Abstract

A key step in malignant progression is the acquired ability of tumour cells to escape immune-mediated lysis. A potential mechanism by which tumour cells avoid immune destruction involves the shedding of MHC Class I Chain-Related Protein A (MICA), a Natural Killer (NK) cell-activating ligand, from the tumour cell membrane. Hypoxia has been shown to cause increased MICA shedding; however, this hypoxia-induced effect can be attenuated by pharmacological activation of the cyclic guanosine monophosphate (cGMP)-dependent nitric oxide (NO)-signalling pathway in cancer cells. The primary objective of the present study was to determine whether treatment of tumour-bearing nude mice with the NO-mimetic glycercyl trinitrate (GTN) attenuates in vivo tumour growth and if so, whether this effect is dependent on the presence of an intact NK cell compartment. Results indicated that continuous transdermal administration of GTN (1.8 µg/h) can significantly attenuate the growth of transplanted human DU-145 prostate tumours but that this effect of GTN is lost in mice whose NK-cells have been depleted. Tumours and serum from the mice in this study were analysed to determine whether GTN treatment had any effect on the expression levels of proteins integral to the proposed MICA shedding mechanism; however, the results of these studies were inconclusive. As phosphodiesterase (PDE) inhibition represents a potential method to enhance NO-signalling, experiments were performed to determine whether treatment with the PDE5/6 inhibitor zaprinast could also attenuate hypoxia-induced MICA shedding and decrease in vivo growth of DU-145 tumours. Results demonstrated that treatment with zaprinast (10 mg/kg) significantly attenuates MICA shedding in DU-145 cancer cells and significantly decreases in vivo tumour growth. Taken together, the results of these experiments indicate that GTN attenuates tumour growth by sensitising tumour cells to innate immunity, likely by increasing membrane-associated tumour cell MICA levels through the
reactivation of NO-signalling, and that zaprinast decreases tumour growth likely through a similar mechanism. These findings are important because they indicate that agents capable of reactivating NO-signalling, such as NO-mimetics and PDE inhibitors, can potentially be used as immunosensitisers in the treatment and/or prevention of cancer.
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# Table of Contents

Abstract..................................................................................................................................................ii  
Acknowledgements............................................................................................................................iv  
Statement of Contribution...................................................................................................................v  
List of Abbreviations............................................................................................................................viii  
List of Figures........................................................................................................................................xii  

Chapter 1 Introduction .........................................................................................................................1  

1.1 Cancer Immunobiology ..................................................................................................................1  
   1.1.1 Cancer Immune Surveillance.................................................................................................1  
   1.1.2 Immunoediting......................................................................................................................4  

1.2 NKG2D-NKG2DL Interactions .........................................................................................................9  
   1.2.1 NK Cells and the NKG2D Receptor ....................................................................................9  
   1.2.2 MHC Class I Chain-Related Protein A (MICA) .................................................................11  
   1.2.3 MICA Shedding and the Role of ERp5 and ADAM10 .........................................................12  

1.3 Tumour Hypoxia ............................................................................................................................13  
   1.3.1 Hypoxia Inducible Factor-1 (HIF-1) ..................................................................................15  
   1.3.2 Cellular Adaptations to Hypoxia .......................................................................................16  

1.4 Nitric Oxide (NO)-Signalling .........................................................................................................17  
   1.4.1 Therapeutic Applications of cGMP-Dependent NO-Signalling Activation .......................19  

1.5 Prostate Cancer & Biochemical Failure .......................................................................................23  
   1.5.1 Preclinical Models of Prostate Cancer ..............................................................................27  
      1.5.1.1 Cell Lines .....................................................................................................................27  
      1.5.1.2 Murine Xenograft Models ..........................................................................................28  
   1.5.2 NO-Mimetics as a Potential Therapy for Biochemical Failure ........................................28  

1.6 Hypotheses ...................................................................................................................................29  

1.7 Objectives .....................................................................................................................................30  

Chapter 2 Materials & Methods .........................................................................................................33  

2.1 Cell Culture...................................................................................................................................33  
   2.1.1 Exposure to Hypoxia ..........................................................................................................33  

2.2 Animal Procedures ......................................................................................................................33  
   2.2.1 Xenograft Implantation ........................................................................................................33
2.2.2 Treatment with Glyceryl Trinitrate (GTN) ................................................................. 34
2.2.3 Treatment with Zaprinast .......................................................................................... 34
2.2.4 Depletion of Natural Killer (NK) Cells ...................................................................... 35
2.2.5 Measurement of Tumours ......................................................................................... 36
2.2.6 Sacrifice and Necropsy ............................................................................................. 36
2.2.7 Isolation and Fractionation of Mononuclear Cell Populations ................................. 37
2.3 Flow Cytometry ............................................................................................................ 38
2.3.1 Dissociation of Tumour Tissue ................................................................................ 39
2.3.2 Cell Cultures Treated with Zaprinast ...................................................................... 39
2.4 Immunoblotting ............................................................................................................ 40
2.5 Enzyme Linked Immunosorbent Assay (ELISA) .......................................................... 42
2.6 Quantitative Polymerase Chain Reaction .................................................................... 43
2.7 Clinical Trial Design .................................................................................................... 43
2.8 Calculations and Statistical Analysis ............................................................................. 45
Chapter 3 Results .............................................................................................................. 46
3.1 NO-Mimetic Studies ..................................................................................................... 46
3.1.1 Effect of GTN and 8-Br-cGMP on HIF-1α Accumulation and ADAM10 Expression in DU-145 Cells ........................................................................................................... 46
3.1.2 DU-145 Tumour Growth in Mice Treated with GTN and Anti-Asialo GM1 .............. 49
3.1.3 Characteristics of Tumours from Mice Treated with GTN and Anti-Asialo GM1 .... 49
3.1.4 Protein levels in Tumours from Mice Treated with GTN and Anti-Asialo GM1 ...... 53
3.1.5 Serum Levels of MICA from Mice Treated with GTN and Anti-Asialo GM1 .......... 53
3.2 PDE Inhibitor Studies .................................................................................................. 58
3.2.1 Effect of Zaprinast Treatment on Surface MICA Expression in DU-145 Cells ........ 58
3.2.2 DU-145 Tumour Growth in Mice Treated with Zaprinast ......................................... 58
3.2.4 Serum Levels of MICA from Mice Treated with Zaprinast ........................................ 60
Chapter 4 Discussion ........................................................................................................... 64
4.1 General Discussion ...................................................................................................... 64
4.2 Summary and Conclusions ......................................................................................... 71
Chapter 5 Future Directions .............................................................................................. 72
References ......................................................................................................................... 74
List of Abbreviations

ADAM: a disintegrin and metalloproteinase
AR: androgen receptor
CaP: prostate cancer
(c)AMP: (cyclic) adenosine monophosphate
(c)GMP: (cyclic) guanosine monophosphate
CTL: cytotoxic T lymphocyte
DAP10: DNAX-activating protein of 10 kDa
DAP12: DNAX-activating protein of 12 kDa
DC: dendritic cell
DETA·NO: diethylenetriamine nitric oxide adduct
ELISA: enzyme linked immunosorbent assay
ERp5: endoplasmic reticulum protein 5
GM-CSF: granulocyte/macrophage-colony stimulating factor
GTN: glyceryl trinitrate (nitroglycerin)
HIF-1: hypoxia inducible factor-1
HLA: human leukocyte antigen
HRE: hypoxic response element
IFN-γ: interferon-γ
IL-10: interleukin 10
IL-12: interleukin 12
LH-RH: luteinising hormone-releasing hormone (agonists)
MAB: maximum androgen blockade
MCA: methylcholanthrene
MHC: major histocompatibility complex
MIC: MHC class I-chain-related proteins
MICA: MHC class I chain-related protein A
MICB: MHC class I chain-related protein B
Mult1: murine UL16-binding protein-like transcript 1
NK cell: natural killer cell
NKG2D: natural killer group 2, member D (receptor)
NKG2DL: NKG2D ligands
NKT cell: natural killer T lymphocyte
NO: nitric oxide
NOS: nitric oxide synthase
NRS: normal rabbit serum
PAI-1: plasminogen activator inhibitor-1
PBL: peripheral blood lymphocyte
PDE: phosphodiesterase
PHD: prolyl hydroxylase domain containing enzymes
PI3K: phosphotidylinositol-3-kinase
PKG: cGMP-dependent protein kinase
PSA: prostate specific antigen
PSADT: PSA doubling time
pVHL: von Hippel-Lindau tumour suppressor protein
RAE-1: retinoic acid early transcript-1
RAG-2: recombinase activating gene-2
RNS: reactive nitrogen species
ROS: reactive oxygen species
RP: radical prostatectomy
SD: standard deviation
SEM: standard error of the mean
sGC: soluble guanylyl cyclase
sMICA: soluble MICA
TAM: tumour-associated macrophage
TGF-β: transforming growth factor-β
Th1: T helper-1 lymphocyte
TNF: tumour necrosis factor
TNF-α: tumour necrosis factor-α
TNFSF: tumour necrosis factor (receptor) superfamily
TRAIL: TNF-α-related apoptosis-inducing ligand
ULBP: UL-16 binding protein
uPAR: urokinase-type plasminogen activating receptor
qPCR: quantitative polymerase chain reaction
XRT: external beam radiation therapy
# List of Figures

Figure 1.1 The Three Stages of Immunoediting.................................................................6
Figure 1.2 Characteristics of a Hypoxic Tumour Mass......................................................14
Figure 1.3 Endogenous Nitric Oxide Synthesis and Signalling.............................................18
Figure 1.4 The Dual Nature of Nitric Oxide........................................................................22
Figure 1.5 Proposed Mechanism of Immune Escape and NO-Mimetic Action......................31

Figure 3.1 Effect of GTN and 8-Br-cGMP on HIF-1α Accumulation and ADAM10 Expression in DU-145 Cells..................................................................................................................47
Figure 3.2 DU-145 Tumour Growth in Mice Treated with GTN and Anti-Asialo GM1............50
Figure 3.3 Characteristics of Tumours from Mice Treated with GTN and Anti-Asialo GM1....52
Figure 3.4 Protein Levels in Tumours from Mice Treated with GTN and Anti-Asialo GM1....54
Figure 3.5 Serum Levels of MICA from Mice Treated with GTN and Anti-Asialo GM1........56
Figure 3.6 Effect of Zaprinast Treatment on Surface MICA Expression in DU-145 Cells........59
Figure 3.7 DU-145 Tumour Growth in Mice Treated with Zaprinast ....................................61
Figure 3.8 Serum Levels of MICA from Mice Treated with Zaprinast .................................62

Figure 4.1 Proposed Mechanism of NO-Signalling-Mediated Attenuation of HIF-1α Accumulation..........................................................................................................................69
Chapter 1

Introduction

1.1 Cancer Immunobiology

1.1.1 Cancer Immune Surveillance

The concept that the immune system acts as an extrinsic tumour suppressor by preventing the proliferation of neoplastic cells was first proposed by Ehrlich [65] in the early 20th century. Half a century later, both Thomas [228] and Burnet [30-33] independently formalized this idea into what is now known as the cancer immune surveillance hypothesis. Due to the immaturity of the field of immunology and the lack of appropriate models at the time, it was initially difficult to test the validity of this idea. By the 1970’s however, the field had advanced to the point where Stutman [219-221] and others [34, 80, 171, 200, 230] were able to conduct the experiments that would ultimately lead to the general abandonment of the theory for over a decade. The former’s experiments were particularly important as they represented the first extensive investigation into physiological cancer immune surveillance using immune-deficient mice.

Stutman’s experiments were designed to determine whether hypothymic mice, the only congenitally immune-deficient mouse strain available at that time, were more susceptible to either carcinogen-induced (methylcholanthrene [MCA]) or spontaneous neoplasms when compared with wild-type controls. The running hypothesis was that if the immune system contributed substantially to the suppression of incipient neoplasms, then the immune-deficient mice should have a higher tumour incidence and/or lower tumour latency period when compared with their
immune-competent counterparts. The results of the studies conducted by Stutman did not support this hypothesis.

