Cytotoxic drugs sensitize tumor cells to immune cell-mediated killing by Interleukin-2 activated peripheral blood leukocytes

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Cytotoxic Drugs Sensitize Tumor Cells to Immune Cell-mediated Killing by Interleukin-2 Activated Peripheral Blood Leukocytes

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DEDICATION

This work is dedicated to my mother Theresia and my father Joachim for their love, continued encouragement, and willingness to send their only child on a long journey, and to my family, especially my grandmother Magdalena, my grandfather Josef and my uncle Heinz, for their unsurpassable support.
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INTRODUCTION

Currently, most cancers are treated with a combination of surgery, chemotherapy and radiotherapy. Recent advances in molecular and tumor biology signal transduction, genomics, proteomics and drug design have accelerated new discoveries and translate into a better understanding of oncology. Physicians still have to deal with a number of seemingly unavoidable obstacles, one of them being the either inherent or acquired chemoresistance of certain tumors.

While for tumors detected in early stages surgery is the most important and outcome-defining modality, metastatic cancers need treatments that exert systemic effects as opposed to only local control. The loss of cytotoxic agents as a treatment option in chemoresistant tumors prompted the search for alternatives and resulted in the rejuvenation of immunotherapy. Despite the fact that immunotherapy was able to achieve responses the rates were usually unimpressive. Long-term survival has, with the exception of a few anecdotal reports, never been described. Need to improve those results brought thoughts about combining multiple regimens to achieve synergistic effects into the discussion. Combination therapy has been a standard for many years as seen in adjuvant and neoadjuvant regimens, in which local treatment (mostly surgery, but also radiotherapy) is combined with systemic treatment (chemotherapy) or in radiation oncology, in which cytotoxic drugs are being used as radiosensitizing agents.
For our project we hypothesize that chemotherapeutic drugs, applied at a dose below that leading to a cytotoxic effect on tumor cells, might elicit responses in affected cells that ultimately lead to a higher sensitivity to lymphokine-activated killer (LAK) cell-mediated lysis (chemosensitization). The mechanisms related to this effect might be up- or downregulation of surface molecules, altered expression of apoptosis-related genes or changes in expression of multifactorial genes resulting in an altered balance between antiapoptotic and apoptotic factors.

Derived from our hypothesis, the objectives of this thesis were 1) to prove the presence of beneficial chemosensitization effects of drugs on tumor cell lines, 2) to determine whether the addition of cytotoxic drugs to existing immunotherapy is achievable in regards to toxicity and efficacy, 3) to elucidate possible molecular mechanisms for increases in tumor susceptibility to immune-cell mediated killing and 4) to use an in vivo mouse model to confirm possible in vitro results and establish a base to translate them into a clinical application.

In manuscript 1, a chemosensitization effect of gemcitabine was confirmed in renal cell cancer (RCC). Further investigations proved that proper scheduling of treatment minimizes toxicity on the immune system while preserving the chemo-sensitization effect on the tumor cells. In addition, we discovered that gemcitabine induced a morphological cell size shift in tumor cells; further sorting of subpopulations also showed a functional effect.

Two currently ongoing studies (gene expression microarray analysis, in vivo mouse model) are described.
Cancer

Overview: Epidemiology and Pathogenesis

Cancer is not only recognized as one of the most prevalent diseases in the world (responsible for one out of four deaths in the US), it is also known that due to the relationship between age and cancer incidence combined with the ongoing epidemiologic shift toward an aging population, it will become an increasingly important factor for public health and for the economy.

As published in the most recent issue of Cancer Statistics 2007 (Jemal et al., 2007), the predicted number of new cases in the US during the year 2007 will be 1,444,920 (incidence) while the number of deaths attributable to cancer is predicted to be 559,650 (mortality). Drawn from numbers published in 2004, cancer represents the second leading cause of death in the US (23.1%), only preceded by cardiovascular diseases (27.2%). Far behind following on ranks three, four and five are cerebrovascular diseases (6.3%), chronic lower respiratory tract diseases (5.1%) and accidents/ unintentional injuries (4.7%), respectively. Data, also from the year 2004, looking at leading causes of death adjusted for gender and age group identifies cancer as the leading cause in males aged 60 to 79 and females aged 40-79. Again, predictions for 2007 indicate that the highest incidence will be found in prostate cancer for men and breast cancer for women while the leading cause of mortality is predicted to be due to lung and bronchial carcinoma in both genders.
The American Cancer Society defines cancer as a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Since spread of abnormal cells refers to metastasis, which is a hallmark of malignant disease, the term cancer cannot be used to describe a benign lesion. Other descriptions used are neoplasia (meaning new growth, usually refers to cancer but can be malignant or benign) and tumor (meaning swelling or abnormal mass, can also be benign or malignant).

At the molecular level cancer is defined as a genetic disease arising from mutations in the deoxyribonucleic acid (DNA) of one cell that confer selection advantages and thereby support the propagation of this cell. These mutations can occur in various ways (substitution, deletion, insertion, point-mutation, frame-shift mutation) and may be inherited or acquired; it is important to note, however, that even if a predisposition is inherited additional acquired mutations are needed to express the cancer phenotype (Meza-Junco et al., 2006). The proliferating cell passes the mutated genetic material to progeny which can continue to acquire new mutations. Since mutations are by definition non-specific, they may turn out to be beneficial or detrimental. At this point, Darwinian selection comes into play: cells with detrimental mutations become extinguished while cells that acquired beneficial mutations survive and get the chance to propagate the trait; this process is called clonal selection (Cahill et al., 1999; Nowell, 1976). Since all cells within a tumor arise from the expansion of a single cell of origin, cancer is considered to be monoclonal (Nowell, 1976).
A vast number of genes have so far been studied that play roles in certain types of cancer. Even though the definition of a fixed gene set for a specific malignancy has not been possible, the insights about underlying mechanisms that lead to cancer formation gathered from those studies allowed the grouping of certain genes that play particular roles in the cancer formation process. Mutations leading to cancer thereby target three classes of regulatory genes: (a) oncogenes; (b) tumor suppressor genes; and (c) DNA repair genes.

Proto-oncogenes are normal genes found in healthy cells; they encode proteins that are critical for many different pathways like cell-cycle regulation, cell differentiation, signal transduction, apoptosis pathways and transcriptional regulation (Krontiris, 1995). A mutation in a proto-oncogene transforms it into an oncogene. The final outcome is often a constitutive activation that results in increased and uncontrolled proliferation. As a rule, mutations in proto-oncogenes yield a gain-of-function effect meaning that a change either results in emergence of a new, or enhancement of an existing function.

Tumor suppressor genes encode proteins that prevent uncontrolled cell growth by exerting a restraining function on other proteins involved in proliferation. Mutations produce a loss of function phenotype; this translates into the inability of the affected tumor suppressor to prevent the development of a neoplasia (Akiyama, 1995). It needs to be mentioned that the connection between mutations in tumor-suppressor genes and the loss-of-function effect represents a simplification applicable to most of those genes. A noteworthy exception is p53; it has been recognized as a tumor suppressor gene but,
besides loss-of-function, mutations can also express a gain-of-function phenotype leading to increases in proliferation rate, antiapoptotic activity, therapy resistance as well as enhancement of invasiveness and tumorigenicity (Blagosklonny, 2000; van Oijen and Slootweg, 2000).

DNA repair genes give rise to all necessary proteins that participate in repair pathways such as mismatch repair, nucleotide excision repair, base excision repair, homologous recombinational repair and non-homologous end-joining (Fleck and Nielsen, 2004). Defective gene products impair those pathways resulting in accumulation of DNA damage over time.

Another approach to characterize genes involved in carcinogenesis is the differentiation between gatekeeper, caretaker and landscaper genes. Gatekeeper genes directly control cell proliferation, and a mutation consequently affects proteins involved in cell-cycle control, signal transduction and transcriptional activation. Caretakers on the other hand do not directly affect cell proliferation but rather encode various proteins involved in DNA-repair pathways, thereby assuring the integrity of the cell genome (Kinzler and Vogelstein, 1997; Levitt and Hickson, 2002; Michor et al., 2004). Mutations in those genes lead to genetic instability, which, in turn, supports the accumulation of more mutations. Genetic instabilities may manifest either as microsatellite (due to DNA-Polymerase errors) or chromosomal instabilities (due to positioning errors); the existence of a third way of occurrence represented by point-mutations leading to instabilities at a single-nucleotide level is hypothesized, but so far difficult to prove (Cahill et al., 1999; Loeb et al., 2003). Since spontaneous mutation rates are not high enough
to explain the observed number of mutations in cancer cells, the discovery of
genetic instabilities leading to a faster accumulation of mutations fostered the
development of a mutator phenotype concept (Loeb et al., 2003). Landscapers,
the last group of genes, exert their influence on the surrounding stroma leading
to a tumor-promoting microenvironment (Bissell and Radisky, 2001).

Keeping in mind that cancer is a genetic disease it becomes apparent that
everything able to either directly cause mutations, to indirectly influence the rate
of mutation occurrence or to impair repair mechanism qualifies to be an etiologic
factor for the initiation of a tumor. But an alteration in one gene does not cause
the clinical picture of cancer, therefore additional changes besides the tumor
initiation need to be acquired, a process called tumor progression (Michor et al.,
2004). The environment plays an important role in inducing mutations: pathogens
(especially viruses), chemicals, toxins, radiation, pollutants and many other
factors can cause the earlier mentioned mutations transforming a proto-
oncogene to an oncogene, rendering a tumor suppressor gene inactive,
inactivating or damaging a DNA repair mechanism or affecting any gene that
causes a selection advantage as well as a disruption of normal cell behavior
(Wogan et al., 2004).

Another notable factor is inheritance of germ-line lesions that confer a
predisposition for cancer development. The presence of early childhood cancers
showing mutations in tumor-suppressor genes, which require both alleles to be
deactivated, made the probability of two somatic mutations being responsible
quite unlikely. Knudson’s two-hit hypothesis, using the example of
retinoblastoma, offered an explanation and introduced the heredity transmission background into cancer development. According to the hypothesis, a cancer predisposition can be inherited if a parent carries a germ-line mutation, which is passed to all cells of the progeny, representing the first hit. The second hit, is acquired during life as a somatic mutation. Knudson et al. point out, that the two-hit hypothesis can either be interpreted as two hits in two dominant genes (oncogenes) or two hits in one recessive gene (tumor suppressor gene). Further work identified the mutational target as a tumor-suppressor gene, thereby favoring the latter interpretation (Knudson, 2001). As a result, genetically inherited cancer predisposition is recessive at the molecular level and shows the observed dominant behavior only at the family level (Turnbull and Hodgson, 2005).

The question as to which genes need be mutated to provide characteristic changes for cancer was thoroughly investigated and led to the discovery of various cancer genotypes; an article published by Hanahan et al. (2000) organized those genotypes into six groups containing essential alterations that ultimately lead to the clinical manifestation of cancer. The groups were labeled 1) self-sufficiency in growth signals; 2) insensitivity to antigrowth signals; 3) evasion of apoptosis; 4) limitless replicative potential; 5) sustained angiogenesis; and 6) tissue invasion and metastasis. These provide the understanding that multiple genetic alterations may be present but all ultimately converge into those six essential groups.
Current Treatment Strategies

Treatment can be sub-classified into the three modalities of surgery, chemotherapy and radiotherapy; it can be of curative or palliative intent. Occasionally, single-modality treatment strategies can be used in curative approaches. This mostly applies to surgery since solid tumors detected at a very early stage have a high probability of cure by complete resection. Chemotherapy alone is rarely used for solid tumors but plays the most important role in hematological malignancies. Diseases like Hodgkin’s lymphoma, some subtypes of non-Hodgkin’s lymphoma and acute lymphoblastic leukemia (ALL) can be cured by a combinatorial chemotherapeutic regimen. Radiotherapy alone is less frequently used than chemotherapy for curative approaches but is an established modality for palliation, local control and as an option for patients that are not able to undergo surgical procedures.

