement of the MAPK pathway in erucin anti-cancer effect. Moreover, the H_2S-donor property of erucin may represent a reliable explanation of its anti-cancer activity that is worthy to be further investigated.

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Hydrogen Sulfide as a Signaling Molecule in The Cardiovascular System: Actions and Interactions with NO.

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Hydrogen sulfide (H_2S) has emerged as an important endogenous gasotransmitter, which regulates homeostasis and affects the function of most organs in the body. Endogenously produced H_2S is generated through cystathionine γ-lyase (CSE) and cystathionine β- synthase (CBS), which use cysteine as a substrate, and 3-mercaptosulfurtransferase (3-MST), which utilizes 3-mercaptopyruvate as a substrate. CBS expression is higher in neuronal tissues, while CSE predominates in the heart and blood vessels. H_2S production and metabolism have been shown to be deregulated under pathophysiological conditions, contributing to the development and progression of disease.

H_2S exhibits a variety of effects in the cardiovascular system that impact on the endothelial, smooth muscle and cardiomyocyte functions. H_2S promotes endothelial cell growth, migration and organization into capillary networks, and enhancing angiogenesis. In addition, H_2S acts on smooth muscle cells to reduce the vascular tone leading to hypotension. Both endogenously produced and exogenously administrated H_2S exhibit cardioprotective actions by exerting anti-apoptotic, anti-inflammatory and anti-oxidant effects. H_2S ameliorates the damage that occurs after ischemia-reperfusion in many organs, in addition to the heart. H_2S also protects against the development of heart failure and atherosclerosis. The actions of H_2S in cells and tissues are mediated by modulation of ion channel activity, inhibition of phosphodiestareases, activation of kinases and nitric oxide synthase activation. H_2S has the ability to trigger posttranslational modifications of proteins by sulfhydrating cysteine residues to modify protein functions.

A better understanding of the regulation of H_2S production and signaling in physiological and phathophysiological conditions will help harness the therapeutic potential of this gasotransmitter.

Nitric Oxide in Cancer Resistance to Cisplatin: Tumor Associated Macrophages as Key Players.

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Tumor-associated macrophages (TAMs) represent the largest population of infiltrating inflammatory cells in malignant tumors, promoting their growth, invasion and immune evasion and chemoresistance. Using different tumor models in vitro and in vivo, we found that nitric oxide (NO) generated at low levels by inducible NO synthase of M2-polarised TAMs is able to protect tumor cells from apoptosis induced by cisplatin (CDDP). The NO effect depends on the inhibition of acid sphingomyelinase (A-SMase), which is activated by CDDP. Mechanistic insights indicate that NO actions occur via the generation of cGMP and activation of protein kinase G (PKG), inducing phosphorylation of syntaxin 4 (synt 4), a SNARE protein responsible for A-SMase trafficking and activation. Noteworthy, phosphorylation of synt4 at serine 78 by PKG is responsible for the proteasome-dependent degradation of synt4, leading to inhibition of A-SMase-triggered apoptosis.

This is the first demonstration that the NO system is a key mechanism through which M2-polarised TAMs protect cancer cells from CDDP-induced apoptosis leading to tumor chemoresistance. Moreover, this is the proof that NO produced at low levels has proneoplastic properties and has to be taken into account when designing a therapeutic strategy based on NO donors.

Emerging Nitric Oxide-releasing Porous Materials for Therapeutic Applications.

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Nitric oxide (NO) is a key signaling molecule involved in innumerable physiological and pathological functions and its exogenous administration offered great potential for advancing NO-mediated therapy of several diseases. NO has therapeutic activity in the maintenance of vascular homeostasis, immune response to infections, regulating neurotransmission and wound healing. These versatile functionalities have brought a rapid increase in research focused on developing successful NO donors and NO-delivery strategies. However, existent homogenous donors are limited due to their systemic nature of delivery, which can cause unwanted effects. Moreover, the most reported NO-releasing materials fail to achieve successful clinical outcomes due to the difficulty in modulating the release NO amounts during a precise time at the target site.

Recently, a novel approach using zeolites and metal organic frameworks (MOFs) have been investigated to store and deliver NO due to their interesting porous framework in which NO can coordinate. These materials provide a safe storage, low toxicity, highly efficient packing of NO within the solid and its delivery in a controlled rate.