Retrospectively, we are able to look back and identify several confounders that likely contributed to what is now recognized to be a falsely negative conclusion. First, hypothymic nude mice represent an imperfect model of immune deficiency: they retain small populations of T lymphocytes and have an intact innate immune system (NK cells had not been well described at the time). The innate immune response itself, in conjunction with its stimulatory effects on the residual (albeit weakened) adaptive immune system, may have conferred a sufficient level of protection in these mice to obfuscate any real difference in susceptibility to neoplastic formation. Furthermore, the mouse strain used in the experiments was subsequently shown to express a highly-active isoform of the enzyme responsible for the conversion of MCA into its carcinogenic form. The highly efficient production of this carcinogen in vivo may have acted to further mask any protective effects of the immune system by increasing the likelihood of cancer arising in the immune-competent mice. Unfortunately, because these confounders were not appreciated at the time, the negative results published by Stutman caused the cancer immune surveillance theory to fall out of favour with the scientific community.

In the mid 1990s, the slowly growing appreciation for the limitations that had plagued the Stutman experiments, coupled with the growing selection of inbred mouse strains with specific immune deficiencies, led to a renewal of interest in the idea of cancer immune surveillance. Experiments similar in design to those originally conducted by Stutman, but with more appropriate models, were now successful in demonstrating that mice insensitive to IFN-γ (a
cytokine integral to the coordination of innate and adaptive immune systems) [110, 217, 218] or lacking perforin expression (a key component of the cytotoxic granules found in immune effectors) [212, 217, 232] were both significantly more susceptible to both spontaneous and chemically (MCA)-induced neoplasms. In 2001, another landmark study was published using RAG-2 knockout mice [203]. RAG-2 (Recombinase Activating Gene-2) expression is required for the rearrangement of antigen receptors on various lymphocyte populations including T, B and NKT cells; mice in which this gene has been knocked out are devoid of all three lymphocyte subpopulations and thus severely immune deficient [207]. The results of the study showed that RAG-2 knockout mice, after exposure to MCA, formed sarcomas significantly more quickly than did wild type mice. The same group was also able to show that these mice were more susceptible to spontaneous intestinal malignancies as well. The results from this sampling of studies contributed substantially to what had now become a growing body of evidence that immune responses played an important role in suppressing cancer. Taken together, the experiments conducted in the 1990s and early 2000s provided strong evidence for the presence of multiple, overlapping mechanisms of IFN-γ, perforin and lymphocyte dependent anti-tumour activity in mice, thus indicating that both the adaptive and innate branches of the immune system likely contributed to cancer detection and elimination (i.e. immune surveillance) in these models.

While by this point a substantial body of evidence indicating that natural immune responses played an important role in suppressing cancer in mice had been compiled, it remained unclear whether corresponding processes existed in humans. Several observational and in vitro studies were performed to address this question and ultimately led to three distinct areas of evidence that together, provide support to the hypothesis that cancer immune surveillance also exists in
humans: first, it was shown that the presence and degree of lymphocytes [47, 48, 155, 168] within a tumour (dubbed “tumour infiltrating lymphocytes”) could be used as a positive prognostic indicator, thus suggesting that these immune cells were involved in combating expanding neoplasms; second, many human tumour-associated or tumour-specific antigens were (and continue to be) identified and it has been shown that spontaneous innate and adaptive immune responses can develop against cancer cells expressing these antigens [22, 118, 194, 233]; third, immune-suppressed transplant recipients were shown to be at increased risk of developing malignancies with no known viral cause, indicating either an increased propensity for de novo tumour formation or the unhampered outgrowth of occult neoplasms that had previously been suppressed by either the donor or recipient’s intact immune system [16, 177, 204].

Despite its controversial past, the data available from the mouse experiments coupled with those from in vitro and observational human experiments have provided convincing evidence that cancer immune surveillance does indeed exist and that it occurs in both mice and humans. In the future, knowledge of the pathways by which cancer develops resistance to immune-mediated killing will be important in the development of new therapeutics able to help our immune system better identify and eliminate these rogue cell populations.

1.1.2 Immunoediting

After it was generally accepted that the immune system acts to protect the host from neoplastic formation, the fact remained that cancers did still arise in immune-competent individuals; accordingly, shortly after the resurgence of interest in the cancer immune surveillance theory, it was realized that the immune system’s relationship with cancer consisted of more than just as an
extrinsic tumour suppressor mechanism. It was theorized that the immune system also served as a
selection mechanism by which more immunogenic clonal variants were destroyed while leaving
their less immunogenic counterparts to flourish and repopulate. A pivotal experiment supporting
this hypothesis was done by Shankaran, et al. [203] in which sarcomas were induced by
administration of a chemical carcinogen in both wild type and RAG-2 knockout mice. Cells from
these tumours were subsequently transplanted into non-tumour bearing wild type mice. While all
the tumours generated in the wild type mice grew after transplantation, only 40% of the tumours
generated in the immune-deficient mice formed tumours after transplantation. These results
supported the hypothesis that cancer cells are influenced by their immunological environment by
demonstrating that tumours grown in the absence of an immune system are more immunogenic
than those that arise in an immune-competent animal. This idea has since been supported by
studies conducted by several groups [66, 212, 223, 224, 232].

The discovery that the immune system acts not only to protect the host from cancer, but also to
sculpt the immunogenicity of the tumour cell population led the development of a theory known
as immunoediting. While this theory incorporates the concept of cancer immune surveillance, it is
broader in scope and focuses on the idea that the immune system plays two different roles in its
relationship with cancer. Immunoediting, as originally proposed by Dunn, et al. [62], consists of
three separate phases: elimination, equilibrium and escape (Fig. 1.1).

The first phase, elimination, represents the original concept of immune surveillance. This stage
begins when the immune system recognizes the presence of non-self or altered-self through
interactions involving tumour-associated or tumour-specific antigens and leading ultimately to the
Figure 1.1 The Three Stages of Immunoediting.

The immunoediting theory models the relationship between the immune system and cancer using three distinct stages. (1) The first stage, elimination, is the successful detection and removal of nascent transformed cells by the host’s immune system (*extrinsic* tumour suppression). This protective process is thought to occur continuously throughout the life of an organism. (2) If elimination fails, the lesion proceeds to the equilibrium stage. Pre-cancerous cells can remain in this stage for years during which the immune system acts as a selective pressure, eliminating more immunogenic clones while allowing less immunogenic variants to flourish. (3) A lesion proceeds to the escape stage when it has acquired the ability to grow unchecked by the host’s immune system. It is at this stage when most cancers become clinically detectable. Adapted from Dunn *et al.* [64].
release of IFN-γ. The second step begins as effectors of the innate immune system (e.g. NK cells) are attracted by IFN-γ and begin to lyse tumour cells through perforin and TRAIL-mediated mechanisms. As step two takes place, dead tumour matter is made available to dendritic cells and other antigen presenting cells (APCs) in the area. These cells endocytose the loose, dead tumour antigens and migrate to regional lymph nodes where, in what is considered the third step, naïve Th1 CD4⁺ T lymphocytes are primed to recognize them. The process is completed as the CD4⁺ T lymphocytes train cytotoxic CD8⁺ T lymphocytes to recognize the same tumour antigens eventually leading to the elaboration of a full adaptive immune response against the tumour cells that express them. In the best-case scenario, this four-step process leads to the complete elimination of the neoplasm and protection of the host. In some cases however, certain tumour cells are able to escape recognition and elimination by the immune system and continue onto the second phase of the immunoediting process, equilibrium.

According to the immunoediting theory, tumour cells that are able to escape elimination are thought to enter a dynamic equilibrium with the immune system. During this time, it is hypothesised that constant, yet ultimately ineffective, immune pressure on these cells forms what has been aptly termed a “crucible of Darwinian selection” [63] in which more immunogenic variants are eliminated and less immunogenic variants are able to more easily proliferate. This phase is imagined to be the longest of the three phases and may occur over the course of twenty years or more, during which the disease would not yet be clinically apparent. It is also possible that the disease will never progress beyond this stage and the host will live to old age and die of unrelated causes. While this phase is the most theoretical and has not been conclusively demonstrated, some evidence in support of such an equilibrium process has been gathered from
the studies on the transmission of cancer from transplant donors to recipients. These studies provide evidence that an immune-suppressed environment, such as that found in transplant recipients, can allow the proliferation of occult tumours that had previously been suppressed by the immune system of the transplant donor.

The final stage of the immunoediting process is escape and occurs when the cancer cells have acquired the ability to proliferate within an immunologically intact environment after continual selection during the equilibrium stage. Due to the evidence supporting the anti-cancer activity of both innate and adaptive branches of the immune system, these selected cells must develop multiple ways in which to evade destruction by immune effectors. There are many indirect and direct methods of immune escape that have been shown to develop in cancer cells: indirect immunosuppression through the manipulation of regulatory T lymphocytes or the elaboration of immunosuppressive cytokines such as TGF-β and IL-10 [115, 195]; alterations in factors affecting tumour cell recognition including loss of antigen expression, loss of Major Histocompatibility Complex (MHC) antigens [149] and shedding of NKG2D ligands [87, 210]; direct evasion of tumour destruction by the over-expression of anti-apoptotic signals [40]; or through defects in death-receptor mechanisms [224]. It is during this phase when cancers most commonly become clinically detectable. Understanding the cellular and molecular basis of the mechanisms by which cancer evades the immune system can help with the development of therapeutics designed to restore immunogenicity to cancer cells and thus augment the natural power of the immune system against them.
1.2 NKG2D-NKG2DL Interactions

1.2.1 NK Cells and the NKG2D Receptor

The innate immune system’s role in cancer immune surveillance is played predominantly by a subset of lymphocytes (CD3^− CD56^+) known as Natural Killer (NK) cells. They were initially discovered in 1975 in mice and were shown to be a distinct subset of immune effectors capable of killing tumour cells without prior sensitisation [93, 183].

In contrast to the adaptive T lymphocyte response, which recognizes potentially troublesome cells through an MHC-dependent antigen-presentation mechanism, NK cells detect their targets through two complementary mechanisms: missing-self and induced-self. Missing-self indicates the ability of NK cells to detect the loss of MHC markers on the surface of damaged and/or infected cells and to target these for elimination, a concept originally demonstrated by an experiment showing that syngeneic tumour cells deficient in MHC expression were selectively rejected by NK cells [72, 111, 144, 258-261]. This ability of NK cells is important because it allows them to identify and destroy cells that are less sensitive to the actions of T lymphocytes due to their reduced MHC expression. The other mechanism used by NK cells in the recognition of potentially dangerous cells is the process known as induced-self. This is the idea that cells can be induced to express NK-activating ligands that specifically trigger NK activity [8, 54-57, 186-188]. Further research along with the characterization of numerous ligands able to inhibit NK activity has led to the development of the current theory that NK activation depends on a careful balance of inhibitory and activating NK ligands [120-123].
Once activated, NK cells have been shown to kill either through a perforin- and granzyme-mediated apoptotic mechanism [136, 234] or through the interactions of members of the tumour necrosis factor (TNF) superfamily (TNFRSF) with their corresponding ligands on target cells (e.g. TRAIL/TRAIL receptor, Fas/CD95) [234, 256]. Activated NK cells also exert secondary effects through the secretion of various chemokines and cytokines (e.g. IFN-γ, TNF, GM-CSF) [17, 58]. Their role as one of the primary sources of IFN-γ is important for the coordination of the innate and adaptive arms of the immune system; specifically, IFN-γ has been shown to be critical in the priming process of T helper 1 (Th1)-biased T lymphocyte responses [153] as well as for the transition of dendritic cells (DC) to an IL12-producing DC1 phenotype required for an effective anti-tumour CD8⁺ T lymphocyte response [2, 153].