Despite the fact that early-stage cancers can be cured with single-modality therapies, the use of multi-modality regimens often carries an endpoint benefit, either in cure/ remission rate, remission duration or symptomatic control. This is largely due to the fact that multiple regimens are designed to complement each other (e.g. surgical removal of the primary and destruction of micro-metastases by chemotherapy) or to exhibit synergisms (e.g. the use of a chemotherapeutic agent to increase the radiation sensitivity of tumor cells).

If more than one modality is used for treatment, scheduling becomes an important aspect. Adjuvant refers to therapies that are employed after successful treatment of the tumor (which may be surgical removal, definitive radiation
therapy or definitive chemotherapy) in patient groups that are found to be subjected to high recurrence rates or if surgery was not able to remove the complete tumor. While most adjuvant therapy plans involve chemotherapy, sometimes radiotherapy is used alone or in conjunction with chemotherapy (radiochemotherapy). Neoadjuvant chemotherapy is employed before surgery and carries the rationale that a patient with an apparent localized disease has a chance of already being subjected to undiscovered systemic disease which translates into the presence of micrometastases. Preoperative neoadjuvant therapy again mostly involves chemotherapy but radiotherapy alone or radiochemotherapy are also possible modalities. In the case of chemotherapy, neoadjuvant regimens carry multiple advantages: a) the early exposure of the tumor to the selected agent allows evaluation of efficacy by observing the magnitude of the cytotoxic effect or its absence; b) micrometastaese are subjected to cytotoxic therapy much earlier than in adjuvant settings; and c) regression of the primary tumor may lead to downstaging and better local management possibilities.

**Manipulation of the Immune System, Immunotherapy and Implementation of Chemotherapy into Existing Immunotherapy Regimens**

The problem with surgical approaches aiming at a cure is that to achieve this goal, the removal of all tumor cells is of utmost importance. This is achievable in the setting of an early cancer with limited extension into surrounding tissues. As soon as further invasion, regional spread to lymph nodes
or even metastases are present, surgery alone will not be able to eradicate the
tumor and additional modalities targeting disseminated tumor cells come into
play. Consequently, the immune system is a very tempting target for
interventions aimed at recruitment of immune cells that could attack tumor cells,
since it represents the body’s natural defenses in contrast to chemo- and
radiotherapy, which introduce potentially harmful insults. As will be discussed in
later sections, the question arises as to why cancer occurs in the first-place,
taking into consideration that the human body possesses an effective immune
system which is able to destroy neoplastic cells. It has been acknowledged that
an anti-tumor activity is definitely present, but, in cases where cancer arises, this
activity is severely impaired and immune cells are inhibited.

Immunotherapy, also known as biologic therapy, exploits possibilities to
elicit or enhance anti-tumor immune responses; in the example of cancer, it can
be used to overcome tumor inhibition of a cytotoxic immune response. Numerous
studies involve the use of cancer vaccines, monoclonal antibodies, cytokine
stimulation and adoptive cell transfers. The focus can either be direct tumor cell
killing (mediated by antibodies or CD8+ tumor specific T cells), increasing tumor
recognition and thereby tumor-specific effector cell frequency (tumor vaccines,
dendritic cells), downmodulation of effector cell inhibition (regulatory T cells,
cytotoxic T lymphocyte antigen 4 [CTLA-4]) or augmentation of immune cell
proliferation and killing capabilities (cytokines). Most studies deal with the
interaction between a therapeutic intervention and the immune system, or, more
precisely, specific immune cells. Possible interactions between therapeutic
interventions and the tumor cells have so far not received comparable attention (with the exception of cytotoxic activity exerted by high-dose chemotherapeutic drugs). Taking into account that even most recently discovered treatments rarely lead to a complete cure and instead only provide small improvements in survival or remission duration, the theoretical approach to combine a variety of these small steps to achieve marked improvements becomes attractive and necessary. However, all decisions must be made with the requirement of acceptable toxicity and better quality of life.

One of the strengths of immunotherapy is the possibility of designing treatment protocols that implement multiple immune modulators, each one acting by a different mechanism or targeting a different activity; the resulting synergism can be achieved at a low price of limited toxicity compared to aggressive chemo- or radiotherapy regimens. Considering the previous statements, two immune interventions, one targeting the activation and disinhibition of immune cells and the other targeting the sensitivity of tumor cells to immune cell-mediated killing, can synergistically achieve a large benefit.

The Immune System

Overview

Immunology studies the defense system of organisms, encompassing physiologic reactions to pathogens and pathologic mechanisms like autoimmune diseases and allergies. Due to a fast-growing body of knowledge it has become a large and steadily expanding area of research integrating new insights from the
fields of medicine, genetics, molecular and cellular biology and signal
transduction.

The human immune system can be divided into two parts, the innate (or
nonspecific) immunity and the adaptive (or specific) immunity. Both are
interlinked on several levels and depend on, but also complement each other.
The terms specific and nonspecific have to be interpreted carefully: in this
context, they emphasize that while immune cells from the adaptive branch
(lymphocytes) possess very specific receptors which only recognize few
antigens, the receptors from immune cells from the innate branch recognize a
wide variety of antigens on pathogens. Despite this, the innate immunity basically
is specific, too; if this was not the case, it would attack our own body, but to
emphasize the different abilities to recognize a wide or narrow antigenic
spectrum and the ability of the adaptive immune system to tailor the response to
the invading pathogen (described below), many textbooks still use the terms
specific and nonspecific.

The innate immunity exists from birth on and eliminates pathogens in a
non-specific way, meaning that the response mechanism is always the same and
does not become tailored to the current insult. Adaptive immunity, on the other
hand, is not available from the beginning of life, only the so-called naïve effector
cells are present in a resting state. Those cells need a combination of stimulatory
signal and foreign antigen which elicits educational processes leading to an
immune response specifically tailored to the invading pathogen.
Before discussing the two forms of immunity and specific alterations of the immune system in cancer patients more deeply, a brief review of immune cells as well as signaling and surface molecules that play a major role in mediating recognition of self and foreign proteins is necessary.

**Immune Cells**

All cells of the immune system arise from a pluripotent hematopoietic stem cell in the bone marrow; this cell gives rise to two main lineages, the common lymphoid progenitor and the common myeloid progenitor. The common lymphoid progenitor can differentiate into T lymphocytes, B lymphocytes and natural killer (NK) cells while the common myeloid progenitor is responsible for the production of granulocytes (neutrophils, basophils, eosinophils), monocytes/ macrophages, mast cells and dendritic cells.

Granulocytes represent the most abundant cell type in the adult immune system and are considered phagocytes serving the innate immunity. They differ in morphology and function which allows division into three subsets: neutrophils are phagocytes involved in killing of pathogens by phagocytosis and subsequent fusion of the resulting phagosome with toxic granules; basophils strongly resemble mast cells and play a role in allergic reactions and store large amounts of mediators like histamine and other vasoactive peptides that are released upon antigen contact with their surface-immunoglobulin (Ig) E; eosinophils are important in defense against parasites but are also involved in allergic reactions.
Monocytes and macrophages are phagocytes of the innate immunity branch that possess many important abilities. Besides being able to carry out phagocytosis and pathogen killing, they also process antigens of engulfed pathogens and present them on major histocompatibility complex (MHC) class II molecules to T cells. In addition, they can secrete a variety of cytokines that function as activators or signaling molecules. After arising from the progenitor, the cells begin circulating in the blood as monocytes; after some time they migrate into tissues and finish their differentiation into macrophages.

Dendritic cells are professional antigen-presenting cells. Their mode of action resembles macrophages but their antigen-processing and –presenting machinery is a lot more effective. Immature dendritic cells are located in all tissues, waiting for pathogen contact; as soon as pathogens enter they are engulfed by dendritic cells. This triggers a maturation response that involves the processing of antigens with presentation in context of MHC class II, the upregulation of costimulatory molecules and the migration to the next lymph node where the, now mature, dendritic cell starts to activate effector cells of the adaptive immune branch.

NK cells are cytotoxic effector cells belonging to the innate immunity. In contrast to CD8⁺ cytotoxic T cells, NK cells do not require antigen priming to kill virus-infected or malignantly transformed cells. They possess a repertoire of inhibiting and activating receptors and mainly recognize aberrant or non-self cells by downmodulated MHC class I surface expression; these molecules act, if present in normal numbers, as a ligand for the inhibitory receptors of NK cells.
thereby preventing their activation. Cytotoxicity is mainly mediated by the perforin/granzyme pathway, but apoptosis-induction by Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been reported.

Mast cells are also cells from the innate immunity. They play a role in protecting mucosal surfaces, but their main function is storage and release of histamine, heparin, leukotrienes, prostaglandins, cytokines and other vasoactive substances; hence, mast cells are involved in orchestrating allergic reactions and simultaneous activation of large numbers can result in anaphylactic shock.

T cells belong to the adaptive immune response and can differentiate into two main effector (CD4+ or CD8+) subsets and their respective memory cells. In general, T cells carry out the functions of cell-mediated cytotoxicity (mediated by CD8+ cells, either by the perforin/granzyme or the Fas/FasL pathway and immune modulation (mediated by CD4+ cells). A special subset of T cells has long been recognized but only recently attracted much interest. Those cells are called regulatory T cells (Tregs) and are characterized by intracellular expression of Foxp3 and a high amount of the surface molecule CD25. Most Tregs are CD4+ but a small subset is found to be CD8+. They function as immunomodulators and play important roles in infectious diseases, autoimmunity, transplant tolerance and tumor biology. Tregs are able to inhibit CD4+ T cells, CD8+ T cells, dendritic cells, NK cells, natural killer T (NKT) cells, and B cells in a contact-dependent manner, and their depletion results in a marked increase of antitumor immunity (Beyer and Schultze, 2006).
B cells, also assigned to the adaptive immunity, can differentiate into plasma cells which are capable of secreting large amounts of antibodies. Antibodies are small proteins whose unique structure allows specific recognition and binding of an antigen. Phagocytes and complement can bind antibody-antigen-complexes and carry out their respective function (phagocytosis, cell lysis).

NKT cells represent a small subset of T cells; they express the cell-surface marker NK1.1 (NK cell marker) and the α:β T cell receptor (TCR). One difference about the TCR in NKT cells is its semi-invariant composition. TCR are composed of an invariant alpha-chain (Vα24JαQ in humans) combined with one of three different β-chains (the TCR will be discussed in the next section). Another important fact is that NKT cells recognize glycolipid antigens presented by the MHC class Ib molecule CD1. Antigen recognition leads to the secretion of large amounts of Interleukin (IL) 4 and interferon γ (IFNγ). NKT cells function as immune regulators and form another bridge between innate and adaptive immunity; their deficiency or malfunction leads to autoimmune diseases.

Comprehensive reviews and discussions about immune cells can be found in (Alam, 1998), (Janeway, 2006) and (Paul, 2003).

**Important Molecules in the Immune System**

The term MHC represents a genomic region spanning roughly four million base pairs found in vertebrates; this region gives rise to MHC molecules by encoding their structural proteins and proteins responsible for peptide generation.
and transport. The term human leukocyte antigen (HLA) is often synonymously used for MHC, but in a strict sense HLA represents genes in the MHC gene region that encode structural MHC complex proteins.

In general, there are two MHC classes (MHC) designated class I and II. MHC complexes are important components of the immune system responsible for antigen presentation to immune cells. While MHC class I presents antigens arising in the cytosol of the cell, MHC class II uses extracellular antigens that have been taken up by phagocytosis, pinocytosis or endocytosis (Paul, 2003).