Our group has been exploring different microporous titanosilicates, namely ETS-4, ETS-10 and modified specimens, which are able to adsorb great amounts of NO (7-11% NOAds) and release it over long periods. Another strategy, based on Co- and Ni-MOFs built from vitamin B3 (nicotinic acid) showed the capability to store and release NO in a slow and reversible manner, releasing all the NO amount absorbed (i.e. 2.6 and 2.0 μmol NO mg-1solid for Ni and Co compound, respectively).

All the studied materials were evaluated in terms of toxicity using primary keratinocyte cells (HEKn) and their stability in biological fluids, showing high biocompatibility for all materials at concentrations of 180 μg/mL and a great stability for titanosilicates. The ability of these new NO donors to regulate biological functions was assessed by controlling the mitochondrial respiration and the cell migration. The latter tests can be regarded as a first evaluation for the potential application in wound healing.

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Therapeutic Potential of Nitric Oxide Modulation in Ocular Diseases: A Focus on Novel NO-Releasing Molecules.

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Nitric oxide is a gaseous small molecule, considered for a long time as an environment pollutant. Since the discovery of the physiological role of NO, NO-donor molecules have been developed as therapeutic agents for the treatment of ischemic cardiac disease [1], and recently for treatment of glaucoma, an ocular neurodegenerative disease [2]. In fact, latanoprostene bunod (NO donor prostaglandin analogue - Vyzulta®) was approved for the treatment of glaucoma, working as an ocular hypotensive agent, with greater efficacy than latanoprost [3]. NO has been proven to regulate the vascular tone of ophthalmic artery through a cross-talk with other gases CO and H_2S [4]. Additionally, several studies suggested the role of NO as a neuroprotectant in preclinical models of glaucoma [5–7], although clinical evidence of the NO-donor molecules neuroprotection in glaucoma have not already proven [3,8]. Additionally, NO can regulate the expression of Heme Oxygenase 1 (HO-1) [9]. HO-1 is also known as heat shock protein 32 (HSP32) and is one of the components of cellular defense mechanisms against oxidative stress-mediated injury. Recently, we explored the effect of novel NO-releasing molecules in an in-vitro model of age-related macular degeneration: i.e caffeic acid phenethyl ester NO-releasing derivative (CAPE-NO) and the curcumin NO-releasing derivative (curcumin-NO) [10]. The CAPE-NO was found to be efficacious in decreasing oxidative stress on the retinal pigmented epithelial cells and to induce Heme Oxygenase -1 (HO-1), through the promotion of Nrf2 translocation. In conclusion, the drug design and development of bitopic ligands (e.g Nrf2 inducers or prostaglandin analogues) with NO-releasing moieties could be a good strategy for the treatment of ocular disease.

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The Roles of the Denitrosylase GSNOR in Tumor Progression.

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The modification of protein cysteine residues by Nitric oxide (NO), namely *S*-nitrosylation, has been linked to tumor development and promotion. *S*-nitrosylation is the prototypic redox modification of thiol groups and affects protein functionality acting as a post-translational modification. Although the biology of NO has been extensively studied in the context of cancer research, the major efforts focus on NO biosynthesis by nitric oxide synthases (NOSs), while the role played by enzymes terminating the signal (denitrosylases) has been overlooked. *S*-nitrosoglutathione reductase (GSNOR) is the main denitrosylating system within the cell. By reducing *S*-nitrosoglutathione (GSNO) to glutathione disulfide (GSSG) and ammonia (NH_3), GSNOR indirectly controls the amount of *S*-nitrosylated proteins (PSNOs). Notably, the aberrant *S*-nitrosylation signaling resulting from GSNOR deficiency has been reported to impair the DNA repair machinery, thus driving cell transformation in human liver cancers. We recently found out that GSNOR declines during aging and results in mitochondrial dysfunctions, a typical feature of cancer cells. In the present study we identify GSNOR as a crucial player in tumor progression. From *in silico* and *in vitro* experiments we revealed that the enzyme is highly down-regulated in several human advanced cancers, this being dependent on cancer-overexpressed miRNAs. We found that GSNOR suppression affects the adhesion properties of malignant cells and promotes the epithelial-mesenchymal transition. Furthermore, GSNOR-deficient cancer cells gain the capability of growing in the absence of substrate attachment becoming resistant to *anoikis,* a critical step driving metastasis. On these bases we propose that protein *S*-nitrosylation has a pivotal role in the aggressive phenotype of cancer cells affecting cell adhesion, anchorage-dependent survival and metabolism adaptation. This supports the hypothesis that GSNOR down-regulation can be exploited as a marker of malignancy.