While several NK receptors on the surface of NK cells have been described, the natural killer group 2 member D (NKG2D) receptor has by far received the most attention. The NKG2D receptor is a homodimeric, C-type lectin-like activating receptor expressed on nearly all NK cells, CD8⁺ αβ T cells and γδ T cells as well as on a minority of CD4⁺ αβ T cells [8]. NKG2D associates with an adaptor protein known as DNAX-activating protein of 10 kDa (DAP10) [74, 242, 244]. Engagement of an activating NKG2D ligand (NKG2DL) leads to phosphorylation of the DAP10 adaptor ultimately resulting in NK cell activation, T lymphocyte co-stimulation and phosphatidylinositol-3-kinase (PI3K) pathway activation [243, 244]. In mice, there is a splice variant of NKG2D, referred to as NKG2D-S, which can associate with the related adaptor protein DNAX-activating protein of 12 kDa (DAP12). Engagement of the NKG2D-S/DAP12 complex also leads to cytokine release and NK activation [55, 77, 191].
One important feature of the NKG2D receptor is the fact that it is able to bind many different ligands. In humans, the ligands of NKG2D include the UL16-binding proteins (ULBP) and two MHC class I chain-related proteins (MICA and MICB) [4, 5, 139, 140]. The ligands bound by NKG2D in mice differs from those in humans and include the Retinoic acid early transcript (Rae)-1 family of proteins (Rae1-α, -β, -γ, -δ, -ε), the H60 ligands [41, 42, 55] and murine UL16-binding protein-like transcript 1 (Mult1) [37, 38, 55]. Despite these differences between NKG2D ligands in mice and humans, it is important to note that the NKG2D receptor itself is highly evolutionary conserved and murine NKG2D has been shown to be capable of interaction with human NKG2DLs, such as MICA [247].

Expression of NKG2D ligands is indicative of cellular stress that can be induced by heat shock, infection, ultraviolet radiation or DNA damage. Furthermore, certain NKG2D ligands, namely MICA and MICB, are expressed by many tumour cell lines and epithelial cancers but not in their corresponding healthy tissues [82, 84, 108, 197, 198, 239]. These observations have led to the theory that NKG2DL expression is a way for the host to counteract neoplastic formation by identifying malignant cells for destruction by the immune system.

1.2.2 MHC Class I Chain-Related Protein A (MICA)

MICA is an important NKG2D ligand, the expression of which contributes to tumour cell recognition and destruction by NK cells. The human MICA gene is located in the human leukocyte antigen (HLA) class I region of chromosome 6 [214]. It codes for an activating ligand of the NKG2D receptor (discussed above). Considered a “nonclassical” HLA class I gene, the MICA sequence shows only around 30% homology with its “classical” counterparts [208]. It is
known to be a highly polymorphic gene with over 50 recognized MICA alleles in the GenBank
data base (http://www.ncbi.nlm.nih.gov) [190]. Like other NKG2DLs, the MICA protein is
considered a stress-induced antigen because its expression can be induced by microbial infection
(bacterial [229, 235] or viral [244]), heat shock [84] and genotoxic stresses incurred by exposure
to chemotherapeutic drugs or ionizing radiation [75]. MICA expression is also increased in cancer
cells, especially in those of epithelial origin [82, 108, 198, 239]. However, there are various
tissues on which MICA is normally constitutively expressed; these include the gastric epithelium,
endothelial cells, and fibroblasts [82, 269]. MICA is an important component of the mechanism
that enables immune effectors to identify and destroy transformed cells; therefore, a decrease in
MICA surface expression on stressed or transformed cells can lead to an impaired immune
response against them.

1.2.3 MICA Shedding and the Role of ERp5 and ADAM10

One potential mechanism used by MICA-expressing tumours to decrease the efficacy of immune
responses against them is by “shedding” surface-bound MICA [242]. This “shedding” hinders
effective immune responses by reducing the amount of MICA available for engagement by NK
cells and other effectors that express the NKG2D receptor; additionally, upon release, this now
soluble MICA (sMICA) can act as a systemic immunosuppressive agent by inactivating NKG2D-
expressing immune effectors, such as NK cells [59, 86, 87, 198]. The process of MICA shedding
begins with the binding of endoplasmic reticulum protein 5 (ERp5), a protein disulfide isomerase,
to the α3 domain of MICA. The formation of the ERp5-MICA complex leads to a conformational
change in MICA that exposes the cleavage site on the MICA molecule [109]. This exposure
allows the proteolytic cleavage of MICA from the cell membrane; a process carried out by
members of the “a disintegrin and metalloproteinase” (ADAM) family of enzymes; the expression of these proteins is commonly up-regulated and it is thought that these enzymes are likely involved in various aspects of malignant progression [242].

1.3 Tumour Hypoxia

The tumour microenvironment plays an important role in malignant progression. Tumour hypoxia (regions of low tissue oxygenation) in particular, defined as an oxygen tension level less than 10 mm Hg [236], has been shown to be associated with various malignant phenotypes including enhanced metastatic and invasive ability and increased resistance to both chemo- and radio-therapy [81, 226]. In fact, studies have shown tumour hypoxia to be an independent indicator of poor outcome in many cancers including those of the cervix [96, 97], pancreas [102], prostate [165], head and neck [237] and breast [147, 237], among others.

Hypoxia develops in tumours as the rapidly proliferating mass outstrips the rate of new vessel formation. As the tumour tissue expands away from the nearest capillary it will eventually exceed the oxygen diffusion limit (~150 µm [68, 233]) leading to oxygen and glucose deprivation which, if too severe, can lead to cell death [25] (Fig. 1.2). This can be appreciated histologically by the presence of necrotic cores in the centres of many solid tumours (evidence of anoxia-induced cell death). In addition, the tumour-associated vasculature tends to be relatively sparse with inefficient distribution and with a large variation in flow velocity [53]. These perfusion issues are also thought to contribute substantially to the development of hypoxia within solid tumours.
A common property of solid tumours is deficient perfusion. The O$_2$ gradient produced by this leads to anoxia-induced necrosis in the tumour core and regions of hypoxic cells in between this core and the well-perfused regions. As the distance increases from the nearest capillary, decreased O$_2$ levels lead to an increase in HIF-1$\alpha$ accumulation and gradual increases in tissue acidity, dependence on glycolytic pathways and resistance to chemo- and radio-therapy. In the above diagram, HIF-1$\alpha$ expression is depicted in a stepwise fashion because its expression behaves like an O$_2$-sensitive on-off switch (i.e. once O$_2$ drops below a certain level, HIF-1$\alpha$ is turned “on”). In contrast to HIF-1$\alpha$, the other characteristics develop more gradually as the level of tissue O$_2$ decreases and are thus depicted as gradients. Adapted from Biel et al. [25].
1.3.1 Hypoxia Inducible Factor-1 (HIF-1)

The primary oxygen sensor involved in the initiation and regulation of cellular responses to hypoxia is the transcription factor hypoxia-inducible factor 1 (HIF-1) [245]. HIF-1 is a heterodimer consisting of a constitutively expressed β subunit (also known as ARNT) and an oxygen-sensitive α subunit. HIF-1α is expressed under normal oxygen conditions; however, it is hydroxylated by prolyl-4-hydroxylase domain (PHD)-containing proteins; this important, oxygen-dependent post-translational modification leads to the degradation of HIF-1α by the von Hippel-Lindau (VHL) tumour suppressor protein [105, 107]. Under hypoxic conditions, there is insufficient free oxygen to act as a substrate for this degradation mechanism enabling the stabilization and accumulation of HIF-1α. Stabilized HIF-1α binds to HIF-1β and migrates to the nucleus where it interacts with regions on the genome known as hypoxia response elements (HREs). This interaction leads to the transcription of HIF-target genes involved in angiogenesis, metabolism, proliferation, metastasis and differentiation [185]. HIF-1α is increased in human bladder, breast, colon, glial, ovarian, pancreatic, prostate, renal and hepatocellular tumours relative to normal tissue controls [225].

Clinical studies have also shown that increased HIF-1α expression is correlated with a poorer outcome in patients with head and neck cancer, nasopharyngeal carcinoma, pancreatic, breast, cervical, osteosarcoma, endometrial, ovarian, bladder, glioblastoma, colorectal and gastric carcinomas [6, 15, 99, 103, 157, 174, 201, 205, 227, 249, 254, 262]. These data, along with the fact that hypoxia is the most well recognized mechanism of HIF-1α activation in tumours [185],
provide evidence that the development of hypoxia and the subsequent accumulation of HIF-1α occur frequently in cancer and likely play an important role in tumour progression.

1.3.2 Cellular Adaptations to Hypoxia

Adaptations to oxygen deprivation are triggered by increased gene transcription caused by the accumulation and stabilization of HIF-1α (the oxygen responsive subunit of the heterodimeric HIF-1 molecule). HIF-1 exerts its effects by inducing the transcription of genes involved in a variety of processes that can contribute to malignant progression including differentiation, metabolism, angiogenesis, metastasis, proliferation and, most relevant to this work, immune escape.

There is evidence indicating that oxygen deprivation and the resultant accumulation of HIF-1α can lead to immune escape via several general mechanisms: hypoxia can (1) induce the shedding of immune recognition ligands (e.g. MICA) leading to reduced sensitivity to NK- and CTL-mediated lysis [203]; (2) lead to the activation of immunosuppressive cells, such as tumour associated macrophages (TAMs) and T regulatory cells (Tregs) [11, 23, 116, 167, 172, 211]; and (3) directly inhibit dendritic cells and T lymphocytes [35, 44, 49, 106, 119, 136, 175, 202, 208, 246, 253, 268].

Of particular interest to this work is the fact that hypoxia can induce the shedding of immune activating ligands (e.g. MICA) from the surface of tumour cells. More specifically, our lab has previously shown that the shedding of the MICA protein, an activating ligand of the NKG2D receptor found on NK and T lymphocytes, is a method used by tumour cells to evade the
activities of the immune system [209]. As previously mentioned, these shed MICA ligands can further impair anti-tumour immunity by acting as an immunosuppressant by binding NKG2D receptors and leading to internalisation of the receptor-ligand complex, decreased levels of NKG2D expression and a reduction in tumour cell lysis [59, 87]. Previous work done in our lab has demonstrated that this hypoxia-induced MICA shedding in both prostate and breast cancer cells can be attributed to the impairment of endogeneous, cGMP-dependent nitric oxide (NO)-signalling.

1.4 Nitric Oxide (NO)-Signalling

NO is a ubiquitous, pleiotropic signalling molecule involved in many physiological processes including neuronal communication, host defense and vascular tone regulation. It is also implicated in certain pathophysiological conditions that arise in tissues exposed to either too much (chronic overproduction exposes cells to NO-mediated toxicity) or too little (decreased bioavailability) of the molecule [159]. Endogenous NO is synthesized by nitric oxide synthase (NOS), an enzyme with three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). These three isoforms are distinguished based upon tissue distribution, degree of activation (constitutive or inducible) and Ca²⁺/calmodulin dependence [160]. Despite these differences, all NOS isoforms serve to catalyze a two-step oxidation reaction that ultimately results in the conversion of L-arginine into L-citrulline and NO (Fig. 1.3); two moles of O₂ are consumed per mole of NO formed [143] (Fig. 1.3).

The best characterized target of NO is soluble guanylyl cyclase (sGC). This enzyme is a heterodimeric, heme-containing molecule distributed in many mammalian tissues. NO exerts
Figure 1.3 Endogenous Nitric Oxide Synthesis and Signalling.

Nitric oxide is produced endogenously from L-arginine by the nitric oxide synthase (NOS) family of enzymes in an oxygen-dependent reaction. Once synthesized, NO can activate cGMP-dependent NO-signalling by activating soluble guanylyl cyclase (sGC) or alternately, form reactive nitrogen species (RNS) through a variety of reactions. Adapted from Huerta et al. [98].
many of its physiological effects by interacting with sGC [28]. NO-activated sGC results in the production of cyclic guanosine monophosphate (cGMP), which produces a wide variety of effects through its interactions with cyclic nucleotide-gated ion channels and the cGMP-dependent protein kinase (PKG) family of enzymes [14, 21, 146].

1.4.1 Therapeutic Applications of cGMP-Dependent NO-Signalling Activation

As previously discussed, molecular oxygen, in a 2:1 ratio, is required for endogenous NO synthesis; therefore, in conditions of low oxygenation (hypoxia), NO production is inhibited. NO exerts many of its effects through the NO-sGC-cGMP signalling pathway; therefore, if NO bioavailability is reduced due to limited substrate (O₂), signalling through this pathway is also decreased. Indeed, experimental evidence has shown that hypoxia in cancer leads to inhibition of cGMP-dependent NO-signalling and the acquisition of multiple malignant phenotypes listed previously. Furthermore, the development of these characteristics under hypoxic conditions has been shown to be preventable by pharmacological reactivation of cGMP-dependent NO-signalling, thereby highlighting the importance of deficient NO-signalling in the development and maintenance of these phenotypes. As hypoxic gradients are known to develop within most solid tumours, it is reasonable to speculate that reactivation of NO-signalling in these oxygen-starved tissues will be of benefit to cancer patients.