Proteins in the cytosol of a cell are subject to recycling processes (new proteins are synthesized, old proteins are being degraded). Degradation is mediated by proteasomes. The resulting peptides need to gain entry into the endoplasmic reticulum (ER) where the MHC class I molecule (consisting of an α-chain and β2-microglobulin) is waiting to load peptides. Adenosine-tri-phosphate (ATP)-dependent transport is mediated by two members of the ATP-binding cassette family called TAP1 and 2 (transporter associated with antigen presentation). Newly synthesized α-chain of MHC class I binds to calnexin which keeps it in a partly folded state. Subsequent binding of β2-microglobulin causes displacement of calnexin and binding to the MHC class I loading complex (consisting of calreticulin, tapasin, TAP and Erp57). This stabilizes the complex and makes it ready to bind peptides. Peptide binding causes dissociation from the loading complex, folding and translocation via Golgi to the plasma membrane. MHC class I:peptide complexes are recognized by CD8+ cytotoxic T cells that mediate cellular immunity by killing cancer or virus-infected cells either
by Fas/FasL pathway or the release of perforins/granzymes (Elliott, 2006; Janeway, 2006; Solheim, 1999).

All proteins taken up by cells either by phagocytosis, endocytosis or pinocytosis are directed into the MHC class II pathway. Endosomes containing proteins or pathogens migrate from the plasma membrane deeper into the cell and become more and more acidic. Fusion with a lysosome releases lysosomal enzymes (acidic proteases) that start to digest the protein or pathogen into peptides. The MHC class II assembly also starts in the ER. To prevent premature peptide binding, MHC class II (consisting of an $\alpha$- and a $\beta$-chain) is assembled with an associated invariant chain. Invariant chains form trimers in which each chain binds one MHC complex, obscuring its peptide binding cleft. Another function of the invariant chain is the delivery of MHC class II from the ER through the Golgi-apparatus to the endosome. After fusion with an endosome, acidic proteases begin to cleave the invariant chain; ultimately, only a small peptide (called CLIP) located in the binding cleft is spared. CLIP is later displaced by the peptide that will be presented; this process is facilitated by HLA-DM which catalyzes the release of CLIP and the binding of the new peptide. The MHC class II:peptide complex is translocated to the plasma membrane where it can be recognized by CD4$^+$ helper T cells. Helper T cells act as facilitators and regulators of the immune response by releasing cytokines which stimulate macrophages, B cells and cytotoxic T cells (Cresswell, 1994).

A special receptor important for the ability of adaptive immune system effector cells to recognize a wide variety of antigens is exclusively expressed by
T cells. The TCR is a heterodimer and consists of two polypeptide chains, both attached to the cell surface and linked by a disulfide bond. The polypeptide building blocks are in 90% of all T-Lymphocytes α and β chains giving rise to the α:β T cell receptor. The other 10% of T lymphocytes possess γ and δ chains resulting in a γ:δ TCR (Davis and Bjorkman, 1988). The main function of TCR is the recognition of antigens presented in context of MHC molecules resulting in induction of the specific T cell effector function. With the help of accessory chains that associate to a structure called CD3 complex, the activated TCR can initiate a downstream signaling cascade. The cascade starts with the Lck and Fyn-mediated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) located on the CD3 complex and the TCR. Lck and Fyn represent tyrosine kinases that belong to the Src family. The phosphorylated ITAMs allow binding of ZAP-70, another tyrosine kinase, which upon activation by Lck phosphorylates various substrates advancing the signaling cascade in many directions. Ultimately, the end result is the activation of transcription factors NFκB, NFAT and AP-1 (Janeway, 2006). The B cell counterpart of the TCR is the Immunoglobulin-family; it will be discussed in the adaptive immunity section.

Fas/Apo1 receptor (CD95) and Fas ligand (CD178) is a receptor-ligand pair that is of special interest to this study. Fas receptor belongs to the tumor necrosis factor (TNF) receptor superfamily. Upon binding to Fas ligand, Fas receptor recruits the adaptor proteins MORT1/FADD which in turn bind and aggregate procaspase-8 molecules. Autoactivation of procaspase-8 yields the active form, caspase-8, which activates other procaspases and induces
apoptosis by cleavage of cellular substrates that are essential for normal cell viability (Wallach et al., 1999). The Fas receptor/ Fas ligand pair is a critical component of the human immune system and functions as a regulator of lymphocyte survival and T cell homeostasis. It also serves an important function in cytotoxic immune cells (CD8+ T cells, NK cells etc.) which express Fas ligand and thereby can induce apoptosis. Recent research also points at its involvement in inflammation and cancer progression (Kim et al., 2004; Marsik et al., 2003; Matute-Bello et al., 2001).

**Innate Immunity**

The effector systems responsible for innate immunity can be divided into three groups. One group is the cellular component, represented by leukocytes and their associated cytokine products. Leukocytes serving the innate immunity are granulocytes (neutrophils, eosinophils and basophils) and monocytes/macrophages, collectively known as phagocytes, mast cells and NK cells. Dendritic cells can also be considered part of the phagocyte population that is involved in innate immunity functions but their main purpose is the presentation of processed antigens to activate the adaptive immunity instead of destruction of pathogens. Another group is made up by all natural barriers the human body possesses, namely mucosal surfaces (respiratory tract, gastrointestinal tract, reproductive tract) and epithelial surfaces (skin). They prevent entry of pathogens, possess areas that function as reservoirs for immune cells and are lined with epithelial cells which can interfere with the attachment of microbes and
secrete antimicrobial substances. The last group is represented by soluble molecules able to aid in host defense which includes the complement system, antimicrobial substances, proteins that are able to sequester iron and zinc and enzymes like lysozyme (Alam, 1998; Paul, 2003).

The innate immunity is the first barrier microorganisms encounter when invading a host. It is also the fastest repelling force, but this quickness is bought at the cost of specificity: while the adaptive response is fine-tuned to the momentarily invading organism mediated by antigen-specific recognition systems, the innate immune response uses genetically predetermined recognition receptors, so called pattern-recognition receptors (PRR) that are able to bind a small, conserved group of structures combined under the term pattern-associated molecular patterns (PAMP) which are widely expressed on the surface of microorganisms but not on human tissues or cells. Important molecules belonging to the PAMP group are lipopolysachharides, peptidoglycan, lipoproteins, flagellin, double-stranded ribonucleic acid (RNA), unmethylated CpG DNA, glycosylphosphatidylinositol, certain sugar molecules found on bacteria and anionic polymers (Pandey and Agrawal, 2006). PPR can be divided into two groups: the first PPR group signals the presence of infection and includes toll-like receptors (TLR). So far, 13 TLR have been identified in mammals, 10 of them are expressed in humans; their ligation causes dimerization of the receptor and activation of the NFκB, JNK, p38 and ERK pathways via binding of the adapter protein MyD88 and subsequent signaling by IRAK (Lasker and Nair, 2006).
A second group is referred to as phagocytic receptors including scavenger receptors, the macrophage mannose receptor and the beta-glucan receptor. Scavenger receptors are expressed on macrophages, dendritic cells and certain endothelial cells and mediate the uptake of microorganisms, apoptotic cells, low-density lipoproteins and other anionic ligands (Peiser et al., 2002). The macrophage mannose-receptor shows the same expression distribution as scavenger receptors and is due to different carbohydrate recognition domains within the receptor able to bind a multitude of carbohydrates including mannose, fucose, n-acetylgalactosamine and glucose (Fraser et al., 1998). The β-glucan receptor, also called dectin-1, is expressed in leukocytes and promotes uptake of carbohydrates with beta-1,3- or β-1,6-glucan linkages (Willment et al., 2001). The innate immune system has been extensively reviewed in (Beutler, 2004).

Adaptive Immunity

Adaptive immunity is mediated by T and B lymphocytes; both cell lines arise from a common lymphoid progenitor, but T cells differentiate in the thymus and acquire the TCR and the phenotypic T-cell markers CD2 and CD3 while B cells differentiate in the bone marrow expressing an IgM antibody instead of a TCR in addition to the B cell markers CD19 and CD45R (Janeway, 2006).

As mentioned before, T cells can further differentiate into CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. CD4⁺ helper T cells have regulatory functions and respond to antigens presented in context of MHC class II molecules found on antigen-presenting cells. Upon activation, immature (Th0) CD4⁺ cells begin to
proliferate and subsequently differentiate into Th1 effector cells, Th2 effector cells or memory cells. The differentiation pathway is regulated by many factors including the type of immune insult, involved cytokines, co-stimulatory molecules and the peptide bound to the MHC complex. Th1 cells drive immune responses towards cell-mediated immunity and are able to activate macrophages. Their cytokine secretion profile includes IL-2, IFN-γ and TNF-α. Th2 cells shift the immune response towards humoral immunity and B-cell activation. Cytokines secreted by Th2 cells are IL-4, IL-5, IL-6, IL-10 and IL-13.

CD8+ cytotoxic T cells are directly able to destroy infected or malignantly transformed cells and carry out immunosurveillance functions. Because of the dangerous cytotoxic potential CD8+ cells possess, the simple contact with a recognized peptide presented by a MHC class I molecule is usually not enough to produce an armed CD8+ effector cell, a co-stimulus (eg. B7 family), provided either by a dendritic cell or an armed CD4+ cell that recognizes the same antigen is necessary. Upon activation, CD8+ cells can kill either by using the perforin/granzyme or the Fas/FasL pathway.

Memory cells can arise from both, CD4+ and CD8+ T cell subsets and are part of the remarkable efficiency of the adaptive immune response. Memory cells will persist in the body long after the initial immune response has been resolved and the associated CD4+ and CD8+ effector cells have disappeared. If the body is attacked by the same pathogen, or, more specifically, is experiencing an insult involving the same antigen, memory cells can instantly be activated and differentiate into effector cells, thereby circumventing the time normally needed
for the adaptive immune system to mount a response. Memory cells also represent the base of vaccination strategies which elicit an immune response to an attenuated or killed organism (or part of it) in order to induce an adaptive immune response resulting in memory cell production and subsequent immunity. B lymphocytes emerging from the bone marrow are, like T cells, considered naïve after production and require a stimulus to be activated. They migrate to lymph nodes where they lay dormant and express IgM and IgD on their surface that are constantly ready to bind the corresponding antigen. Upon antigen contact with the surface Ig molecules, the B cell starts the differentiation process and secretes IgM. Shortly thereafter, gene rearrangements occur which lead to an immunoglobulin switch to IgG, IgA or IgE synthesis. Cytokines secreted by other cells influence the outcome of the rearrangement in terms of secreted Ig. Immunoglobulins are divided into five classes, IgG, IgA, IgM, IgD and IgE, that all differ in their structural composition and function. IgM is either bound to the B cell surface as antigen-recognition structure or secreted as an early-response antibody. It is able to activate complement. IgG is the most abundant antibody and can activate complement and opsonize cells marking them for phagocytic clearance. It is the only antibody able to cross the placental barrier. IgA is mostly found on mucosal surfaces and body fluids and prevents pathogens from binding to and penetrating host barriers. IgE is involved in allergic reactions and immune responses against parasites. It binds to mast cells and basophilic granulocytes and causes them to release mediators like histamine upon antigen contact. IgD serves as an antigen receptor on B cells.
T and B cells share the same requirement in order to carry out the respective effector function: they need to be activated. In most cases (with some exceptions), the activation signal consists of two components. One is an antigenic stimulus that interacts with the TCR (T cells) or the surface IgM (B cells). The other is mediated by co-stimulatory molecules from the B7 family; it ensures that the antigen is recognized as foreign and an immune response is triggered. Antigens presented without co-stimulatory molecules (a situation that occurs all the time in the body due to low background uptake of self-proteins from dying cells by antigen-presenting cells [APC]) induces anergy, a state in which the T cell becomes tolerant to this antigen.