Cancer and Beyond: Discovery and Development of NO-releasing Therapeutics.

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Nitric oxide, the endogenous vasodilator, is a key signaling molecule that has been implicated in many biological processes and disease states. The manipulation of local nitric oxide concentration using drugs that deliver NO has been exploited for the treatment of many disease indications ranging from heart and circulatory disorders through to the treatment and prevention of cancers. Herein, we focus on three drugs that have different mechanisms of NO release, eliciting distinct biological effects leading to contrasting potential applications. NBS-1120, an enhanced NSAID (eNSAID), based on the aspirin scaffold that releases nitric oxide and hydrogen sulfide, JS-K an arylating NO donor, and RRx-001 an immune stimulatory agent that induces NO release indirectly. All three compounds impact the local concentrations of NO, however their ultimate mechanisms of action are complex, arising from the multi-faceted nature of NO and its context-specific activity. Here, we describe the known activity and safety of these NO donors, current hypotheses on their mechanism of actions and their developmental status.

Pro-Apoptotic Effects of S-Nitrosothiols O-Chloro and M-Chloro S-Nitroso-Aryl-Amides in Human Breast Cancer Cell Lines.

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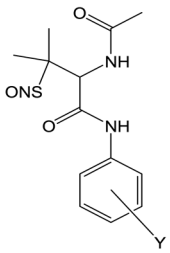
Breast cancer affects women worldwide. Breast cancer tumors are classified as estrogen receptor positive (ER+) or negative (ER-). ER- tumors are generally more aggressive and patients with ER- tumors have a poor prognosis due to unresponsiveness of their tumors to drugs that interfere with estrogen production. Nitric oxide (NO) plays an important role in regulating tumor signaling processes associated with aggressiveness and progression. Increased expression of inducible NO synthase is a poor prognostic marker for breast cancer. Control of intracellular NO production and/or exposure of breast cancer cells to NO donors are potential chemotherapeutic strategies. S-Nitrosothiols, a specific class of NO donors, react with GSH/Cysteine and thiol proteins making them potential chemotherapeutic agents in the treatment of various cancer types. A series of S-nitroso-aryl-amides derivatives of penicillamine that are fairly water soluble were synthesized and used for biological testing (**Scheme I- Derivatives 1-4**) [1]. Conformational analysis of the compounds indicated that the o-chloro (**2**) and m-chloro (**3**) derivatives were the most effective as NO donating compounds. Release of NO by these compounds in PBS, pH 7.4 confirmed this.



Compound	Y	$C_{Nitrosothiol}$ (g/ml)	C_{NO} (g/ml)	% NO
1	H	4×10^{-4}	8×10^{-6}	19.0
2	o-Cl	5×10^{-4}	13.1×10^{-6}	27.0
3	m-Cl	8×10^{-4}	14.4×10^{-6}	30.5
4	MeO	17×10^{-4}	12.8×10^{-6}	7.9

Scheme I

O-chloro and m-chloro derivatives were evaluated for their in vitro cytotoxicity against human breast cancer cells, (MCF7 (ER+) and MDA-MB-231 (ER-)) and non-tumor human mammary epithelial cells (MCF10), and human umbilical vein endothelial cells (HUVEC). The m-chloro derivative displayed a potent cytotoxic effect against both breast cancer cell lines. However, the compound did not affect the viability of MCF10 non-tumor epithelial cells. After 2hs of treatment with the o- and m-chloro derivatives, the cancer cells lines were positive for Annexin V and propidium iodide labeling. HUVEC were negative for Annexin V/propidium iodide. Both compounds produced higher intracellular levels of NO in breast cancer cells when compared to normal cells. Apparently these high concentrations of NO are major contributors to the differential cytotoxicity displayed by these compounds. These findings suggest that the chloro-derivatives of the nitrosothiols s-nitroso-aryl-amides may aid in the design of chemotherapeutic agents used for the treatment of human breast cancer.

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Carbonic Anhydrase Inhibitor – NO Donor Hybrids and Their Pharmacologic Applications.