Potential targets for clinical reactivation of this pathway include increasing oxygen delivery (pro-angiogenics), increasing sGC activity with NO-independent allosteric effectors [67], providing an exogenous source of NO (NO-mimetics) or by increasing intracellular cGMP levels through
phosphodiesterase (PDE) inhibition. The latter two classes of agents are particularly attractive due to the wide availability and established safety of NO-mimetics and PDE inhibitors.

NO-mimetics are a class of agents that mimic the activity of NO, usually through the in vivo production of NO itself during catabolism; i.e., they act as an exogenous source of NO. One of the best known NO-mimetics is glyceryl trinitrate (GTN), or nitroglycerin, which has been used medicinally for over a century in the treatment of myocardial infarction, heart failure, hypertension, and angina pectoris [98]. More recently, GTN has been shown to be useful in non-cardiovascular scenarios such as preservation of organs for transplantation [9, 145], pain management [94, 130], treatment of chronic anal fissure [142] and for lengthening the time to progression in patients with small cell lung cancer [256].

GTN, an organic nitrate, is a pro-drug which requires denitrification in order to generate NO. The mechanism by which this occurs in vivo is still disputed [176]. Numerous studies have already shown GTN’s anti-neoplastic and chemosensitizing properties; for example, GTN can attenuate hypoxia-mediated metastatic potential in murine melanoma cells [179], sensitisise colon cancer cells to Fas-mediated apoptosis [156] and decrease hypoxia-induced chemoresistance in prostate cancer [71], breast cancer [123], and lung cancer [256, 257]. GTN is also readily available in continuous release transdermal patch form and is well tolerated [176], making it a practical choice of NO-mimetic for use in cancer therapy.

NO has been shown to increase the activity of sGC more than 200 fold [101, 135], a signalling cascade that is counterbalanced by the action of PDEs. The latter are enzymes that catalyze the
hydrolysis of the second-messengers cGMP and cAMP to their inactive metabolites, GMP and AMP respectively. While multiple PDEs are capable of cGMP hydrolysis, only PDE5 has been shown to function exclusively to catalyze cGMP [50]. Thus, the action of PDEs, in particular PDE5, decreases intracellular cGMP levels and activity along the NO-sGC-cGMP signalling axis. Intuitively it makes sense that, since PDE inhibitors function by preventing the degradation of intracellular cGMP, that the drug’s effects would be more pronounced when cGMP formation is elevated; i.e., if there is little or no cGMP to begin with, the effect of PDE inhibitors would be less noticeable. In fact, evidence already exists suggesting that this is the case, at least in erectile dysfunction [51].

A final issue relevant to potential NO-mimetic treatments that is currently the subject of some controversy, is the seemingly opposing effects of NO on cancer progression depending on the administered dose (Fig. 1.4). Many studies have shown NO to be tumourigenic by inhibiting apoptosis [18, 78, 138, 265], stimulating proliferation [133] and angiogenesis [113, 173, 264] and by causing direct [10, 196] and indirect genotoxicity [131, 132, 170, 248]. Conversely, there is also literature demonstrating the anti-tumour effects of NO via increased apoptosis [20, 24, 69, 78, 137, 151, 152], inhibition of proliferation [122, 124, 206], attenuation of angiogenesis [92, 129, 178], protection against metastasis [161], chemosensitisation [222], immune sensitisation [210] and anti-oxidant-based cytoprotection [122]. From a survey of these studies, it is generally suggested that low concentrations of NO can be tumourigenic and contribute to tumour angiogenesis while at high concentrations it displays its anti-neoplastic properties [45].
Figure 1.4 The Dual Nature of Nitric Oxide.

Controversy exists in the literature regarding the effect of NO on tumours. This confusion stems from the fact that NO has been shown to have different effects depending on the administered dose as well as other variables such as redox status. The issue is further complicated by the fact that the physiological concentration of NO is difficult to measure as well as by the uncertainty surrounding how exogenous NO is stored and selectively distributed to tissues “in need” of it (e.g. hypoxic tissues). Adapted from Huerta et al. [98].
An added complication is the fact that the normal physiological concentrations of NO (and thus what is considered “high” or “low”) are difficult to quantify as well as the poorly understood mechanisms by which NO is stored in the body and delivered to tissues in need (e.g. hypoxic tissues). Overall, the microenvironment, amount and duration of NO exposure, and cellular background, all contribute to the final effects of this molecule.

1.5 Prostate Cancer & Biochemical Failure

Prostate cancer (CaP) represents a major healthcare problem throughout North America due to its high incidence and mortality rates. It is the most common non-cutaneous malignancy and the second most common cause of cancer-related mortality for men in both the United States [3] and Canada [184] and takes a substantial toll on the population through premature mortality and associated morbidities [91]. In the United States alone, nearly 220,000 individuals are newly diagnosed with prostate cancer each year and over 27,000 deaths per year are attributed to this disease [3, 164]. A similar scenario, albeit on a smaller scale, exists in Canada [36]. An impending demographic shift due to longer life spans and the ageing “baby boomer” population will lead to a substantial increase in elderly men in the near future [13] resulting in further inflation of these incidence and mortality rates.

The incidence of prostate cancer has increased significantly over the past few decades; this increase is largely due to the changes in prostate cancer screening brought about in large part by the prostate specific antigen (PSA) blood test in the late 1980s [3]. This blood test has become a standard component of the routine physical exams performed in men over the age of 40. The capability for early detection and monitoring of serum PSA levels in middle-age men has led to a
large increase in the number of men presenting with clinically-localized, *i.e.* potentially “curable,” disease [39]. While this stage-migration is a positive development for many men, it also has led to prostate cancer being over-diagnosed and many will now receive radical therapies for disease that would never have become clinically significant during their lifetimes [46, 60]. Despite this increase in younger men being diagnosed with prostate cancer, the tumour entity remains primarily a disease of the elderly with over 65% of new cases diagnosed in men older than 65 years [13].

There is a variety of treatment options available to men presenting with clinically-localized prostate cancer; however, many will choose either radical prostatectomy (RP) or radiation therapy (XRT) as a definitive primary therapy [153]. In fact, over two-thirds of the individuals diagnosed with prostate cancer each year in the US are treated with one of these two methods [164]; furthermore, many patients who initially undergo radiation therapy as a first-line primary treatment will later undergo salvage radical prostatectomy in the event of treatment failure. A high percentage of men who receive radical prostatectomy will eventually experience disease recurrence in the form of rising PSA levels with some estimates as high as 40% [164, 181]. This form of recurrence is commonly known as biochemical failure. Due to the naturally prolonged course of prostate cancer, biochemical failure generally precedes radiological or clinical evidence of disease by a significant period. These rates and percentages reveal that as many as 62,000 men experience biochemical failure each year in the US alone [164] demonstrating that this scenario is not an uncommon one (220,000 diagnosed in US/yr, 70% treated with RP or XRT, 20-40% recur ≈ 31,000 - 62,000/yr).
Due to the stage migration afforded by early detection of rising PSA levels, many of the men now experiencing biochemical failure are relatively young and otherwise healthy [164]. In addition, with the growing elderly population, the number of older men experiencing biochemical failure with slow growing disease or significant co-morbidities is rising. This fact leads to an increased need for treatments that improve clinical outcomes as well as preserve the patient’s quality of life.

Currently, treatment options for men with biochemical failure are limited and are generally separated into two general categories: “salvage” local therapies and systemic therapies. Salvage local therapies will typically involve salvage external beam radiotherapy to the prostate bed (for men who received primary RP) or salvage radical prostatectomy or cryotherapy [164] (for men who received primary XRT). Unfortunately however, these salvage therapies have been shown to only be effective in individuals with very early-stage biochemical failure and for whom there is little reason to suspect distant metastases [114, 164]. This leaves the second category, systemic therapies, which usually refers to hormonal therapy. Indeed, the current standard for systemic treatment of biochemical failure is known either as “traditional hormonal therapy” or “androgen ablation therapy” and can include orchietomy, luteinizing hormone-releasing hormone (LH-RH) agonists, estrogen therapy and/or maximum androgen blockade (MAB) [52, 162, 267].

Although it is the current standard, systemic hormonal therapy remains a “last-ditch” treatment avenue for biochemical failure and is associated with various concerning issues. First, the side effects of androgen ablation therapy can be quite severe. The prospect of hot flashes, loss of libido, decreased muscle mass, mild anemia and long term risk for osteoporosis is sure to instill consternation in the mind of any patient considering surgical or chemical castration as a potential treatment for biochemical failure. Second, due to the generally long clinical course of prostate
cancer and the age of most patients, many individuals presenting with biochemical failure have a low likelihood of developing clinical disease within their life time [19] (e.g. patients with low PSA velocity) and thus should not need to endure the side effects associated with systemic hormonal therapy [189]. Due to these concerns, hormonal therapy is not usually recommended until there is clinical evidence of disease recurrence [181, 189].

Observation or surveillance/watchful waiting in many patients with biochemical failure can be justified due to the long clinical course of prostate cancer and the absence of second-line therapies that are not also associated with significant co-morbidities [183]. Watchful waiting is particularly appropriate in those patients who are older, have multiple co-morbidities and/or are deemed to have slow disease progression [13, 163]. A retrospective study done at Johns Hopkins Hospital in 1999 tracked the natural history of biochemical failure in patients after primary therapy and revealed that the median time to the development of distant metastases in these patients was eight years and median time from detection of metastasis to death from prostate cancer was an additional five years [181]. The same study found a significant correlation between the time required for PSA levels to double (PSA doubling time or PSADT) and the amount of time before the appearance of metastases and eventual patient death (longer PSADT was associated with a longer time before clinical progression).

For these reasons, it is clear that new therapies with the potential to delay the clinical progression of prostate cancer and prolong the interval from biochemical failure to clinical treatment failure would be of significant benefit to patients experiencing biochemical failure [176], regardless of their age. Research done in this lab has indicated that NO-mimetics, such as GTN, may be
successful in fulfilling this need as was recently demonstrated in a clinical trial that revealed GTN’s ability to significantly extend PSADT in patients with biochemical failure [209].

1.5.1 Preclinical Models of Prostate Cancer

1.5.1.1 Cell Lines

In the past few decades, there has been an explosion of new prostate cancer cell lines and clonal derivatives of the “classics” created; however, many of these newer cell lines have subsequently been shown to contain intraspecies contamination [100, 148] or even that they are from a completely different origin or species than initially claimed [234]. Due to the uncertainty surrounding these newer lines, as well as the absence of any compelling reason to use them instead of the tried-and-true variants, the work conducted for this thesis was conducted using DU-145 cells.

DU-145 was the first established prostate cancer cell line [213]. These cells are epithelial cells derived from a dural prostate metastasis excised from a 69-year-old Caucasian male [216]. The originating tumour was described as moderately differentiated adenocarcinoma (in contrast with the 100% undifferentiated, aneuploidal PC-3 cell line [231]). DU-145 cells are not androgen sensitive (they do not express androgen receptor) and do not produce prostate specific antigen [213]. The cells produce good-sized colonies when seeded at $10^3$ to $10^5$ cells per dish and have a doubling time of approximately 34 hours [207]. Subcutaneous injection of DU-145 cells into nude mice has previously been demonstrated to produce tumours that resemble their parental cells in both phenotype and genotype [154] and these tumours tend to metastasize to the lung, spleen, liver, adrenal glands, kidney, lymph nodes and diaphragm [7].
1.5.1.2 Murine Xenograft Models

Like any other model, murine xenograft models have their limitations; however, they remain an important method for developing therapeutics and examining regulatory pathways of tumour growth and progression. Nude mice, first described in 1962 by Isaacson and Cattanach [104] are athymic and thus deficient in T lymphocyte-mediated immune responses. For this reason, they are capable of harbouring tumours grown from human cancer cell lines [182, 187]. In these models, the human cells are often co-injected with human reconstituted basement membrane (Matrigel) due to its growth-promoting effects [73, 117]. One limitation of this model in particular is the different microenvironment the tumour cells are exposed to when injected subcutaneously. This problem can be resolved through orthotopic injection; however, orthotopically growing tumours are often more difficult to monitor.