B cells are special in the way that they can also act as an APC by presenting engulfed antigens on MHC class II. Furthermore, they can either be activated by the combination of antigen and co-stimulatory molecules expressed on armed helper T cells or by certain microbial antigens alone.

A special subset of cells called APC is able to provide the necessary stimuli for lymphocyte activation. Monocytes, macrophages, dendritic cells and B cells belong to this subset; with the ability to engulf pathogens (phagocytosis) or smaller molecules in solution (pinocytosis), process the antigens and present them in context of MHC class I and II molecules with the necessary co-stimulatory molecules they serve as the important link between innate and adaptive immunity.

Bearing in mind that every single T and B cell in the body can specifically recognize one antigen the question arises how we can be protected against the
vast number of pathogens that all have their own set of expressed antigens. If we, for example, consider the Ig repertoire of a human (the total number of producible antibodies that can recognize different antigenic sequences) which is calculated to be at least $10^{11}$, it becomes apparent that we cannot possess a specific DNA-sequence coding for the recognition molecule of each possible antigen; the way nature circumvented this problem is by somatic diversification. Humans inherit a small, defined number of genetic sequences that encode parts of the recognition molecules (TCR and Ig). By combining those gene sequences in many different ways, a process called genetic rearrangement, a large variety of TCR and Ig with different antigenic specificities can be created.

The Role of Interleukin-2

IL-2, a cytokine composed of 133 amino-acids in monomeric form, is secreted by T lymphocytes in response to activation upon antigen contact with appropriate co-stimulatory molecules. It acts as a T cell growth factor and stimulates immune responses (Janeway, 2006).

Besides induction of IL-2 synthesis and secretion, activation of T cells also results in the surface-expression of a high-affinity Interleukin-2-receptor (IL-2R). The IL-2R is a heterotrimeric molecule composed of an $\alpha$- and $\gamma$-chain in the intermediate-affinity state, and an additional $\alpha$-chain (CD25) in its high-affinity state (Liu et al., 1996; Minami et al., 1993). IL-2 interaction with the $\beta$ or $\gamma$ subunit leads to phosphorylation of the receptor itself and associated JAK1 and JAK3 molecules. The subsequent binding of STAT5 results in its phosphorylation
causing STAT5 to dissociate, dimerize and translocate into the nucleus. Other downstream IL-2 signalling actions include phosphorylation of the src-family protein kinases LCK and SYK, upregulation of BCL-2 expression and induction of the PI3K-Akt pathway and the RAS-RAF MAPK pathway (Miyazaki et al., 1995; Smith, 2006).

The molecular actions translate into various effects exerted on other stimulated immune cells. T cells are subjected to proliferation induction and a differentiation shift to effector function producing cytotoxic T lymphocytes (CTL) (Rizzo et al., 1987). B cells experience facilitated synthesis of Ig and also show increased proliferation (Miyawaki et al., 1987). NK cells also show increases in proliferation rate and are stabilized, resulting in increased persistence (Sitnicka and Hansson, 1992). T cell and NK cell proliferation and activation gives rise to an immune cell population called LAK cells which will be discussed in the next section. In addition, IL-2 promotes survival and growth of regulatory T cells, thereby maintaining peripheral tolerance (Fehervari et al., 2006) and promotes elimination of self-reactive T cells by the mechanism of activation-induced cell death (Dai et al., 1999).

Various toxicities that arise with the application of IL-2 have been investigated and are mostly linked to the immune cell activation with subsequent release of large amounts of cytokine and acute phase reactants. Those released substances act as chemoattractants for lymphocytes, cause lymphocyte infiltration and increase the permeability of capillary endothelial cells leading to the capillary leak syndrome with associated hemodynamic instabilities (Anderson
et al., 1996; Fraker et al., 1989; Jin et al., 2006). In addition, IL-2 appears to
directly act on ion channels located in the heart causing arrhythmias, increased
left-ventricular pressure and increased coronary blood flow (Wang et al., 2001).
Gastrointestinal toxicities also result from lymphocytic invasion and subsequent
action of the secreted cytokines (Kaufman et al., 2002).

The finding that IL-2 application caused T cell proliferation and CTL
differentiation rejuvenated immunotherapy; many clinical trials were conducted
using IL-2 in various administration routes, dosage regimens and as an ex vivo
activating agent for adoptive immune cell transfer strategies. Although responses
were noticed, they were rather infrequent. Therefore, despite being a significant
improvement, it became obvious that research had to focus on finding
alternatives or to complementing additions to this treatment in order to achieve
better outcomes. Shortly after receiving the first results, the inability of IL-2 to
meet the high expectations lead to a decline in enthusiasm for
immunotherapeutic approaches. Recent advances in the understanding of
underlying mechanisms, crosstalks, the discovery of modulating factors and
problems associated with effective immune responses in cancer patients give
rise to the hope that in the future we might be able to manipulate all variables to
harness an efficient immune system-based response to malignantly transformed
cells (Steinman and Mellman, 2004).

Today, IL-2 has an established role in treatment of metastatic renal cell
carcinoma and metastatic melanoma; for both applications IL-2 is FDA-approved.
Despite relatively low response rates (16% overall response rate, 6% complete
responses) physicians occasionally observe stable complete responses (Atkins et al., 1999, 2001; Lens, 2003; McDermott et al., 2005; Rosenberg et al., 1994). A major drawback of IL-2 therapy is the severe side effects profile including capillary leak syndrome, hypotension, respiratory alkalosis, cardiac toxicity, neurologic toxicity, gastrointestinal toxicity, hepatobiliary toxicity, chills, nausea, vomiting and diarrhea which connect the use of IL-2 to a low but significant mortality rate (Dutcher et al., 2001; Vial and Descotes, 1992).

**Lymphokine-activated Killer Cells**

LAK cells are a heterogeneous population of cytotoxic lymphocytes that are able to lyse tumor cells without prior antigen-priming (Yang et al., 1986). They arise after in vitro or in vivo exposure to IL-2 (Grimm et al., 1983); studies suggest that their progenitors are of T cell and NK cell origin (Chadwick et al., 1993; Tilden et al., 1987). During the activation period, a variety of cells emerge which differ in their surface antigen expression and their lytic capabilities. While some studies reported that tumor killing activity was mainly a function of the NK cell compartment (Phillips and Lanier, 1986; Tilden et al., 1987), other studies using different tumor models point at the major contribution of T cells (Kalland et al., 1987). Roussel et al. (1990) described the interesting finding, that the LAK cell phenotype is influenced by the duration of IL-2 activation. LAK-cytotoxicity can be divided into short-term effects, mediated by perforin/granzyme and Fas/FasL pathways, and long-term effects attributable to soluble and membrane-bound TNF (Lee et al., 1996).
Clinical approaches to harness the tumor-killing capabilities of LAK cells either employ IL-2 application to generate LAK in vivo or use the adoptive cell transfer strategy that consists of peripheral blood mononuclear cell isolation with ex vivo activation in IL-2-supplemented cell cultures and re-infusion into the patient (Hoffman et al., 2000; Rosenberg, 2001; Yang and Rosenberg, 1988).

**Immunosurveillance and Cancer Immunoevasion**

The question, whether the human immune system can fight cancer effectively or not elicited numerous debates and views that were subject to great changes over time. Parish presented a comprehensive review about the changing opinions regarding this topic in his article about cancer immunotherapy (Parish, 2003).

Currently, immune reactions against tumors are an established fact and the presence of immunosurveillance is widely recognized. Furthermore, deeper understanding of acting mechanisms and appreciation of the dynamic interplay between the immune system and the tumor itself led to the introduction of the immunoediting concept. Immunoediting consists three phases, namely elimination (which represents the past understanding of immunosurveillance), equilibrium and escape. In Immunoediting, it is accepted that an arising tumor is attacked by the immune system in the elimination phase which leads to the destruction of many tumor cell clones. Eradication of all tumor cell clones leads to successful defense against this insult. If not all clones are eradicated, surviving clones can enter the phase of equilibrium in which they may either be held in
check by the immune system (establishing a chronic presence) or immunologically sculpted to render new clones which might possess survival advantages that allow entry in the escape phase. In the escape phase, the new tumor variants are able to evade the immune system and establish a detectable disease. Evidence for this model have been extensively reviewed by Dunn et al. (2004a), Dunn et al. (2004b) and Smyth et al. (2006). The focus of research has therefore experienced a shift away from the question “is the immune system capable to combat cancer” to the question “why is the immune response in cancer patients present but ineffective.”

Over the past years the scientific community gained deeper insights into immune-evasion mechanisms employed by tumor cells; even though researchers are beginning to connect various dots a complete understanding that is necessary to translate basic knowledge into powerful immunomodulation therapies has still not been achieved. An important contributor to tumor immunoediting is Darwinian selection. As explained in the section about cancer, each tumor cell is subjected to high mutation rates leading to the accumulation of many different mutations with a random distribution among the tumor. If a mutation confers a survival advantage represented by a lowered susceptibility against immune system–mediated killing this tumor cell will survive and pass the mutation to its progeny, while other cells without the mutation are being killed. In a slow progress, the tumor population balance shifts from mostly susceptible cells to mostly resistant cells.
The review of Smyth et al. (2006) differentiates between two categories of tumor escape: intrinsic mechanisms, focusing on events that influence the tumor cell directly, and extrinsic mechanisms, dealing with the interaction of the tumor cell with the host immune system. Smyth et al. organized intrinsic mechanisms as follows: (a) lack of expression of MHC class II molecules and co-stimulatory molecules; (b) downregulation or loss of expression of MHC class I molecule proteins; (c) downregulation or expression of genes associated with antigen presentation (such as transporter associated with antigen processing (TAP), low-molecular-weight protein (LMP) and β2-microglobulin); (d) low level of expression of tumor-associated antigens at early phases of tumor growth; (e) loss of antigenic epitopes; (f) physical barrier preventing effector cells accessing tumors; and (g) loss of response to IFNs (Smyth et al., 2006).

Extrinsic mechanisms are: (a) ignorance; (b) tolerance of T cells to tumor-specific antigens resulting from anergy or deletion caused by host APC, myeloid cells, or regulatory T cells; (c) suppression of T cells caused by tumor-derived factors [e.g., transforming growth factor (TGF-β), IL-10, vascular endothelial growth factor (VEGF), FasL, galectin, indoleamine 2,3-dioxygenase (IDO)], immunosuppressive myeloid or regulatory T cells; (d) secretion of soluble ligands that block lymphocyte activation (e.g. NKG2D-L); (e) defects in antigen presentation by professional APC; and (f) impaired APC maturation (Smyth et al., 2006).
**Selection of Tumor Types for our Project**

**Rationale for Selecting Specific Cancer Cell Lines**

We selected two epithelial tumors for our project: renal cell adenocarcinomas and ovarian adenocarcinomas. In renal cell carcinomas, IL-2 has an established role as first-line agent in metastatic disease. Due to this existing protocol the proof of a chemosensitizing effect mediated by low-dose chemotherapy would allow its easy implementation as a supplemental agent into existing immunotherapy regimens. The rationale for this implementation is derived from the high immunogenicity of the cancer and the primary aim of therapy (stimulating the immune system to attack the cancer); consequently, the adjunct use of an agent that boosts the effectiveness of the immune response while keeping a low toxicity profile would clearly be beneficial.

Chemosensitization in general refers to the ability of a compound to increase the sensitivity of an exposed target to a second treatment; in our project the compound is represented by a chemotherapeutic drug, the exposed target is the chosen cancer cell line and the second treatment is the addition of activated lymphocytes.