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Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloproteins widespread in organisms all over the phylogenetic tree. Some of them, such as the isoform CA IX, a membrane-bound, hypoxia-inducible enzyme, are highly expressed in many types of solid tumors, but show a very restricted expression in normal tissues [1]. Many new classes of CA IX inhibitors were reported in the last years, among which sulfonamides, sulfamates, coumarins, sulfocoumarins, polyamines, together with some hybrids also incorporating NO-donating moieties [1-6]. CA IX plays an important functional role in processes critical for tumor growth and metastasis, including pH regulation, survival, adhesion and migration [1, 2]. The tumor-specific expression of CA IX and its association with cancer progression and poor treatment outcome has led to interest in targeting this enzyme for cancer therapy. The development of pharmacologic inhibitors that selectively target this isoform without "off-target" inhibition of cytosolic CAs is critical for their use as cancer therapeutics [1,2]. One of these ureidosulfonamides possessing such properties, SLC-0111, entered in Phase II clinical trials for the treatment of solid metastatic tumors [7].

Sulfonamide CA inhibitor-NO donors belonging to several classes were also shown to possess a significant antiglaucoma activity in animal models of the disease [8,9]. The possibility to use such hybrid drugs in other pathologies such as neuropathic pain [10], arthritis [11] or cerebral ischemia [12] will also be discussed.

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Light Guided Production of Nitric Oxide and Singlet Oxygen for the Multi-modal treatment of cancer.

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Combined cancer therapies exploit either synergistic or additive effects arising from appropriate drug combinations [1]. These treatment modalities have long been adopted as the standard of care for improving the therapeutic efficacy against many cancer types [2]. However, there are significant drawbacks that still limit the use of combination regimens, such as the inadequate control of the delivery process, the insurgence of Multidrug Resistance (MDR) and, most importantly, the different pharmacokinetic properties of the co-administered drugs, which strongly limit the therapeutic effectiveness. Indeed, these problems require the design of novel multi-modal treatments that possibly overcome the paradigm of targeted anti-oncogenic treatment, moving towards a more selective but generalized damaging effect of tumor tissue.

In this respect, the light-controlled generation of reactive oxygen and nitrogen species, such as singlet oxygen ($^1O_2$) and nitric oxide (NO) represents a fascinating alternative.

In recent years, we have explored the possibility of delivering both singlet oxygen and nitric oxide through different chemical and (nano)-technological approaches,[3,4] both comprising the use of light as a tool for the production and control of the cytotoxic effect [Figure 1]. In this conference, we will provide an overview of our most recent results in this field highlighting the potential impact of our approach on cancer therapy.

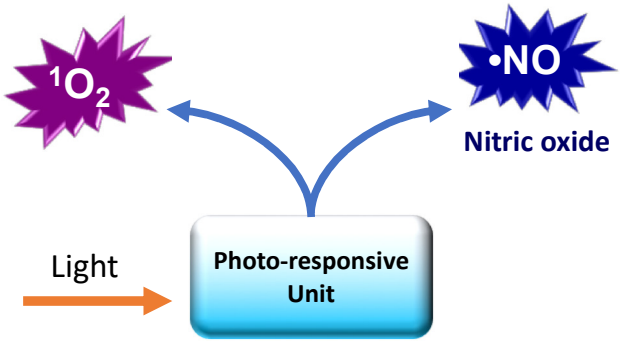

Figure 1. Schematic representation of a molecular or macro-molecular entity able to produce both nitric oxide and single oxygen upon light irradiation

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The Role of Nitric Oxide on Vascular Dysfunction During Aging and Alzheimer's Disease.

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Nitric oxide (NO) is an endogenously synthesized free radical involved in a plethora of physiological phenomena affecting cardiovascular homeostasis and neural functions. Its unregulated bio-availability has been recognized as a key factor in neurodegenerative disorders, especially in relation to the mechanisms through which NO-mediated vascular impairment accentuates the effect of reactive oxygen species. Interestingly, the recent literature indicates the pivotal role of NO and oxidative stress to both early and advanced stages of neurodegenerative disorders, as well as promoting their progression. Alzheimer's disease (AD) is the most common form of dementia, characterized by extracellular amyloid (Aβ) plaques and intracellular neurofibrillary tangles coupled with reactive microgliosis, loss of neurons and synapses in the cortex. However, the recent research supports the hypothesis of the pivotal role of NO depletion in the reduction of extracranial blood flow and impairment of cortical and peripheral circulation with a consequent detriment of cognitive function in humans with AD. It is worth mentioning that AD, the leading form of dementia, continues to elude the scientific 'armada' devoted on fighting this disruptive neurodegenerative disease, particularly with respect to its multifaceted origin. Although research on the biological mechanisms underlying the cause and the progression of AD is advancing, we are far to discover effective disease-modified approaches. Currently, there is no absolute cure for AD: the few drugs available simply lessens the clinical symptoms. Therefore, new therapeutic approaches, including NO homeostasis, should be considered and might be useful as therapeutic targets.