1.5.2 NO-Mimetics as a Potential Therapy for Biochemical Failure

Previously, our lab addressed the question of whether hypoxia can affect the acquisition of resistance to cells of the innate immune system by prostate cancer cells. Two commonly used prostate cancer cell lines, DU-145 and PC-3, were incubated in hypoxia for 24 hours prior to co-incubating them with activated peripheral blood lymphocytes (PBLs) in chromium release assays. The results of the study showed that pre-exposure to hypoxia can significantly reduce the anti-tumour effects of innate immune cells and that this effect is, at least in part, due to inhibition of endogenous NO-signalling [210]. This study led our group to postulate that the hypoxia-associated acquisition of immune-resistance by prostate cancer cells was due to a change in the expression of cell surface ligands on the cancer cells. Additional in vitro work demonstrated an attenuation of the hypoxia-induced metastatic ability of prostate cancer cells incubated with GTN.
and an in vivo study revealed that GTN can slow the growth of xenografted prostate tumour (PC-3) in male Swiss nude mice [204].

In addition to this preclinical evidence, our group recently completed a Phase II prospective, open-label clinical trial of men with biochemical failure after radical prostatectomy or radiation therapy [209]. The objective was to evaluate the effect of low-dose GTN on PSADT in men with biochemical failure after primary treatment failure. The results of the trial demonstrated that continuous transdermal delivery of low-dose (0.03 mg/h) GTN may be effective in slowing the clinical disease progression of prostate cancer as judged by the increased PSADT of the participants. The PSADT of the participants was calculated before and after the initiation of treatment and was compared with a matched control group that received no immediate treatment for their PSA-only recurrence. The end-of-trial analysis was carried out according to the intent to treat principle and the PSADT of the group receiving the intervention was found to be 31.8 months compared to the 13.3 months of the matched control group (P < .001). These results indicate that GTN has an inhibitory effect on PSA progression in men with biochemical failure possibly through a mechanism leading to increased immunogenicity in the tumour cells. This trial was the first trial of a GTN patch in patients with prostate cancer. The observed prolongation of the PSADT coupled with the intervention’s well-documented safety record and low cost give reason for further testing in a larger scale stage III placebo-controlled study.

1.6 Hypotheses

1. GTN attenuates in vivo DU-145 tumour growth but these effects are dependent on an intact NK cell compartment.
2. If the effects of GTN are linked to the activation of NO-signalling, alternate methods of activating or enhancing signalling along this pathway lead to similar effects including the attenuation of hypoxia-induced HIF-1α accumulation, ADAM10 upregulation and MICA shedding (Figure 1.5).

3. Treatment of prostate cancer patients with low-dose transdermal GTN decreases the levels of soluble MICA in their blood (relative to individual pre-treatment values).

### 1.7 Objectives

1. To determine whether the NO-mimetic GTN can attenuate *in vivo* DU-145 tumour growth. If GTN is shown to be able to attenuate DU-145 tumour growth, it would confirm and expand previous results that showed that GTN can attenuate PC-3 tumour growth.

2. If GTN is able to attenuate *in vivo* DU-145 tumour growth (Objective 1), to determine whether this effect is dependent on the presence of NK cells. If the ability of GTN to attenuate tumour growth is lost in the absence of NK cells, it would indicate that GTN exerts its growth-attenuating effects via an immune mediated mechanism.

3. To test whether PDE5/6 inhibition with zaprinast can attenuate hypoxia-induced MICA shedding in DU-145 cancer cells *in vitro*. If zaprinast is shown to decrease the hypoxia-induced shedding of MICA, an effect previously shown to be achievable with GTN, it would strengthen the hypothesis that this effect is a consequence of NO-signalling activation.
Figure 1.5 Proposed Mechanism of Immune Escape and NO-Mimetic Action.

The pictured mechanism of immune escape proposes that hypoxia leads to immune escape by increasing the shedding of MICA, an important NK cell-activating ligand from the tumour cell surface. This increased shedding is caused by a hypoxia-induced increase in membrane-bound ADAM10 expression. This increase in ADAM10 expression, as well as the increase in MICA shedding that follows from it, is dependent on the accumulation of HIF-1α that occurs under hypoxic conditions. NO-mimetics can interfere with this mechanism of immune escape by preventing this hypoxia-induced accumulation of HIF-1α. By doing so, NO-mimetics are able to sensitise tumour cells to the cytolytic actions of innate immune effectors (e.g. NK cells).
4. To test whether PDE5/6 inhibition with zaprinast can attenuate in vivo DU-145 tumour growth. Previous studies in our laboratory revealed that the NO-mimetic GTN attenuates the growth of xenotransplanted human PC-3 prostate tumours. To further elucidate whether the growth inhibitory effect of GTN is in part (or wholly) mediated by cGMP-dependent NO-signalling, inhibition of cGMP degradation should lead to maintenance of NO-signalling and attenuation of tumour growth. Administration of zaprinast to tumour-bearing mice may shed some light on the mechanism of action of GTN.

5. To determine whether 8-Br-cGMP, a cGMP analogue, can block hypoxia-induced HIF-1α accumulation and upregulation of ADAM10 expression in DU-145 cancer cells. If 8-Br-cGMP is shown to block hypoxia-induced HIF-1α accumulation and the hypoxia-induced increase in ADAM10 expression, effects previously shown with GTN, it will strengthen the hypothesis that these effects are a consequence of NO-signalling activation.

6. To assess the effects of various doses of GTN treatment on PSADT and serum MICA levels in a placebo-controlled trial of men with prostate cancer. If GTN treatment can significantly decrease serum MICA levels, it would provide evidence in support of the hypothesis that GTN can attenuate hypoxia-induced MICA shedding.
Chapter 2

Materials & Methods

2.1 Cell Culture

Human DU-145 prostatic adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured as a monolayer and maintained in 5% FBS (Invitrogen, Burlington, ON, Canada)-supplemented RPMI 1640 medium. The culture plates were stored in a standard Thermo Forma CO2 incubator (5% CO2 at 37º C).

2.1.1 Exposure to Hypoxia

Hypoxic conditions (0.5% O2) were established by placing the culture plates in airtight plastic chambers that were subsequently flushed with a 5% CO2/95% N2 gaseous mixture (BOC, Kingston, ON, Canada). Pro-Ox Model 110 O2 regulators (Lacona, NY, USA) were used to maintain experimental oxygen tensions within these chambers. After the initial culture period, cells received fresh medium prior to being placed under different oxygen concentrations (0.5% or 20%).

2.2 Animal Procedures

2.2.1 Xenograft Implantation

Six- to eight-week old male NIH Swiss nude mice (Taconic, Hudson, NY) were anaesthetized by forced inhalation of isofluorane gas and injected s.c. in the left hind flank with approximately 2 x 10⁶ DU-145 cells suspended in 200 µL of human reconstituted basement membrane (Matrigel;
BD Biosciences, Franklin Lakes, NJ). After implantation, the tumours were allowed to reach a volume of 100 mm³ before treatment was started.

2.2.2 Treatment with Glyceryl Trinitrate (GTN)

Minitran 0.4 mg/h GTN patches (3M Pharmaceuticals, St. Paul, MN) and placebo patches were cut to a size of 0.25 cm². A GTN patch of this size delivers approximately 1.8 µg/h. Mice were treated with the appropriate type of patch based upon random group assignment at the beginning of the experiment. For the duration of the trial, patches were changed daily to ensure continuous delivery of GTN. To discourage removal of the patches, mice were given their own cages and the patches were secured to the napes of their necks using a thin layer of New Skin Liquid Bandage (Medtech Products, Irvington, NY). We have previously shown that such a delivery strategy yields detectable levels of GTN and its metabolites in the plasma of treated mice.

2.2.3 Treatment with Zaprinast

The PDE5/6 inhibitor zaprinast (Tocris Biosciences, Ellsville, MO) was suspended in peanut oil by sonication. Zaprinast primarily inhibits the activity of PDE5 and PDE6 and also weakly inhibits PDE9, PDE10 and PDE11 [76]. Mice in the treatment group were injected i.p. with 10 mg/kg of this zaprinast suspension (a volume between 90 and 130 µL) every five days. Mice in the placebo group received sonicated peanut oil. Fresh zaprinast suspension was prepared immediately before each round of injections due to the low stability of zaprinast in suspension.
2.2.4 Depletion of Natural Killer (NK) Cells

Ganglio-N-tetraosylceramide (asialo GM1) is a neutral glycoprotein expressed in high concentrations on the surface of mouse and rat NK cells [215]. After the antibody binds to this glycoprotein, the NK cells are lysed through interactions with Fc receptor-expressing cells of the immune system, such as macrophages and neutrophils. While the ability of anti-asialo GM1 antibody (Wako Pure Chemical Industries, Richmond, VA) to selectively deplete NK cells in nude mice has been described previously [112], this was verified internally prior to the commencement of the primary study. Mice received i.p. injections every five days with either 100 µL of anti-asialo GM1 antibody or 100 µL of normal rabbit serum. This regimen was selected based on information included with the product indicating that in the manufacturer's experiments, splenocyte-mediated lysis of YAC-1 cells was reduced from 19.3% to 0.2% three days after a single 100 µL antibody injection and that this reduction would last 4 days before a gradual diminution. Normal rabbit serum was chosen as the control because the anti-asialo GM1 antibody was produced from a mixture of IgA, IgG and IgM immunoglobulin fractions. This experiment was conducted for three rounds of injections. The mice were sacrificed three days after the final injection and the relative NK cell population sizes were assessed using the protocol described in section 2.2.7. An ~80% reduction in NK cells was measured in mice that were injected with anti-asialo GM1 antibody thus confirming its ability to deplete NK cell populations in this model. The fact that the reduction in NK cells was less than 100% may be due to the gradual re-expansion of these cell populations between injections. NK cell depletion was not verified during the experiment due to the additional stress that would have been placed on the mice while gathering blood samples; furthermore, this antibody's ability to deplete NK cell populations in mice has been previously reported in numerous studies.
2.2.5 Measurement of Tumours

Tumours were measured using electronic callipers. In general, tumours were roughly oval in shape. To calculate the volume, the long axis (length) and the short axis (width) of the “oval” were measured and the values were entered into the following equation: \( \text{Volume} = \frac{\text{length} \times \text{width}^2}{2} \). To minimize the potential for unintentional measurement bias, multiple individuals were involved in tumour measurement and occasionally, multiple measurements were acquired in one day (by different people) and the average of the measurements was used.

2.2.6 Sacrifice and Necropsy

Mice were sacrificed after approximately 60 days on treatment or once the tumour had become too large (i.e. the humane endpoint). One hour before sacrifice, mice were injected i.p. with 60 mg/kg of pimonidazole hydrochloride (Hypoxyprobe-1; Millipore, Billerica, MA) in saline. This is a small molecule that selectively binds to proteins in hypoxic cells and can be detected later with a monoclonal antibody included with the kit.

Mice were anaesthetized with isofluorane gas prior to drawing blood by cardiac puncture. Immediately following cardiac puncture, whole blood was centrifuged for 10 minutes at 1500 g. Serum was then aspirated, placed into a new microcentrifuge tube, and frozen at -80º C.

Prior to necropsy, cervical dislocation was performed to ensure mice were dead. The tumour was assessed in situ for evidence of invasion and degree of adherence to the peritoneum. The tumour was then removed, weighed and cut into three sections. After removal, one portion of the tumour was snap frozen in liquid nitrogen, another was fixed in paraformaldehyde, and the third piece
was immediately dispersed (see section 2.3.1), labelled and subjected to flow cytometric analysis for MICA expression. Sections of lung, liver, diaphragm, kidney, mesentery and abdominal lymph node were taken and fixed in paraformaldehyde for later histological analysis. A standardized necropsy sheet was completed for each mouse noting the organs that were removed, the mouse weight at sacrifice, the final tumour volume/weight, the relevant tumour characteristics and presence of any metastatic lesions.

### 2.2.7 Isolation and Fractionation of Mononuclear Cell Populations

To verify NK depletion, splenocytes were isolated from fresh mouse spleens treated with or without anti-asialo GM1 antibody and analysed by flow cytometry. Total NK cells counts were then compared between treated and untreated animals.