Ovarian cancer is currently not being treated with IL-2; owing to the late stages this particular cancer is presenting at upon detection, regimens are being investigated that can effectively deal with metastatic disease. Here, immunotherapy might play a very important role.
Renal Cell Carcinoma

Renal cell cancer includes the group of renal cell carcinomas, making up 80-85% of all cases, and the transitional cell carcinomas of the renal pelvis accounting for 15-20% (Motzer et al., 1996). The prevalence for the year 2005 was 2.6% in the US (Jemal et al., 2005); predicted numbers for incidence and mortality during the year 2007 are 51,190 and 12,890, respectively (Jemal et al., 2007). Renal cell carcinomas are not extremely common, they account for only 2% of all malignancies (McLaughlin and Lipworth, 2000). Nevertheless, cancer statistic data over the last years shows a steady increase in incidence, which is most probably due to improved imaging techniques and their routine employment in medical practice (Drucker, 2005; McLaughlin and Lipworth, 2000), and a relatively constant mortality (Greenlee et al., 2001, 2000; Jemal et al., 2002, 2003, 2004, 2005, 2006).

Acknowledged risk factors for development of kidney cancer are, besides genetic inheritance of germ-line mutations, smoking, obesity, hypertension, unopposed estrogen therapy, diet, exposure to petroleum, asbestos or heavy metals and acquired cystic kidney disease (Cohen and McGovern, 2005; Motzer et al., 1996; Murai and Oya, 2004).

Renal cell carcinomas can be classified according to their histology into clear-cell carcinomas (75%), papillary carcinomas type I and II (15%), chromophobe carcinoma (5%) and oncocytoma (5%); the histologic types of collecting duct carcinoma and medullary carcinoma are very rare (<1%) (Linehan et al., 2003). Recent discoveries established a link between certain mutated
genes and the resulting histological cancer types. Mutations in the von Hippel-Lindau (VHL) gene are linked to clear-cell carcinomas, the Met gene is involved in the development of type I papillary carcinomas while the FH gene accounts for type II papillary carcinomas. Chromophobic carcinomas and oncocytomas are associated with BHD gene mutations. The mentioned genes were long known for their role in inherited disease syndromes (like von Hippel-Lindau disease) and the increased incidence of kidney cancer linked to those syndromes, but recent studies showed that those genes are also often involved in sporadic cases (Linehan et al., 2003).

While the classic presentation of renal cell carcinoma involves hematuria, flank pain and a palpable flank or abdominal mass, most cases are currently detected during the asymptomatic stage which is a result of increases in use of sophisticated diagnostic imaging (McLaughlin and Lipworth, 2000; Motzer et al., 1996). Therefore 50% of cases present in stage I or II upon diagnosis (McLaughlin and Lipworth, 2000). Diagnosis is usually established by ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI); those modalities have mostly replaced intravenous urography (Motzer et al., 1996). CT, chest X-ray and bone scans are used for tumor staging which is based on the revised TNM system published by the American Joint Committee on Cancer (AJCC) (Drucker, 2005). Stage I and II represents tumor of different dimensions but confined to the kidney, stage III represents tumor extension into neighboring structures but confined to Gerota’s fascia and/or lymph node involvement and stage IV involves tumor invasion beyond Gerota’s fascia or distant metastasis
Metastases are most commonly found in lungs, lymph nodes, brain, bone and liver (Drucker, 2005).

The mainstay of treatment in stages I through III remains radical nephrectomy (Cohen and McGovern, 2005). It is important to notice that adjuvant therapy has so far no proven benefit in patients with complete resection (Clark et al., 2003; Cohen and McGovern, 2005; Messing et al., 2003), but, owing to the high recurrence rates, the need for identification of effective adjuvant therapies is recognized (Bleumer et al., 2006). While total nephrectomy includes the kidney, Gerota's fascia, ipsilateral adrenal gland and regional lymph nodes (Cohen and McGovern, 2005), a different approach termed nephron-sparing surgery, which includes techniques like laparoscopic partial nephrectomy, thermal ablative techniques or open partial nephrectomy (Fergany, 2006), is indicated in patients who would be rendered functionally anephritic after radical nephrectomy because of conditions like insufficient function of the contralateral kidney, bilateral synchronous renal cell carcinoma or current condition after prior nephrectomy (Novick, 1995). Studies show that nephron-sparing surgery can safely be used in low-stage tumors with same recurrence rates like radical nephrectomies (Hollingsworth et al., 2006).

Management of patients with metastatic disease is challenging and includes evaluation with approaches tailored to each individual. Possible treatment options include palliative nephrectomy with metastasectomy, cytoreductive surgery followed by systemic therapy or systemic therapy only in patients not qualifying for surgical approaches. While palliative surgery can
improve quality of life, cytoreductive surgery shows benefits in terms of disease-free survival (Flanigan et al., 2004; Kavolius et al., 1998; Russo, 2004). Systemic therapy has been expanded over the past years; the first immunomodulatory agents showing effectiveness were IFNα and IL-2. While for IFNα overall response rates of 14% were observed, IL-2 did not show much higher overall response rates (21% in high-dose regimens, 13% for low-dose regimens) but was able to induce more stable remissions (Fisher et al., 2000; Yang et al., 2003). Sorafenib and sunitinib, two agents from the class of kinase inhibitors which inhibit VEGF, platelet derived growth factor (PDGF) and c-kit receptor tyrosine kinases (Larkin and Eisen, 2006), were successfully tested and are now being included in phase III trials and management plans (Motzer et al., 2006; Ratain et al., 2006). Other therapies currently being investigated include bevacicumb (antibody against VEGF), panitumumab (antibody against epidermal growth factor receptor [EGFR]), gefitinib/ erlotinib (EGFR tyrosine kinase inhibitors), temsirolimus (mTor kinase inhibitor), tumor vaccines and allogeneic stem-cell transplantation (Cohen and McGovern, 2005).

Chemotherapy is mostly ineffective in renal cell carcinomas; Vinblastine was used in the past but might be increasingly replaced by regimens containing gemcitabine, capecitabine and / or floxuridine (Milowsky and Nanus, 2003; Stadler et al., 2006; Wenzel et al., 2002). Radiotherapy plays a small role in local symptom control but is not included in regular curative or palliative treatment approaches (Drucker, 2005).
The relative 5-year survival rate reported for this malignancy is 65% (Jemal et al., 2006).

**Ovarian Carcinoma**

Statistics for cancer of the ovary report a prevalence of 22,430 predicted new cases and 15,280 predicted deaths for the year 2007 (Jemal et al., 2007). Data over the last years shows a decline in incidence while the numbers for mortality are mostly constant with slight oscillations around 15,000. Ovarian cancer has been, and still is, the leading cause of death from gynecologic malignancies (Bhoola and Hoskins, 2006).

Epidemiologic studies have identified factors that can increase or decrease the risk of developing cancer of the ovary. Factors that decrease the risk are young age at first pregnancy, young age at first birth, oral contraceptives and breast feeding. The converse conditions, old age at first pregnancy and birth as well as nullipara, age, polycystic ovary disease, pelvic inflammatory disease and high-fat diet contribute to a risk increase (Guppy et al., 2005). Genetic mutations in BRCA1 or 2 account for 10% of all cases (Bhoola and Hoskins, 2006) and confer a strong increase in susceptibility leading to an estimated risk of 20-60% for developing an ovarian carcinoma by the age of 70 years (Narod and Boyd, 2002).

According to the WHO, ovarian neoplasms are classified into three main groups describing their tissue of origin; these groups are surface epithelial-stromal tumors, sex cord-stromal tumors and germ cell tumors. Various
histological subtypes can be found in each of the main groups. Ovarian epithelial carcinomas are by far the most common and account for 90% of all cases (Guppy et al., 2005). Diagnosis of this malignancy regarding the time-point in the course of the disease remains a major problem. The majority (68%) of diagnosed cases present with distant metastases translating into stage IV disease (Jemal et al., 2007). Much effort has been devoted to the discovery of a successful screening method but so far techniques like serum CA-125, which is the only FDA-approved screening method, transvaginal ultrasound and the combination of both, although being better than one modality alone, have not been able to show the expected results (Hakama et al., 1996; Rosenthal and Jacobs, 1998; Roupa et al., 2004). A promising approach for the future might be proteomic profiling which shows higher sensitivity and specificity values (Kong et al., 2006; Lin et al., 2006).

While early-stage disease is often asymptomatic, advanced disease can produce symptoms related to the mass effect of the tumor like constipation and abdominal distension as well as change in bowel habits, bloating, pain, bleeding, vaginal bleeding and ascites (Guppy et al., 2005). Ultrasound or CT along with a physical examination, laboratory studies and CA-125 represent the primary work-up for undiagnosed pelvic masses and can, depending on the situation, be complemented with chest X-ray and gastrointestinal imaging studies (Im et al., 2005). Staging is done during surgical exploration and uses the Federation Internationale de Gynecologie et d’Obstetrique (FIGO) system: stage I describes tumor limited to the ovary, in stage II the tumor extends to the pelvis, stage III
can either be tumor in one or both ovaries with peritoneal metastases, tumor extends over limit of pelvis or lymph node involvement. Stage IV is reserved for metastasized tumors (except peritoneal metastases which are stage III). There is also a AJCC TNM staging system but the most commonly used system remains FIGO (Greene, 2002).

In ovarian cancer, all patients receive surgery as an initial therapy. A unique characteristic of this type of cancer is that the initial intervention, surgery, is carried out for both treatment and staging purposes (Greene, 2002). The surgical approach consists of the removal of the omentum, sampling of iliac and para-aortic lymph nodes, blind biopsies of neighboring tissues and the removal of uterus, adnexae and both ovaries (Trimbos et al., 2003). If the patients wishes to retain fertility, a unilateral salpingo-oophorectomy can be considered in stage I diseases (Rao et al., 2005).

Adjuvant chemotherapy is used after surgery for certain risk levels in early stage disease and for advanced stage disease. The standard regimen includes paclitaxel plus carboplatin (Ozols et al., 2003); alternatively docetaxel plus carboplatin (Vasey et al., 2004) or paclitaxel plus cisplatin (McGuire et al., 1996) can be considered. Stage IA and IB patients in the low risk category are exceptions who can omit adjuvant chemotherapy and be followed by observation (Young et al., 1990). The role of radiotherapy is difficult to assess but it might become incorporated in future management of stage III disease as consolidation therapy after surgery and chemotherapy (Einhorn et al., 2003; Sorbe, 2003). Localized disease carries a good prognosis with 5-year survival rates of 94%;
these rates rapidly drop down to 69% for regional spread and 29% for distant metastasis (Jemal et al., 2006).

Chemotherapeutic Drugs

Rationale for Selecting Specific Chemotherapeutic Drugs

A wide variety of chemotherapeutic drugs is currently available for treatment. Many approaches to assign those agents into groups, often separating mode of effect or origin, face the difficulty of overlap; nevertheless grouping is done for easier reference and study purposes. A commonly used approach recognizes (a) alkylating agents; (b) antimetabolites; (c) platinum complexes; (d) antimicrotubule agents; (e) antitumor antibiotics; (f) topoisomerase inhibitors; and (g) miscellaneous agents (Holland-Frei, 2005).

For our study we selected gemcitabine as our main drug; the main reason for this decision was its high chemosensitization efficacy in preliminary screening experiments. In addition, the low toxicity profile, the frequent use in various metastatic disease settings and the solid amount of evidence for its efficacy based on a large body of studies increased its attractiveness as a candidate drug. To extend the study and to test whether observed effects could also be applied to other agents we chose two more drugs to be examined. We selected drugs from the class of antimicrotubule agents with small but important differences in their mode of action: vinblastin, a vinca alkaloid, represents a drug that has been in use for a long time and the fact that it is still employed in a large number of
treatment regimens and clinical trials underlines its efficacy. Paclitaxel, in contrast, is a relatively new drug and belongs to the taxane subclass.

**Gemcitabine**

Gemcitabine (2’,-2’-difluorodeoxycytidine) belongs to the group of antimetabolites and can further be described as a nucleoside analogue. The chemical structure of this synthetic drug strongly resembles deoxycytidine and cytosine arabinoside with the difference of having two fluorine atoms at position 2’ of the sugar (Storniolo et al., 1997).