Potential for Chemoprevention with Hydrogen Sulfide-Releasing Drugs?

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Nitric oxide derived from inducible NO synthase has been suggested to promote colon cancer by stimulating angiogenesis, but NO has also been identified as a potential anti-oncogenic agent. Hydrogen sulfide (H2S), another naturally occurring gaseous mediator, has similarly been shown to exert apparently opposing actions with respect to colon cancer development. We undertook a range of studies to determine the utility of H2S-releasing non-steroidal anti-inflammatory drugs (NSAIDs) in animal models of colon cancer. Nonsteroidal anti-inflammatory drugs have been shown to reduce the incidence of gastrointestinal cancers, but the propensity of these drugs to cause gastrointestinal ulcers and bleeding limits their use.

First, we explored the possibility that a H2S-releasing derivative of naproxen (ATB-346) would be effective in a rat model (azoxymethane) of aberrant crypt foci (ACF). Weekly administration of azoxymethane over a 4-week period resulted in formation of an average of 50 aberrant crypt foci in the colon. Twice-daily treatment with naproxen at high doses significantly reduced the number of aberrant crypt foci. However, a significantly greater effect was observed with ATB-346 and it was also effective at much lower doses, where naproxen was ineffective. Administration of the H2S-releasing moiety alone did not significantly affect the number of aberrant crypt foci, suggesting that both the inhibition of cyclooxygenase activity and release of H2S were necessary for the enhanced chemopreventive effect. ATB-346 suppressed colonic prostaglandin synthesis and whole blood thromboxane synthesis as effectively as naproxen, but in contrast to naproxen, it did not induce gastrointestinal injury.

To extend these studies, we tested the effects of the H2S-releasing naproxen derivative in a murine model of hereditary intestinal cancer, the APCMin+ mouse. Daily oral administration of ATB-346 was significantly more effective at preventing intestinal polyp formation than naproxen. Significant beneficial effects were seen with a treatment period of only 3–7 days, and a reduction of size/number of existing polyps was observed after treatment with ATB-346. A significant decrease in the expression of intestinal cancer-associated signaling molecules (cMyc, β-catenin) was observed after treatment with ATB-346, but not with naproxen. Transcriptomic analysis identified 20 genes that were up-regulated in APCMin+ mice, 18 of which were reduced to wild-type levels by one week of treatment with ATB-346.

ATB-346 is a novel anti-inflammatory drug that exhibits chemopreventive effects in animal models of colon cancer. In contrast to conventional NSAIDs, ATB-346 does not induce significant gastrointestinal injury, as demonstrated in a recent phase 2 clinical trial.

H_2S Metabolism in Colon Cancer Cells Exposed to Hypoxia.

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Hydrogen sulfide (H_2S), after nitric oxide (NO) and carbon monoxide (CO), is known as the third gasotransmitter [1]. Involved in antioxidant protection and regulation of different physiological functions, H_2S is synthesized by cystathionine β-synthase (CBS) and few other enzymes, and is oxidatively catabolized in the mitochondrion, where H_2S-derived electrons are transferred to coenzyme Q by sulfide quinone oxidoreductase (SQR), stimulating respiration and ATP production. Metabolic and oncologic diseases are related to dysregulation of the H_2S metabolism. Hence, the importance of understanding how H_2S bioavailability is regulated under (patho)physiological conditions. We previously reported that both CO and NO negatively modulate CBS with high affinity [2] especially in the presence of its allosteric activator S-adenosyl-L-methionine [3], and that a CBS variant responsible for classical homocystinuria shows an unusual high propensity to CO inhibition, suggesting a novel pathogenic mechanism [4]. Here, by investigating the effect of hypoxia on H_2S catabolism in colon cancer, we report that hypoxia-treated SW480 cells have overall reduced ability to detoxify H_2S and less mitochondria but enriched in SQR. These data suggest that under hypoxic conditions, while the lower sulphide-detoxifying activity contributes to ensure higher protective H_2S levels against O_2 deprivation, the mitochondria get enriched in SQR to afford protection from H_2S poisoning.

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