Mice were euthanized as described above and their spleens were removed. Each spleen was placed into its own petri dish with PBS and placed on ice. To prepare a cell suspension, each spleen was placed on wire mesh, rinsed with PBS and crushed using the blunt plunger end of a 10-mL syringe. Care was taken to minimize the amount of solid spleen pieces in the resulting suspension. 15 mL of cell suspension was then drawn into a pipet and placed in a labelled 20 mL tube. Clean equipment was used for each spleen to avoid cross-contamination. Samples were then centrifuged for 10 minutes at 400 g and the liquid portion was removed. The cell pellet was then resuspended in 2 mL of red blood cell (RBC) lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.2) for 12 minutes. After this time elapsed, 8 mL of PBS was added to each sample to stop the RBC lysis reaction. The solutions were then transferred to new tubes and again centrifuged for 10 minutes at 400 g. Media was removed and the cell pellets were resuspended in
1 mL of PBS. Two microliters of each sample were then transferred into a 1.5 mL tube for cell counting. Ninety-eight microlitres of PBS and 100 µL of trypan blue were then added to each 2 µL sample and cells were counted using a hemocytometer. Using the counts obtained in this step, a 1-mL sample was then diluted in order to achieve a concentration of 1 x 10^7 cells per tube. Potential non-specific binding sites on the cell membranes were then blocked by mixing 90 µL of each sample with 10 µL of normal rat serum and placed in the refrigerator for 30 minutes. Upon completion of this blocking step, the appropriate antibodies were added to each sample using the following guidelines: anti-CD3 antibody (eBioscience, San Diego, CA) 0.6 µL/10^6 cells, anti-CD122 antibody (eBiosciences) 1.2 µL/10^6 cells and DX5 (anti-CD49b) antibody (eBiosciences) 1.0 µL/10^6 cells. These antibodies were used in combination to identify the activated NK cell subpopulation. The samples were then refrigerated for 30 minutes. Single-labelled, unlabelled and isotype control samples were prepared at this point as well. Cells were then prepared for three-colour flow cytometry. Activated NK cells were identified as CD122+/DX5+/CD3- cells: CD3 is expressed only on T lymphocytes whereas DX5 (CD49b) is expressed on both T- and NK-lymphocytes; therefore, by excluding CD3^+ cells but including those positive for DX5, NK cell populations were identified. This population was further restricted by counting only those NK cells (CD3^-/DX5^+) that were also positive for CD122 (the IL-2 receptor) as this indicates their IL-2 activation status.

2.3 Flow Cytometry

Flow cytometry was used to measure the surface expression levels of MICA on DU-145 cell cultures and dissociated tissue from tumours grown in nude mice. The details of each flow
cytometry experiment performed, along with associated procedures, are described in the subsections below.

### 2.3.1 Dissociation of Tumour Tissue

Each harvested tumour was placed in a glass petri dish in 5 mL of RPMI-1640 culture medium supplemented with 5% FBS and placed on ice. Under a tissue culture laminar flow cabinet, the culture medium was replaced with 5 mL of 37°C digestion buffer (0.2 mg/mL collagenase, 0.072 mM CaCl₂, 20% FBS, 1X PBS) and the tumour was vigorously chopped into small pieces using #22 scalpel blades. The pieces were then transferred to a 60-mm plate using a glass Pasteur pipette. This plate was then incubated at 37°C for 3 hours. Every 30 minutes, the contents of the plate were thoroughly pulverized using a 5-mL pipette tip to encourage dissociation. Upon completion of the digestion period, the homogenate was passed through a tissue sieve placed over a 50 mL conical tube. Ice cold PBS with 1% PBS was then used to wash the plate. The contents of the wash were added to the sieve. This wash step was repeated twice. The resulting solution was centrifuged at 55 g for 5 minutes at 4°C. The supernatant was then decanted and the pellet was resuspended in 5 mL of PBS with 1% BSA. Samples were then transferred to 10 mL polystyrene tubes and labelled for flow cytometry.

### 2.3.2 Cell Cultures Treated with Zaprinast

DU-145 cells were plated and allowed to reach 70%-80% confluence. They were then placed in conditions of either 20% or 0.5% O₂, with or without zaprinast (1 µM) for 24 hours (details of how these oxygen conditions were established can be found in section 2.1). Zaprinast was prepared by dissolving the solid in alkaline saline and then titrating with HCl until a pH of 7.2
was reached. After treatment, samples were harvested using 5 mM EDTA, briefly (5 minutes) washed three times in a buffer of PBS supplemented with 2% FBS and then incubated with mouse anti-human MICA monoclonal antibody (Santa Cruz Biotechnology, CA) at a 1:250 ratio. After one hour of incubation with primary antibody at room temperature, samples were again washed three times. Goat anti-mouse antibody conjugated to HRP (Dako, Mississauga, ON, Canada) was prepared in a 1:2500 dilution and added to the samples, which were then allowed to incubate at room temperature for another hour. Finally, samples were placed in 10 mL polystyrene tubes and analysed with a Beckman Coulter EPICS Altra HSS flow cytometer (Beckman Coulter, Brea, CA). Excitation was performed using an argon laser operating at a wavelength of 488 nm and emitted fluorescence was detected in the range of 505 to 545 nm with at least 1 x 10^4 events collected per sample.

2.4 Immunoblotting

Samples were snap frozen in liquid nitrogen and then lysed using RIPA buffer (25 mM TrisHCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with Protease Inhibitor Cocktail (Roche Diagnostics, Laval, QC, Canada). Resulting lysates were sonicated for 5 seconds at 50 Hz and centrifuged for 10 minutes at 850 g and 4º C. After centrifugation, lysates were transferred to new microcentrifuge tubes to guarantee the removal of precipitate. Protein concentration in each sample was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). For this assay, an acidic dye is added to a protein sample; upon addition to the sample, the dye undergoes a color change depending on the sample’s protein concentration. The change in absorbance that results from this color change can be measured using a spectrophotometer. Finally,
the absorbance values can be compared to a standard curve in order to determine the amount of protein in the sample. After protein determination, samples were stored at -20º C.

Based upon the results of the protein determination assay, 1 µg of total protein from each sample was resolved on an 8% SDS-polyacrylamide gel and transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) using a semi-dry transfer apparatus. After transfer, the membranes were blocked in Tris-buffered saline Tween-20 (TBST; 140 mM NaCl, 50 mM Tric HCl, pH 8.0) with 5% BSA overnight at 4º C.

After blocking, membranes were incubated with primary antibody in TBST with 1% BSA for 1 hour at room temperature. The following primary antibody concentrations were used: HIF-1α at 1:250 (BD Transduction Laboratories, Oakville, ON, Canada), ADAM10 (R&D Systems) at 1:500, ERp5 (Santa Cruz) at 1:500, MICA (R&D Systems) at 1:500, α-tubulin at 1:5000 (Sigma-Aldrich Canada Ltd.) and β-actin at 1:5000 (Sigma-Aldrich Canada Ltd.).

Membranes were then washed three times for five minutes each in 7 mL of TBST. After washing, they were incubated at room temperature for 1 hour with either anti-goat (Bio-Rad Laboratories, Mississauga, ON, Canada) or anti-mouse (Bio-Rad Laboratories) horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) in TBST with 1% BSA. After incubation with secondary antibody, membranes were again washed using the same procedure described above; this time, however, they were washed six times each to ensure complete removal of secondary antibody. Finally, secondary antibodies were detected using ECL enhanced chemiluminescence reagent.
(Amersham Biosciences, Baie D’Urfe, QC, Canada) and then membranes were exposed onto Kodak X-Omat Blue film.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

The DuoSet MICA ELISA kit (R&D Systems, Minneapolis, MN) was used to measure levels of soluble MICA in mouse serum. A 96-well plate was prepared for sample addition with the following steps: wells that were to be used were coated with 100 µL of the provided mouse anti-human MICA antibody (capture antibody) diluted in PBS to the working concentration of 2.0 µg/mL. The plate was then incubated overnight at room temperature. The following day, the wells were washed three times with 400 µL wash buffer (0.05% Tween 20 in PBS, pH 7.2). The wells were then blocked by adding 300 µL of reagent diluent (1% BSA in PBS, pH 7.2) to each well and incubated overnight at 4º C. Upon completion of blocking, the wells were again washed three times. After this washing step, 100 µL of each sample was added to the appropriate well. The plate was then covered and incubated for 1 hour at room temperature. At the completion of the sample incubation, the wells were again washed three times and 100 µL of biotinylated goat anti-human MICA antibody (detection antibody), diluted in reagent diluent to a working concentration of 400 ng/mL, was added to each well. The plates were again covered and incubated for two hours at room temperature. Upon completion of this step, washing was repeated and 100 µL of a 1:200 dilution of the provided Streptavidin-HRP was added to each well, covered, and allowed to incubate for 20 minutes at room temperature (the plate was protected from direct light during this and all subsequent steps). 100 µL of substrate solution (a 1:1 mixture of H2O2 and Tetramethylbenzidine) was added to each well and allowed to incubate for another 20 minutes at room temperature. Finally, 50 µL of 2 M H2SO4 (stop solution) was added to each
well. Upon addition of the stop solution, the optical density of each well was immediately
determined using a microplate reader set to 450 nm.

2.6 Quantitative Polymerase Chain Reaction

Total RNA was isolated using a High Pure RNA Isolation Kit (Qiagen) according to the
manufacturer’s protocol. One µg of total RNA was then reverse-transcribed with Transcriptor
Reverse Transcriptase (Roche, Mississauga, ON, Canada) using a random hexamer (Cortec,
Kingston, ON, Canada). Real-time PCR was performed with a Corbett Research Rotor Gene 3000
real-time cycler (Corbett Robotics Inc, San Francisco, CA, USA). Reported values are ratios
between the levels of ADAM10 and β-actin RNA.

2.7 Clinical Trial Design

The proposed trial is a small, randomized, placebo-controlled correlative study of two years
duration; the primary goal of the study is to investigate the correlation between treatment with
GTN and levels of PSA and serum MICA. The levels of other molecules will also be measured
including uPAR, PAI-1, ULBP2, B7-H1 and TGF-β. uPAR and PAI-1 were selected for analysis
as they are key molecules in a pathway thought to contribute to the development of increased
metastatic ability in cancer. ULBP2, B7-H1 and TGF-β were chosen as they represent either
ligands important to innate (ULBP2) and adaptive (B7-H1) immune responses or are
immunosuppressive cytokines (TGF-β). By analyzing the effects of GTN treatment on the serum
levels of these molecules, it is hoped to gain insight into how GTN affects their expression in a
humans.
The population of interest includes active surveillance/watchful waiting patients with primary tumours that have not received primary curative therapy as well as patients with disease recurrence (biochemical failure) following curative therapy (radiotherapy or radical prostatectomy).

This is a three-arm study with a control group and two test groups; the test groups are distinguished based on the dose of GTN. Participants will undergo 2:1, test to control randomization (i.e. there will be one patient placed in the control group receiving a placebo patch for every two patients placed into the test groups) Patients in the test groups will receive intervention in the form of a transdermal patch delivering GTN at a rate of 0.033 mg/h or 0.066 mg/h.

The target for recruitment is a total of 60 patients: 20 will receive the placebo patch, 20 will receive GTN at 0.033 mg/h (“low” dose treatment arm) and 20 will receive GTN at 0.066 mg/h (“high” dose treatment arm). Initial blood and urine collection will occur upon acceptance into the trial as well as at follow-up appointments conducted at 3 months, 6 months, 12 months, 18 months and at trial completion (24 months).

Using the serum and urine collected during the trial, levels of serum MICA, PSA and the other molecules mentioned above will be measured. Post-trial concentrations of MICA will be compared with pre-trial measurements from the same individual. Values obtained from subjects treated with GTN will be compared with those of subjects receiving placebo.
2.8 Calculations and Statistical Analysis

Statistical calculations were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). For comparisons between two groups, Student’s t-test was used followed by Welch’s correction. For comparisons between more than two groups, a one-way ANOVA was conducted followed by the Bonferroni post-hoc multiple comparisons test.

An independent biostatistician (Mr. Andrew Day, Kingston General Hospital-Clinical Research Centre) conducted the tumour growth analyses using the SAS/STAT™ software program (SAS Institute, Inc., Cary, NC). Briefly, each mouse’s growth curve was distilled to a single value by taking the average of the log of the volume for each measurement during the treatment period. This approach is equivalent to comparing the area underneath that mouse’s growth curve. The final log volume averages were then compared using the exact Wilcoxon-Mann-Whitney test.