After uptake into cells, gemcitabine is phosphorylated by deoxycytidine kinase resulting in the active metabolites difluorodeoxycytidine diphosphate and triphosphate (Heinemann et al., 1988). Difluor diphosphate acts as an inhibitor of ribonucleotide reductase leading to depletion of deoxynucleotides used for DNA synthesis (Storniolo et al., 1997). The triphosphate is incorporated into DNA directly and causes masked chain termination by first allowing another nucleotide to be added by DNA polymerase and then blocking the enzyme (Huang et al., 1991). This allows gemcitabine to avoid the proofreading function which is dependent on the 3’-5’exonuclease activity of the polymerase complex (Kunkel, 1988).

The main influence is exerted on DNA synthesis by the mechanism of chain termination; RNA synthesis is also inhibited, although to a much lesser degree. A combined effect of ribonucleotide reductase inhibition and action as a competitive substrate for deoxycytidine kinase (thereby preventing the activation
of deoxycytidine) results in depletion of substrate pools (Ruiz van Haperen et al., 1993). Additionally, radiosensitizing properties (Kal et al., 2006; Lawrence, 2003) and synergy with other chemotherapeutic agents (Robinson et al., 2004) have been reported. One noteworthy aspect of gemcitabine is the self-potentiating potential mediated by its metabolites and effects on various enzymes: the inhibition of ribonucleotide reductase results in a decreased pool of deoxycytidine triphosphate (dCTP) which shifts the favored substrate for DNA polymerase from dCTP to gemcitabine (dFdCTP). Furthermore, the decrease in dCTP inhibits dCMP deaminase, an enzyme responsible for the metabolism of gemcitabine. dCMP deaminase is regulated by the dCTP:dTTP ratio which is lowered owing to the depletion effect on dCTP resulting in inhibition and subsequent impairment of gemcitabine metabolism. The last self-potentiating effect is exerted on deoxycytidine kinase which is negatively regulated by dCTP. Again, the dCTP depletion results in increased activity of deoxycytidine kinase trapping and activating more gemcitabine in the cell (Gandhi et al., 1991).

The adverse effect profile of gemcitabine includes gastrointestinal symptoms like nausea, vomiting and diarrhea and myelosuppression; overall it is reported to be relatively mild, making it useful for palliative treatment in people of older ages or reduced performance status (Gallelli et al., 2004).

Resistance to gemcitabine is conferred by mutations in enzymes like deoxycytidine kinase (decreased activation) and cytidine deaminase (increased deactivation) (Bergman et al., 2002; Neff and Blau, 1996).
In the US the first FDA-approval for gemcitabine was granted in 1996; today it is approved for treatment of nonresectable stage II/III and metastatic adenocarcinoma of the pancreas, treatment in combination with cisplatin in inoperable stage IIIA/IIIB or metastatic non-small cell lung cancer, treatment of relapsed ovarian cancer and treatment in combination with paclitaxel in metastatic breast cancer (U.S. Food and Drug Administration, 2006) but it is currently investigated, mostly in combination with other chemotherapeutic agents, in various clinical trials including renal cell carcinoma, bladder cancer, head and neck cancer, non-Hodgkin Lymphoma, prostate cancer and others (U.S. National Institutes of Health, 2006).

**Vinblastine**

Having been discovered over 40 years ago, vinblastine is a well-known chemotherapeutic drug whose effectiveness in treatment has allowed it to remain in use up to now. Vinblastine belongs to the class of vinca alkaloids which represent one group of the antimicrotubule agents in chemotherapy and can be extracted, along with other alkaloids, from the periwinkle leaves (*Catharanthus roseus G. Don*) (Jordan and Wilson, 2004; Mukherjee et al., 2001).

Drugs in the vinca alkaloid group exhibit their anticancer effect by binding to a distinct region on the β-subunit of tubulin named vinca-binding domain (Bai et al., 1990) and altering microtubule dynamics. Low concentrations induce mitotic arrest by suppression of microtubule dynamics (Jordan, 2002) leading to subsequent apoptosis (Jordan et al., 1996). High concentrations rapidly
depolymerize microtubules and destroy the mitotic spindle locking cells in mitosis (Jordan et al., 1996).

Side effects are manageable and include myelosuppression which manifests mostly as leucopenia and also represents the dose-limiting factor, gastrointestinal disturbances, alopecia, neurotoxicity, nausea and vomiting (Grosh et al., 1983; Schulman et al., 1982; Sorenson et al., 1983).

Resistance development is reported to be due to various mechanisms: efflux of the drug mediated by p-glycoproteins (MDR genes), mutational changes in tubulin-subunits, changes in expression levels of different tubulin-subunits, mutations in MAP or apoptosis-promoting and –inhibiting genes and alterations in microtubule-stability that produce hyperstable microtubules confer resistance to depolymerizing agents like vinblastine (Dumontet and Sikic, 1999; Jordan and Wilson, 2004; Pellegrini and Budman, 2005).

Vinblastine got its first FDA-approval in 1965 and is currently being used for treatment of breast cancer, choriocarcinoma, cutaneous T cell lymphoma (CTCL), Hodgkin’s disease, Kaposi’s sarcoma, Langerhans’ cell histiocytosis, mycosis fungoides, non-Hodgkin’s lymphoma (NHL), testicular cancer (U.S. Food and Drug Administration, 2006).

Despite its old age, over 20 clinical trials are currently recruiting patients for evaluation of activity against neoplasias like gliomas, melanoma, Hodgkin’s lymphoma, bladder cancer and others (U.S. National Institutes of Health, 2006).
**Paclitaxel**

Paclitaxel is a naturally occurring compound discovered in 1963. It was isolated from the pacific yew *Taxus brevifolia* and showed significant cytotoxic activity against multiple solid tumors in screening programs performed by the National Cancer Institute (Rowinsky and Donehower, 1995). One serious problem was the low abundance and the slow growth of the plant making large scale isolations impossible. Owing to the complex chemical structure simple synthetic manufacturing was also not possible, but the discovery of a precursor in other yew species from which paclitaxel (and later another semi-synthetic agent of the taxane class, docetaxel) could be synthesized made high-volume synthesis of this group possible (Mukherjee et al., 2001).

Paclitaxel is an antimicrotubule agent and belongs to the subclass of taxanes. In contrast to the other antimicrotubule subgroup, the vinca alkaloids, taxanes bind to the taxane site located on the inside of the β-tubulin subunit (Nogales et al., 1995) and act, depending on their concentration, by microtubule dynamics suppression due to inhibition of depolymerization (low concentration), inhibition of polymerization and depolymerization (intermediate concentrations) or inhibition of depolymerization and stimulation of polymerization (high concentrations) (Derry et al., 1995; Rowinsky and Donehower, 1995). The ensuing mitotic arrest progresses to apoptosis (Kelling et al., 2003). Studies also show that paclitaxel is able to sensitize cancer cells to radiotherapy (Marangolo et al., 1996; Preisler et al., 1999; Steren et al., 1993), to enhance expression of
TNFα, IL-1 and nitric oxide in macrophages (Allen et al., 1993; Pae et al., 1998) and to inhibit angiogenesis (Belotti et al., 1996).

Side effects related to dosing regimens used in treatment are dose-limiting myelosuppression (mostly neutropenia), hypersensitivity reactions and neuropathy. Occasionally, gastrointestinal symptoms like nausea and vomiting, mucositis and cardiac rhythm disturbances can occur.

Tumor resistance is increasingly observed and reported to be related to efflux of the drug mediated by multidrug resistance glycoproteins, mutations in the binding site of the β-tubulin subunit, mutational changes in the apoptotic and cell-cycle regulation pathways, lipid composition changes of the plasma membrane and overexpression of IL-6 (Orr et al., 2003; Yusuf et al., 2003).

Paclitaxel received its first FDA-approval in 1992 and is now in schedules for treatment of AIDS-related Kaposi-Sarcoma, treatment of metastatic ovarian carcinoma alone or in combination with cisplatin, treatment of metastatic or relapsed breast carcinoma, adjuvant treatment of lymph node positive breast carcinoma and treatment of non-small cell lung carcinoma in combination with cisplatin for patients who cannot receive curative surgery or radiotherapy (U.S. Food and Drug Administration, 2006). Paclitaxel is also part of over 200 clinical trials investigating its effect alone or in combination in various malignancies like head and neck cancer, pancreatic cancer, melanoma, bladder cancer and others (U.S. National Institutes of Health, 2006).
MATERIALS AND METHODS

Cell Lines

The renal cell adenocarcinoma cell line 769-P was purchased from ATCC (Manassas, VA) and grown according to the company recommendations in RPMI culture medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10mM HEPES (Invitrogen, Grand Island, NY), 1.0mM sodium pyruvate (HyClone, Logan, UT), 0.05mg/ml gentamicin (Invitrogen, Grand Island, NY), 4.5g/L glucose (J.T. Baker Chemical Co., Phillipsburg, NJ) and 2mM glutamine (HyClone, Logan, UT).

The ovarian adenocarcinoma cell line OVCAR3 was purchased from ATCC (Manassas, VA) and grown according to the company recommendations in RPMI culture medium supplemented with 20% fetal bovine serum, 0.01mg/ml insulin (Invitrogen, Grand Island, NY), 10mM HEPES, 1.0mM sodium pyruvate, 0.05mg/ml gentamicin, 4.5g/L glucose and 2mM glutamine. Culture incubation conditions were 37°C, 100% humidity and 5% CO₂.

Drugs

Recombinant human IL-2 was purchased from eBioscience (San Diego, CA) and diluted in sterile RPMI culture medium.

Gemcitabine (Eli Lilly, Indianapolis, IN) was purchased from the inpatient pharmacy in powder form (200mg) and reconstituted by adding 6mg to 10ml of culture medium yielding a 2000µM concentration working stock. The working stock was stored in the freezer.
Vinblastine Sulfate (Bedford Laboratories, Bedford, OH) was purchased from the inpatient pharmacy in powder form (10mg) and reconstituted by adding 1.818mg to 10ml of culture medium yielding a 200µM concentration working stock. The working stock was stored in the freezer.

Paclitaxel (Bedford Laboratories, Bedford, OH) was purchased from the inpatient pharmacy in liquid form (30mg/5ml). 284.6µl were added to 10ml of culture medium yielding a 200µM concentration working stock. The working stock was stored in the freezer.

**Tissue Culture**

**Starting with Frozen Cells**

The purchased cells were stored in a liquid nitrogen tank. To establish working cell lines, cryotubes containing cells were thawed in a water bath and the content was added to 4ml media in a T25 tissue culture flask. The cells were kept at a sub-confluent state and medium was renewed every 3-4d.

**Harvesting and Passage of Cells**

After discarding the media the flasks were washed twice with PBS/EDTA. 0.5% trypsin/EDTA was then added and the flask was placed in the incubator for 3 min. Media was added to stop the trypsin activity and the suspension was transferred to a 15ml tube for centrifugation (200G, 4min, room temperature). If the cells were harvested to be used in assays 0.14% collagenase/ 0.1% DNAse (Sigma, St. Louis, MO) was added and the cell pellet was dissolved. The
suspension was passed through a 27G needle once and incubated for 20 min to obtain a single cell suspension. After centrifugation the pellet was dissolved in complete media. Before reaching 100% confluence, the cells were harvested as described and split. The contents of one flask were equally split into 4-12 flasks (RCC) or 2-4 flasks (OVCAR3).

**Long-term Storage of Cells**

For long-term storage of cells, suspensions containing 3-4 x 10^6 cells/ml in complete media were prepared. Cryotubes were filled with 50μl of 5% DMSO. After addition of 950μl cell suspension, the crotubes were closed, gently mixed, placed into a styrofoam box for protection, put into a -20°C freezer for 12-24h and subsequently transferred into a liquid nitrogen tank.

**MTT Assay**

The MTT ((3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was adapted in a modified form from Ruben et al. (Ruben and Neubauer, 1987). Cells were harvested as described in the ‘Harvesting and Passage of Cells’ section. After washing, re-suspending in complete media and counting, the volume was adjusted to obtain a concentration of 50,000 cells/ml. A volume of 100μl of the cell suspension was distributed into a flat bottom 96-well plate in quadruplicates for each condition. The first four wells only contained 200μl of complete media for the blank reading. The 96-well plate was placed in a
humidified incubator for 12h to allow the cells to attach. Two extra rows of cells were distributed into another 96-well plate to obtain the baseline absorbance.

On the next day, the separate plate was processed in a way described in the next paragraph and the baseline absorbance value was recorded. The chemotherapeutic drug was prepared, diluted in another 96-well plate and transferred to the assay plate (100µl of diluted drug + 100µl of cell suspension). A volume of 100µl of complete media was added to the second row for the media control condition. The plate was placed in a humidified incubator for 3d.

After the incubation, 20µl of a 5mg/ml MTT stock (USB Corp., Cleveland, OH) were added to each well and the plate was placed in a humidifier incubator for 4h. In the next step the fluid of each well was carefully aspirated using a multichannel pipetter, 100µl DMSO (Sigma, St. Louis, MO) was added to dissolve the formazan crystals and the plate was placed on a shaker for 20 min. Absorbance values were obtained with a plate reader (BioTek Instruments) at a wavelength of 580nm. The percentage of growth compared to the media control (100%) was calculated as follows:

\[
\% \text{ growth} = \frac{\text{experimental absorbance} - \text{baseline absorbance}}{\text{media control absorbance} - \text{baseline absorbance}} \times 100
\]

Where experimental absorbance is the value determined at 72h drug exposure and baseline absorbance is the value at the beginning of culture prior to addition of drug. “Negative” growth percentages denote cytotoxic activity.
Chromium Release Cytotoxicity Assay

Isolation and Activation of Peripheral Blood Leukocytes

To isolate peripheral blood mononuclear cells (PBMC), blood was drawn from healthy subjects, gently mixed with the same amount of phosphate-buffered saline and layered over lymphocyte-separation media (Mediatech, Herndon, VA). After centrifuging at 400g for 30 min (room temperature) the serum supernatant was carefully aspirated and the PBMC band was collected. The cells were washed in PBS and grown in a T25 culture flask in RPMI culture medium supplemented with 10% fetal bovine serum, HEPES, sodium pyruvate, gentamicin, glucose, glutamine and 200 IU/ml IL-2 for the indicated amount of days.

Assay Setup

The chromium ($^{51}$Cr) release cytotoxicity assay was adapted in a modified form from Staren et al. (Staren et al., 1989). After a defined exposure time to a chemotherapeutic drug the tumor cells were harvested as described in the “Harvesting and Passage of Cells” section. The cells were counted and the volume was adjusted to obtain a concentration of $1 \times 10^6$ cells/ml. After washing once in media, 100µl of labeling media (RPMI without fetal-bovine serum) and 80µl of chromium (Amersham Pharmacia) was added and the cells were incubated for 1h at 37°C in a CO$_2$ incubator. The cells were then washed three times and re-suspended in complete medium. After counting the volume was adjusted to 50,000 cells/ml.
Effector cells were harvested, washed in complete medium once, re-suspended in 4ml of media and counted. To obtain effector to target (E:T) ratios of 20:1, 10:1, 5:1 and 2.5:1 the cells were adjusted to 1,000,000 cells/ml (20:1) and serial dilution was done for the remaining E:T ratios. Effector and target cells were then dispensed into a round-bottom 96-well plate (Corning Inc., Acton, MA).

For each treatment condition, 24 wells (two rows) were used. For spontaneous release values, the first six wells contained target cells only while for total release values the following six wells contained target cells to which 100µl 10% SDS (Invitrogen, Grand Islands, NY) was added at the end of the incubation period. The second row contained target cells and effector cells of the different E:T ratios in triplicates yielding the experimental release values.

The 96-well plate was placed in a humidified CO₂ incubator for 4h at 37°C. After addition of SDS, the fluid of each well was harvested with a special harvesting frame (Molecular Devices, Sunnyvale, CA). The harvested supernatant was read in a gamma-counter (Beckmann Coulter Inc., Gamma 5500B, Fullerton, CA) and percentage cytotoxicity was calculated as follows:

\[
\text{% cytotoxicity} = \frac{\text{experimental }^{51} \text{Cr release} - \text{spontaneous }^{51} \text{Cr release}}{\text{total }^{51} \text{Cr release} - \text{spontaneous }^{51} \text{Cr release}} \times 100
\]

Where spontaneous release values were obtained by RCC incubated in media, and total release values by RCC treated with 100µl of a 10% SDS solution.
Cytotoxicity values were determined by averaging the $^{51}$Cr release from the triplicate wells. Lytic units (LU) were calculated as previously described by Staren et al. (Staren et al., 1989). One LU was defined as the number of effector cells needed to lyse 20% of the 5000 target cells and expressed as the number of LU / $1.0 \times 10^7$ effector cells.

**Flow Cytometry**

**Fas and MHC ABC Surface Expression Analysis**

Tumor cells were harvested as described in the "Harvesting and Passage of Cells" section and washed twice in 1ml cold buffer (PBS without Ca$^{2+}$ and Mg$^{2+}$ supplemented with 2% fetal calf serum and 0.1% sodium azide). Cells were counted, suspended in cold buffer and adjusted to the concentration of $1 \times 10^7$ cells/ml. Flow tubes for staining were prepared by adding 50µl cold buffer and 20µl antibody: anti-human Fas (CD95) antibody (Clone DX2, 0.025mg/ml, BioLegend, San Diego, CA) conjugated to PE; anti-human MHC ABC antibody (Clone W6/32, 0.1mg/ml, AbD Serotec, Raleigh, NC) conjugated to FITC. After adding 50µl of the cell suspension, the tube was briefly vortexed and incubated for 30 min on ice in the dark. After incubation the cells were washed twice with 1ml cold buffer and re-suspended in 1ml cold buffer for analysis.

For the negative controls, we used anti-mouse IgG1 conjugated to PE (Clone X40, 0.05mg/ml, Becton Dickinson Immunocytometry, San Jose, CA) and anti-mouse IgG2a negative control conjugated to FITC (Clone MRC OX-34, 0.1mg/ml, AbD Serotec, Raleigh, NC). Two plots were used for the analysis. A
gate was drawn around all viable cells on a forward scatter (x-axis) and side
scatter (y-axis) plot and all cells in this gate were analyzed on a second plot
using fluorescence intensity (x-axis) and cell count (y-axis).

**Cell Sorting**

Tumor cells were harvested as described in the “Harvesting and Passage
of Cells” section. The cell pellet was dissolved in 1ml PBS/EDTA and passed
through a filter into a flow cytometry tube. The tube was kept on ice until start of
the cell sort.

Cells were sorted with a Coulter Epics ELITE ESP flow cytometer
(Beckman Coulter Inc., Fullerton, CA) according to the parameters forward and
side scatter. After the start of counting by pressurizing the system, the flow
cytometer was allowed to equilibrate for 2 min before begin of the sort. Circular
gates were set on a forward scatter (x-axis) and side scatter (y-axis) plot: from
the main cell population, approximately 15-20% of both ends of the forward
scatter distribution were gated. Sorted cells were collected in cooled flow tubes
with 2ml complete media and kept on ice until start of the following assay.

**Cytospin, Histology and Microscopy**

**Preparation of Cells**

Two T75 flasks with RCC were prepared. After reaching 50% confluence,
media was exchanged to normal media (control) or 20µM Gemcitabine (GEM20)
and incubated for 24h. Tumor cells were harvested as described in the
“Harvesting and Passage of Cells” section, adjusted to a concentration of 15,000 cells/ml and kept on ice until start of the cytospin preparation. It is important to carefully do the disaggregation step to obtain a single cell suspension.

Cytospin Slide Preparation

After setting up the cytospin 200 µl of cell suspension was added to each slide assembly. After spinning for 5 min, the slides were retrieved and stored in ice-cold 70% ethanol.

Histology

Slides were left in 70% ethanol and placed in the refrigerator for 24h. The following staining procedure was used: hematoxylin (4 min), running tap water (three changes), acid ethanol (five dips), running tap water (three changes), ammonia water (5 min), running tap water (three changes), 95% ethanol (1 min), eosin (20 sec), 95% ethanol (two dips), 100% ethanol (2 min, three times, each time in new rack), xylene (5 min). The slides were then coverslipped and left outside to dry.

Image Acquisition

Digital pictures were obtained at 20x magnification on a BH-2 microscope (Olympus Imaging America Inc., Center Valley, PA) and stored as TIFF files for further processing. Pictures were processed in Adobe Photoshop Elements and
analyzed with NIH image (National Institutes of Health, Bethesda, MD) where cell area measurements were performed.

**Data Analysis**

The average and standard deviation of cell size measurements were calculated for each group. A paired student’s t-test was used to compare the different groups.

**Gene Expression Microarray**

**Preparation of Cell Populations by Sorting**

Two T75 flasks with RCC were prepared. After reaching 50% confluence, media was exchanged to normal media (control) or 20µM gemcitabine (GEM20) and incubated for 24h. Tumor cells were harvested as described in the “Harvesting and Passage of Cells” section. In addition to collagenase/DNAse treatment, the cells were suspended in PBS/EDTA and filtered through a nylon mesh into a flow tube. We used approximately 4-5 x 10⁶ cells in 1.5ml PBS/EDTA for flow sorting. Sorted cells were collected in flow tubes filled with 2ml complete media and stored on ice. Cell suspensions were transferred to cryotubes and centrifuged. The media was discarded and the cryotubes were stored in a -80°C freezer.
Preparation of mRNA

RNA extraction was performed with the Qiagen Rneasy Mini Kit (Qiagen Inc., Valencia, CA) according to the supplied protocol. The RNEasy Mini Kit was a generous gift from Dr. Manohar Ratnam (University of Toledo Health Science Campus, Toledo, OH).

Quality Control

The quality of the extracted RNA was assessed with a Beckman Coulter USA DU640 spectrophotometer (ratio 260nm/280nm) and northern blotting with the RNA 6000 Nano LabChip. For the spectrophotometer, each sample was diluted 1:100 in water, pipetted into a cuvette and read at 260nm and 280nm. The RNA 6000 Nano Labchip analysis was conducted on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) using the RNA 6000 Nano LabChip kit (Agilent Technologies, Foster City, CA). The RNA 6000 Nano LabChip was a generous gift from Dr. Khew-Voon Chin (University of Toledo Health Science Campus, Toledo, OH). The experiment was performed according to the supplied protocol.

Submission of Samples

The following steps in the gene expression microarray experiment were conducted by the company Imhoteq Biotechnologies (Berkeley Heights, NJ): RNA quality control, RNA labeling and purification, hybridization to human whole genome array (over 30,000 60-mer polynucleotide probes) on one glass slide;
one gene per probe; based on sequences and annotation information from Unigene (version 163), RefSeq, and the Cancer Genome Anatomy Project (CGAP), microarray scanning, image processing and data filtering and report of raw and filtered data. The samples were mailed on dry ice.

Data Analysis

The raw data was analyzed with the Cluster v2.11 and Treeview v1.60 software (EisenLab, University of California at Berkeley, CA).

**In vivo Renal Cell Carcinoma Mouse Model**

**Mice**

A total of 40 BALB/c mice (age 6-8 weeks, male) were purchased from Harlan (Indianapolis, IN) and kept in cages with four mice each on a regular diet.