For all experiments, the significance level (α) used to determine whether or not to reject the null hypothesis was 0.05. Error bars for all figures represent standard error of the mean (SEM) unless otherwise indicated.
Chapter 3

Results

3.1 NO-Mimetic Studies

3.1.1 Effect of GTN and 8-Br-cGMP on HIF-1α Accumulation and ADAM10 Expression in DU-145 Cells

Western blot and quantitative polymerase chain reaction (qPCR) analysis were used to determine the effect of GTN and 8-Br-cGMP treatment on HIF-1α and ADAM10 expression in DU-145 cells cultured under either standard (20%) or hypoxic (0.5%) oxygen conditions for 24 hours. Treatment with 1 µM GTN or 10 nM 8-Br-cGMP was shown to significantly attenuate the hypoxic-induced accumulation of HIF-1α protein (Fig. 3.1A). Attenuation of hypoxia-induced ADAM10 upregulation was also seen at the mRNA level with 1 µM GTN (Fig. 3.1B – left panel) and protein level with 10 nM 8-Br-cGMP (Fig. 3.1B – right panel). The results shown are representative of three independent experiments.
A.

<table>
<thead>
<tr>
<th>Condition</th>
<th>HIF-1α</th>
<th>β-Actin</th>
<th>αTubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% O₂</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>0.5% O₂</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>0.5% O₂ + GTN</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Relative Protein Levels

B.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ADAM10</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% O₂ + 1μM GTN</td>
<td>![Image]</td>
</tr>
<tr>
<td>0.5% O₂ + 1μM GTN</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

10 nM 8-Br-cGMP:

- precursor (100 kDa)
- partially processed (80 kDa)
- active (50 kDa)

Normalized RNA levels (ADAM10)
Figure 3.1 Effect of GTN and 8-Br-cGMP on HIF-1α Accumulation and ADAM10 Expression in DU-145 Cells.

The effects of hypoxia and treatment with 1 µM GTN or 10 nM 8-Br-cGMP on HIF-1α accumulation and ADAM10 expression in DU-145 cancer cells was analysed using western blot (protein) and quantitative polymerase chain reaction (mRNA). An increase in both HIF-1α accumulation and ADAM10 expression was seen when cells were incubated in hypoxic (0.5% O₂) conditions. Furthermore, treatment with 1 µM GTN or 10 nM 8-Br-cGMP attenuated the hypoxia-induced accumulation of (A) HIF-1α protein, ADAM10 transcript (B – left panel) and ADAM10 protein (B – right panel). The blots pictured are representative of three independent experiments and the densitometry figures were generated using data from all experiments *p < 0.05, **p < 0.01.

The experiments that generated the results shown in (A – left panel) and (B – left panel) were conducted by Dr. Ivraym Barsoum and are shown here with his permission.
3.1.2 DU-145 Tumour Growth in Mice Treated with GTN and Anti-Asialo GM1

A two-round study was conducted with a total of 65 nude mice to determine the effects of continuous transdermal GTN administration on DU-145 tumour growth and the role that the innate immune system, particularly NK cells, plays in these effects. Nude mice have a mutation in the FOXN1 gene that results in hairlessness and thymic aplasia. The latter leads to a severe deficiency in T lymphocytes and an impaired adaptive immune response. We selected a model deficient in adaptive immune responses in order to determine the contribution of innate immune responses to GTN's in vivo effects. A small study with five mice was initially conducted in order to verify the ability of anti-asialo GM1 antibody to deplete activated NK cells. The results showed a reduction in activated NK cell populations after treatment with anti-asialo GM1 when compared with mice treated with normal rabbit serum (3.2A). Results of the primary study showed that GTN was able to attenuate the in vivo growth of human DU-145 prostate tumours (p = 0.01); additionally, it was shown that this effect of GTN was dependent on the presence of an intact NK cell compartment (p = 0.04); i.e. the growth-attenuating effect of GTN was lost in mice whose NK cells were depleted by anti-asialo GM1 antibody administration (Fig. 3.2B).

3.1.3 Characteristics of Tumours from Mice Treated with GTN and Anti-Asialo GM1

Relevant characteristics of the tumours harvested from the mice used in experiment 3.1.2 were recorded. These characteristics were analysed to determine whether there was a correlation between the treatments administered during the study and certain tumour properties such as presence of metastatic lesions, extent of local invasion, presence of necrosis and degree
A.

Activated NK Cells

Normal Rabbit Serum

Anti-asialo GM1

B.

Placebo/NK Competent

GTN/NK Competent

Placebo/NK Deficient

GTN/NK Deficient

Tumour Volume (mm$^3$)

Day

50
Figure 3.2 DU-145 Tumour Growth in Mice Treated with GTN and Anti-Asialo GM1.

The ability of anti-asialo GM1 to deplete NK cells was confirmed prior to the start of the main study. (A) Dot plot showing depletion of CD122+/CD49b(DX5)+ activated NK cells. (B) Mean tumour volume per treatment group from start of treatment until sacrifice. Tumour growth curves were analysed by comparing the slopes of the linear curves of the log_{10}-transformed volumes across all measurements. Each treatment group consisted of 10-20 mice. Increased variability is apparent in the smaller groups. Statistical differences between the slopes of the log_{10}-transformed volumes were determined using the exact Wilcoxon-Mann-Whitney test. Results indicate that tumour growth was attenuated only in the group of NK cell-competent mice treated with GTN patches. *P < 0.05.
Figure 3.3 Characteristics of Tumours from Mice Treated with GTN and Anti-Asialo GM1.

The relative frequencies of tumour and mouse properties by group, treatment type and NK-depletion status are displayed here. The tumour characteristics (adherent, invasive, necrotic, metastatic) are important properties that were tracked to determine whether treatment had any effect on their frequency. No significant correlation between treatment received and any of the tracked properties was found.
of adherence (Fig. 3.3). Each characteristic was evaluated as a binary outcome during gross dissection immediately after the mouse was sacrificed. A tumour was considered adherent if it was attached to the skin and peritoneum. A tumour was considered invasive if there was any evidence of penetration into the peritoneal cavity. If any metastases were found within the abdominal or thoracic cavities, the tumour was considered to be metastatic. No significant correlations were found between treatment group and any of the tracked characteristics.

3.1.4 Protein levels in Tumours from Mice Treated with GTN and Anti-Asialo GM1

Sections of tumours harvested from mice in experiment 3.1.2 were analysed using Western blot to determine whether treatment with GTN and/or anti-asialo GM1 has any effect on tumour protein levels of ADAM10 (Fig. 3.4A), ERp5 (Fig. 3.4B) or MICA (Fig. 3.4C). Empirical and densitometric analysis showed no significant differences between the groups (ADAM10, p = 0.72; ERp5, p = 0.44; MICA, p = 0.63). The p value reported for MICA is based upon all bands combined. This experiment was conducted twice. The blots shown are representative of the sample population as a whole.

3.1.5 Serum Levels of MICA from Mice Treated with GTN and Anti-Asialo GM1

Serum isolated from mice in experiment 3.1.2 was analysed using an ELISA kit for the presence of sMICA (Fig. 3.5). No significant difference was found between groups using ANOVA (Fig. 3.5A; p = 0.61) and no correlation was found between treatment type and sMICA concentration after linear regression analysis (Fig. 3.5B; p = 0.67, R² = 0.006). This experiment was conducted once due to the limited volume of serum that could be obtained from a single mouse. Serum from non-tumour-bearing mice was used as a negative control.
A. ADAM10

<table>
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<th>Group</th>
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</tr>
</thead>
<tbody>
<tr>
<td>B1 (ADAM10)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>B3</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>β-actin (42 kDa)</td>
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Tumour Blot Densitometry (ADAM10)

B. ERp5

<table>
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<tr>
<td>ERp5 (48 kDa)</td>
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<td></td>
</tr>
<tr>
<td>β-actin (42 kDa)</td>
<td></td>
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</tbody>
</table>

Tumour Blot Densitometry (ERp5)

C. MICA

<table>
<thead>
<tr>
<th>Group</th>
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<th>GTN-+/NK-</th>
<th>GTN-+/NK-</th>
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<tr>
<td>MICA (65 kDa)</td>
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<tr>
<td>Glycosylated MICA</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>β-actin (42 kDa)</td>
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Tumour Blot Densitometry (MICA)
Figure 3.4 Protein Levels in Tumours from Mice Treated with GTN and Anti-Asialo GM1.

Western blots were used to compare the relative levels of ADAM10, ERp5 and MICA protein in the tumours harvested from the mice used in experiment 3.1.2. No significant difference was found between groups for ADAM10 (p = 0.72), ERp5 (p = 0.44) or MICA (p = 0.63). Blots shown are representative of the overall results and the densitometry results include values for all tumours. All experiments were conducted twice.
A. Serum MICA

B. Correlation of Tumour Volume to Serum MICA
Figure 3.5 Serum Levels of MICA from Mice Treated with GTN and Anti-Asialo GM1.

Serum from mice used in experiment 3.1.2 was analysed with ELISA for levels of serum MICA. (A) No significant difference was found between groups (p = 0.61) using ANOVA. (B) Linear regression analysis did not show a significant correlation between final tumour volume and serum MICA (p = 0.67, R² = 0.006). (C) The standard curve used to calculate serum MICA concentrations was generated using a purified MICA protein included with the assay.
3.2 PDE Inhibitor Studies

cGMP is a key intermediary in the endogenous NO-signalling pathway and PDEs normally act to degrade it (thus acting as a negative regulator of signalling); therefore, the inhibition of PDE activity should promote increased NO-signalling activity. As NO-mimetics and PDE inhibitors activate the same pathway (albeit by different mechanisms), it was hypothesised that both agents would yield similar effects on cellular phenotype.

3.2.1 Effect of Zaprinast Treatment on Surface MICA Expression in DU-145 Cells

DU-145 cells were cultured under standard (20% O_2) or hypoxic (0.5% O_2) conditions with or without 1 µM of the PDE5/6 inhibitor zaprinast and then analysed using flow cytometry for surface MICA expression. Results showed a significant decrease in MICA expression in cells cultured in hypoxia relative to their counterparts in standard oxygen conditions (p = 0.01). Furthermore, incubation with zaprinast for 24 hours was able to significantly attenuate this hypoxia-induced decrease in surface MICA expression (p = 0.02) (Fig. 3.6). This experiment was conducted three times in triplicate.

3.2.2 DU-145 Tumour Growth in Mice Treated with Zaprinast

A study was conducted with 20 male, Swiss nude mice (Taconic) to determine the effects the of PDE5/6 inhibitor zaprinast on in vivo DU-145 tumour growth. After tumour implantation, mice were randomly assigned to either the treatment group (10 mg/kg zaprinast in vehicle) or control group (vehicle alone).
Figure 3.6 Effect of Zaprinast Treatment on Surface MICA Expression in DU-145 Cells.

Flow cytometric analysis was conducted to measure the effect of hypoxia on the expression of surface MICA in DU-145 cancer cells. Incubation overnight in hypoxic conditions was shown to significantly reduce (p = 0.009) the levels of surface MICA. Additionally, incubation for 24 hours with 1 µM of the PDE5/6 inhibitor zaprinast was shown to significantly attenuate (p=0.02) this hypoxia-induced reduction in MICA expression.
Results showed that zaprinast was able to significantly slow the *in vivo* growth of human DU-145 prostate tumours (p = 0.01) (Fig. 3.7).

### 3.2.4 Serum Levels of MICA from Mice Treated with Zaprinast

Serum isolated from the mice in experiment 3.2.2 was analysed using ELISA for the presence of sMICA (Fig 3.8). No significant difference was found between groups using ANOVA (Fig. 3.8A; p = 0.49) and linear regression analysis did not detect a significant correlation between treatment type and sMICA concentration (Fig. 3.8B; p = 0.29, $R^2 = 0.07$). This experiment was only conducted once due to the limited volume of serum that could be obtained from a single mouse. Serum from non-tumour-bearing nude mice was used as a negative control.
Figure 3.7 DU-145 Tumour Growth in Mice Treated with Zaprinast.

Mean tumour volume per treatment group from start of treatment until sacrifice. Tumour growth curves were analysed by comparing the slopes of the linear curves of the $\log_{10}$-transformed volumes across all measurements. Each treatment group consisted of 10 mice. Statistical differences between the slopes of the $\log_{10}$-transformed volumes were determined using the exact Wilcoxon-Mann-Whitney test. Results indicate that tumour growth was significantly attenuated by zaprinast treatment. $*P < 0.05$. 

A. Serum MICA

B. Correlation of Tumour Volume to Serum MICA
Figure 3.8 Serum Levels of MICA from Mice Treated with Zaprinast.

Serum from mice used in experiment 3.2.2 was analysed with ELISA for levels of serum MICA. (A) No significant difference was found between groups (p = 0.49) using Student’s T-test. (B) Linear regression analysis did not show a significant correlation between final tumour volume and serum MICA (p = 0.29, $R^2 = 0.07$). Levels of MICA were determined using a standard curve that was generated from known concentrations of purified MICA protein (Fig. 3.5C).
Chapter 4

Discussion

4.1 General Discussion

Previous studies have shown that the growth of PC-3 prostate tumour xenografts in nude mice could be attenuated with transdermal GTN treatment [210]. Furthermore, data gathered from prior in vitro experiments provide evidence supporting the hypothesis that this tumour growth inhibition was due to sensitisation of cancer cells to innate immunity through the attenuation of hypoxia-induced MICA shedding. Until now, an important link had remained unexplored: could it be shown in an in vivo model that the growth-attenuating effect of GTN was, in fact, dependent on an intact innate immune compartment? If the ability of GTN to slow tumour growth was lost when innate immunity was suppressed, it would support the concept that these effects are a result of GTN’s ability to sensitise tumour cells to innate immune destruction.

The provision of this link is the primary contribution of this thesis: the data presented here confirm and expand, through the use of an alternate cell line, the previous observation that GTN can attenuate the growth of prostate tumour xenografts in vivo and more importantly, demonstrate that this effect is significantly inhibited when GTN-treated mice are rendered NK-deficient through treatment with anti-asialo GM1 antibody. These data indicate that an important mechanism by which GTN slows tumour growth is through an enhancement of the innate immune system’s ability to recognize and destroy tumour cells. While multiple pathways are likely involved, this observation supports the concept that a mechanism by which GTN exerts its
anti-tumour effects is through the restoration of MICA on the surface of tumour cells, thereby increasing their susceptibility to innate immune effectors.

Tumours harvested from mice used in the primary \textit{in vivo} study were analysed for expression of ADAM10, MICA and ERp5 protein. The selection of these proteins for investigation was rationalised by their role in the hypoxia-induced immune escape mechanism discussed in detail earlier in this thesis. Prior \textit{in vitro} studies and work presented in this thesis showed that GTN can attenuate hypoxia-induced MICA shedding in cancer cells [210], and that this effect is due to inhibition of hypoxia-induced expression of ADAM10; therefore, it was hypothesised that both ADAM10 and MICA protein levels would be lower in the tumours grown in GTN-treated mice when compared with those from mice treated with a placebo. ERp5, as discussed previously, is required for the ADAM10-mediated cleavage of MICA; its expression level was measured in order to determine whether GTN might modulate MICA shedding by decreasing levels of ERp5 protein. Interestingly, when comparing the levels of these molecules in tumours from mice in different treatment groups, results did not reveal statistically significant differences.

A potential confounder that might have led to these inconclusive results was the fact that, following tumour harvest, the tumour was cut in three portions, of which only one third was used in the Western blot. As hypoxic regions within a solid tumour are not evenly distributed, it is possible that the sample used for the immunoblot was not representative of the overall state of hypoxia within the tumour mass. This could be prevented in future experiments by using a sample from a homogenate that was prepared from the \textit{whole} tumour and not just a small portion of it.
Elevated levels of serum MICA have been reported in cancer patients with a wide variety of malignancies [242]; this increase in serum MICA levels may be the result of the increased MICA shedding that occurs in hypoxia. In light of this information, in the present study, sMICA levels were measured in serum isolated from tumour-bearing and control mice. It was predicted that tumour-bearing mice treated with GTN would have lower levels of sMICA because we postulated that GTN-treatment should attenuate MICA shedding in the hypoxic regions of the tumours grown in them. No significant difference in serum MICA concentration was found between the groups and a correlation could not be found between final tumour volume and sMICA levels; however, the fact that we were able to detect sMICA in these samples at all was interesting because mice do not produce MICA; this observation provides evidence that DU-145 xenografts continue to produce and shed MICA after implantation. We were able to conclude this because sMICA was not detectable in the serum of the non-tumour bearing mice. It is currently unknown how large of a difference in sMICA is needed to represent a “clinically relevant” effect. If the required difference is small, it may be that the study conducted here required a larger number of mice.

Another in vivo study was conducted to determine the effect of PDE5/6 inhibition on DU-145 xenograft growth. As discussed previously, it was hypothesised that MICA shedding in tumours is due to a hypoxia-induced inhibition of cGMP-dependent NO-signalling. GTN is thought to activate this pathway by providing an exogenous source of NO; activation of sGC by NO results in the conversion of GTP into cGMP. By inhibiting the enzymes that normally act to degrade cGMP (PDEs), it should be possible to activate the NO-signalling pathway downstream of NO and achieve effects similar to those observed with GTN. Our results supported this hypothesis by
demonstrating that administration of the PDE5/6 inhibitor zaprinast significantly attenuates the *in vivo* growth of DU-145 tumours. Zaprinast was chosen for this study based on the results of currently unpublished data showing that PDE5 is predominantly responsible for cGMP-mediated PDE activity in prostate cancer tissue [90]. It remains to be determined whether this attenuation of tumour growth is indeed a consequence of decreased cGMP hydrolysis and immune sensitisation; however, based on the *in vitro* findings showing that zaprinast can significantly inhibit hypoxia-induced MICA shedding, it is hypothesised that PDE inhibition also slows tumour growth through immune sensitisation.

An additional contribution made by this thesis was the demonstration that treatment of DU-145 cells with agents able to reactivate NO-signalling can significantly attenuate HIF-1α protein accumulation under hypoxic conditions. This effect was seen with both 1 µM GTN and 10 nM 8-Br-cGMP. The fact that this effect was observed with 8-Br-cGMP strengthens the hypothesis that GTN’s effects are due to its ability as an NO-mimetic to activate NO-signalling. Additionally, it was shown that treatment with the same concentrations of GTN or 8-Br-cGMP that were able to inhibit HIF-1α accumulation could also attenuate the hypoxia-induced upregulation of ADAM10 expression. These results provide further evidence that the immunosensitising properties of GTN are due to activation of NO-signalling. The mechanism by which GTN and 8-Br-cGMP inhibit HIF-1α accumulation under hypoxic conditions remains to be elucidated.

Studies have shown that NO can inhibit HIF-1α accumulation through a mechanism that involves inhibition of mitochondrial respiration and a re-distribution of intracellular oxygen [89]. However, it is unlikely that the GTN-mediated inhibition of hypoxia-induced accumulation of
HIF-1α observed in the studies described in this thesis occurs via this mechanism. This is because HIF-1α accumulation in DU-145 cells was also abrogated by 8-Br-cGMP, a non-hydrolyzable analogue of cGMP that does not inhibit mitochondrial respiration. As shown in Fig. 4.1, cGMP is a second messenger that is generated through the action of sGC following activation by NO. Furthermore, there is evidence that GTN is not an effective inhibitor of mitochondrial function (S. Moncada, personal communication).

It is possible that activation of NO-signalling inhibits HIF-1α accumulation through a pVHL-independent mechanism of HIF-1α degradation involving calpain (a Ca^{2+}-dependent protease). Calpain has been shown to bind HIF-1α and directly cause its degradation [266]. The proteolytic activity of calpain was shown to be significantly increased when cytosolic Ca^{2+} levels were elevated through the pharmacological inhibition of certain intracellular calcium channels. Interestingly, these calcium channels have been shown to be inhibited through the activation of cGMP-dependent protein kinase (PKG) [70]. Taken together, this information indicates that the pharmacological activation of NO-signalling may attenuate hypoxia-induced HIF-1α accumulation by increasing the proteolytic activity of calpain via a mechanism involving the PKG-mediated inhibition of intracellular Ca^{2+} channels (Fig. 4.1).

Historically, cancer therapies have focused on “curing” cancer with surgery and/or relatively indiscriminant cytotoxic drugs, treatments that are associated with significant side-effects and reductions in quality of life [166]. Furthermore, while advances in cancer treatment in the past century have led to improvements in the management of certain malignancies, particularly those of hematological origin (e.g. the story of imatinib and CML), the interpretation of changes in
Figure 4.1 Proposed Mechanism of NO-Signalling-Mediated Attenuation of HIF-1α-Accumulation.

A potential mechanism describing how activation of NO-signalling may lead to the attenuation of hypoxia-induced HIF-1α accumulation and the deleterious effects associated with that build-up (e.g., immune escape). Items in green are the most theoretical components of the pathway and represent a potential avenue for further research.
mortality rates in the treatment of solid malignancies has proven to be more difficult to interpret. Since the realization that the immune system can suppress cancer, significant effort has been put forth towards finding treatments that can assist the immune system in its role as an extrinsic tumour suppressor; these therapies, many of which focus on thwarting the attempts of cancer to evade or enervate immune effectors, have become known as immunotherapies. This class of cancer therapeutics is promising because, through them, many of the problems associated with poisonous, cytotoxic therapies can be avoided \( (e.g.) \) increased risk of secondary malignancy, off-target toxicities). The last century of marginal progress in the treatment of cancer indicates that alternate strategies for cancer treatment should be considered; perhaps cancer is so intertwined with aging that the expectation of a “magic bullet” capable of curing all cancers is unrealistic. Research efforts may be better focused on finding tumourstatic agents able to maintain transformed cells in the “equilibrium” stage proposed by the immunoediting theory for longer periods. Since escape from this stage is associated with escape from the immune system, bolstering the immune system with immunotherapies could play an important part in the pursuit of this approach to cancer therapy. The first step towards the goal of effective immunotherapies is the identification of how cancer cells escape from the immune system. It is only after the identification of these mechanisms that novel methods can be conceived to reverse or attenuate these processes. This thesis contributes to this goal through the description of one such immune escape mechanism along with the investigation of several potential immune sensitising agents that target it.
4.2 Summary and Conclusions

The data collected from the studies conducted for this thesis provide additional evidence in support of the central hypothesis that activation of NO-signalling can immunosensitise hypoxic cancer cells by increasing the amount of MICA expressed on their surface. The single most important result contributed by this thesis is the observation that the growth attenuating effect of GTN is lost in NK-deficient mice. Taken together, the results presented here are important because they provide further evidence indicating that NO-mimetics and PDE inhibitors could be used as immunosensitizing agents in the treatment and prevention of cancer.
Chapter 5
Future Directions

The studies conducted on GTN’s immunosensitising properties so far have focused on the contribution of the innate immune system. Potential avenues for future research include the determination of whether hypoxia can lead to evasion from *adaptive* immune effectors, such as cytotoxic T lymphocytes, and whether activation of NO-signalling with GTN can attenuate those effects. Preliminary findings in the lab have already shown that a molecule known to cause T-cell anergy (B7-H1) is upregulated in hypoxic conditions and that GTN can prevent this hypoxia-induced upregulation. This may indicate that GTN can immunosensitise cancer cells via multiple mechanisms.

As both GTN and zaprinast can slow tumour growth, likely by activating the same NO-signalling pathway but in different ways, it would be interesting to determine whether their effects are additive, or even synergistic.

Studies should be conducted to test the hypothesis proposed above that attempts to explain how activation of NO-signalling blocks HIF-1α accumulation. Initial *in vitro* experiments should be designed to determine whether calpain activity and/or calcium are required to see the anti-cancer effects of GTN. Additional studies would follow based upon the results of these preliminary experiments, with the ultimate goal of showing a full pathway from GTN administration to the prevention of MICA shedding and immune escape.
From a clinical perspective, further trials will be highly dependent on the results gathered from the currently in progress study that will measure whether GTN treatment in men significantly affects serum MICA levels and whether initial sMICA measurements can predict the degree of response to GTN therapy. Assuming a positive result is obtained from this study, the next step would be a large, placebo-controlled, double-blinded phase-III randomized controlled trial.
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