**Cell Line**

The mouse renal cell carcinoma cell line RENCA was a generous gift from Dr. Thomas Sayers (National Cancer Institute at Frederick, Frederick, MD). The cells were grown in RPMI culture medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta biologicals, Lawrenceville, GA), 10mM HEPES (Invitrogen, Grand Island, NY), 1.0mM sodium pyruvate (Hyclone, Logan, UT), 0.05mg/ml gentamicin (Invitrogen, Grand Island, NY), 4.5g/L glucose (J.T. Baker Chemical Co., Phillipsburg, NJ) and 2mM glutamine (Hyclone, Logan, UT). Tumor cells were harvested as described in the
“Harvesting and Passage of Cells” section. After collagenase/DNAse treatment, the cells were suspended in PBS and adjusted to a concentration of $1 \times 10^6$ cells/ml.

**Drugs**

Mouse recombinant Interleukin-2 was purchased from eBioscience (San Diego, CA) and diluted in PBS. Gemcitabine (Eli Lilly, Indianapolis, IN) was purchased from the inpatient pharmacy in powder form (200mg) and reconstituted in PBS.

**Experimental Design**

Mice were divided into five treatment groups with eight mice in each group. Groups were labeled: (1) control, (2) gemcitabine only, (3) IL-2 only, (4) gemcitabine, followed by IL-2 and (5) IL-2, followed by gemcitabine. Each treatment group was further divided into two cohorts (for a total of 20 mice in each cohort) with cohort B starting one day later. This resulted into each cohort having four mice in each of the five treatment groups.

Tumor inoculation was scheduled for day 0. A volume of 100µl of the cell suspension prepared as described in the “Harvesting and Passage of Cells” section was injected subcutaneously into the right flank of each mouse. One course of Interleukin-2 treatment included a total of five intraperitoneal injections with 100,000 IU in 200µl. One course of gemcitabine treatment included one
intraperitoneal injection with 150ml/kg body weight in 100µl. Mice were subjected to a total of two courses of treatment.

The treatment schedule is depicted in Figure 1.

**Figure 1: Mouse Model Treatment Schedule**

<table>
<thead>
<tr>
<th>DAY</th>
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<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>5</td>
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<td>10</td>
</tr>
<tr>
<td>15</td>
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<tr>
<td>20</td>
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| 1. Control | X X X X X | X X X X X X | X |
| 2. IL-2   | X X X X X | X X X X X |
| 3. Gemcitabine | ↑ |
| 4. Gemcitabine followed by IL-2 | ↑ X X X X X ↑ X X X X X |
| 5. IL-2 followed by Gemcitabine | X X X X X ↑ X X X X |

- ☀ = tumor inoculation (1x10⁶ cells, injected SQ in flank)
- X = IL-2 (1x10⁶ IU i.p.)
- X = Vehicle control (PBS)
- ↑ = Gemcitabine (150mg/kg i.p.)
- ⊀ = Buprenorphine (0.05mg/kg SQ BID)

The condition of mice was monitored daily and weight was measured every three days. The animals were monitored for the criteria that necessitate euthanasia prior to the scheduled end of the study which included inappetence, weakness/inability to obtain food and water, moribund state, infection and signs of severe organ dysfunction. Tumor growth was monitored by measuring the three orthogonal dimensions and calculating the tumor volume by using the
formula for a prolate spheroid \( V = \frac{4}{3} \pi a^2 b \). We also monitored the body condition score according to guidelines published by Ullman-Cullere et al. (1999). A body condition score cutoff of <2 was used as the limiting parameter; if the tumor burden leading to this condition was reached before day 21, the mouse was scheduled for early termination.

The standard practice of including 15% weight loss proved to be a problem during a preliminary experiment. Weight loss was expected to occur as a result of the aggressive treatment with chemo- and immunotherapy and the 15% cutoff point lead to early euthanization of a large number of mice in the combined regimen treatment groups, a lesser amount of animals in the single-agent treatment group and almost no animal at all in the control group. Due to the different termination dates the obtained data could not be analyzed without problems. The surrogate marker “weight” for well-being proved to be a problem and lead to experimental bias so that we decided to disregard a 15% weight loss, focus on the other mentioned signs for determination of the need for early euthanization and included analgesia with buprenorphine (0.05mg/kg SQ) given twice daily from day 10 for all groups (to eliminate analgesia as an influencing variable).

At day 21, the mice were euthanized using CO2 narcosis and cervical dislocation. Autopsies were performed on each mouse to harvest the tumor specimen and to look for metastases. The tumor was stored in liquid nitrogen for further analysis.
Statistical Analysis

Student’s t test was used for assessing the significance of tumor volume differences among differential treatment groups.
Chemosensitization of renal cell carcinomas to lysis by IL-2 stimulated human peripheral blood leukocytes with Gemcitabine

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Chemosensitization of renal cell carcinomas to lysis by IL-2 stimulated human peripheral blood leukocytes with Gemcitabine

Running title:
RCC chemosensitization with gemcitabine

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Condensed abstract:
The efficacy of current immunotherapy regimens for RCC may be enhanced by the addition of gemcitabine. The importance of sequential therapy is highlighted by the fact that adverse effects on the patient and his immune system can be controlled by the design of proper treatment schedules.
Abstract:

BACKGROUND:
The chemoresistance of renal cell carcinomas (RCC) makes the therapeutic use of cytotoxic agents ineffective. Immunotherapy (IT) represents an alternative and is the current standard-of-care. Response rates of around 20% that have been reported with Interleukin-2 (IL-2) justify the need for improvement. The current study examined the capacity of gemcitabine (GZ) to increase the susceptibility of RCC to killing by IL-2 activated lymphocytes.

METHODS:
Tumor sensitivity to anti-proliferative and cytostatic effects of different cytotoxic drugs was assessed with MTT assays. Tumor sensitivity to the cytolytic activity of lymphokine-activated killer (LAK) cells was determined by the Chromium-51 (\(^{51}\text{Cr}\)) release assay.

RESULTS:
Gemcitabine pretreatment for 24h prior to the addition of LAK cells increased the lytic sensitivity of renal cell carcinoma cells by 24-48%. Increased lytic sensitivity was also produced with only a 4h gemcitabine pretreatment period. Induction of lytic function in IL-2-stimulated LAK cells was severely impaired by 24h co-incubation with IL-2, but extended IL-2 activation schedules employing IL-2 for
up to 6d preserved the lytic function of cells treated with gemcitabine for 4h prior to collection and testing.

CONCLUSIONS:
The results of the current study demonstrate the feasibility of enhancing IL-2 based IT for RCC by addition of gemcitabine. Thus, beneficial effects of chemotherapy (CTX) may be achieved even against chemoresistant tumors. The mild adverse effect profile of gemcitabine makes it an ideal candidate for incorporation into IL-2-based IT regimens for RCC patients.

Keywords:
Renal cell carcinoma, immunotherapy, chemosensitization, gemcitabine, interleukin-2, LAK cells
INTRODUCTION

The inherent chemoresistance exhibited by certain human cancers limits therapeutic options necessitating the need for alternative therapies. Immunotherapy (IT) has emerged as one way to address this problem. Supported by a substantial body of recent evidence demonstrating that the human immune system is able to recognize and attack tumors\(^1\), IT has gained wide-spread acceptance as an important modality for clinical cancer treatment.

Renal cell adenocarcinoma (RCC) represents one of the inherently chemoresistant tumors that is predicted to cause approximately 12,890 deaths during the year 2007 in the US\(^2\). With an (again for the year 2007) estimated incidence of around 51,190, that has risen steadily over the last 10 years, RCC is not one of the most common cancers but still has a major impact on the health care system\(^2\). While good control and even cures can be achieved with surgery for disease in early stages\(^3\), disseminated disease remains a major problem owing in part to the ineffectiveness of chemotherapy (CTX). In that situation, however, RCC serves as one of the best models to demonstrate the clinical efficacy of IT in humans.

Regarding IT, one of the oldest and best-studied areas focuses on the use of the biologic response modifier interleukin-2 (IL-2) to activate lymphocytes and induce differentiation into lymphokine-activated killer (LAK) cells which are able to kill
tumor cells without prior antigen priming. IL-2 proved its efficacy in various clinical studies and is currently used in the treatment of malignant melanoma and RCC \(^4\); recently extension of its use to colon and pancreatic carcinomas has come under consideration \(^5\).

Despite the fact that metastatic RCC responds to IL-2, its efficacy is very limited. Various studies reported overall response rates of 16-23\(^\%\)\(^6-8\) with occasional complete responses. Nevertheless, long-term survival is rare.

The data presented in this study show that gemcitabine exhibits a chemosensitizing effect on RCC tumor cell lines. It also simulates clinical conditions and assesses the feasibility of combinatorial therapy in regards to achievable serum concentrations, exposure time, cytotoxic effects of gemcitabine on immune cells and possible treatment schedules that maximize the benefit of chemosensitization while minimizing immune cell suppression.

**MATERIALS AND METHODS**

**Tumor cell lines**

The RCC cell line 769-P was purchased from ATCC (Manassas, VA) and grown according to the company recommendations in RPMI culture medium (Mediatech, Herndon, VA) supplemented with 10\% fetal bovine serum (Atlanta
biologicals, Lawrenceville, GA), 10mM HEPES buffer (Invitrogen, Grand Island, NY), 1.0mM sodium pyruvate (Hyclone, Logan, UT), 0.05mg/ml gentamicin (Invitrogen, Grand Island, NY), 4.5g/L glucose (J.T. Baker Chemical Co., Phillipsburg, NJ) and 2mM glutamine (Hyclone, Logan, UT). Culture incubation conditions were 37°C, 100% humidity and 5% CO₂.

**Lymphocyte isolation and activation**

Blood was obtained from healthy subjects and peripheral blood mononuclear cells (PBMC) were isolated with lymphocyte-separation media (Mediatech, Herndon, VA). LAK-induction cultures consisted of PBMC (2-4 x 10^6 cells/ml) and were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, HEPES buffer, sodium pyruvate, gentamicin, glucose, glutamine and 200 IU/ml IL-2.

**Drugs**

Recombinant human IL-2 was purchased from eBioscience (San Diego, CA) and gemcitabine was purchased from Eli Lilly (Indianapolis, IN). Both drugs were diluted in sterile RPMI 1640 culture medium.

**MTT**

RCC cells were harvested and dissociated with 0.14% collagenase/ 0.1% DNAse (Sigma, St. Louis, MO). The MTT assay was carried out as previously described with some modifications: 20μl of a 5mg/ml MTT stock (USB Corp., Cleveland,
OH) were added to the 200µl of cell suspension in each well without prior removal of medium; after 4h of incubation all fluid was removed from each well and 100µl DMSO (Sigma, St. Louis, MO) was added. We used 5000 cells/well, allowed the cells 12h to attach and recover after plating and used a drug exposure assay time of 72h.

The percentage of growth compared to the media control (100%) was calculated as follows:

\[
\% \text{ growth} = \frac{(\text{experimental absorbance} - \text{baseline absorbance}) \times 100}{(\text{media control absorbance} - \text{baseline absorbance})}
\]

where experimental absorbance is the value determined at 72h drug exposure and baseline absorbance is the value at the beginning of culture prior to addition of drug.

**51Chromium-release cytotoxicity assays**

A Chromium-51 (\(^{51}\text{Cr}\)) release assay was performed as previously described \(^{10}\) using RCC as target cells. In brief, RCC were labeled with 100µl \(^{51}\text{Cr}\) (Amersham Pharmacia) in 100µl labeling media (normal media without FBS) for 1h. After washing x3 in media with FBS, cells were adjusted with media containing FBS to a concentration of 5 x 10^6 cells/ml. 100µl media containing 5000 target cells were mixed with 100µl media containing LAK cells (concentrations adjusted to obtain E:T ratios of 20:1, 10:1, 5:1 and 2.5:1) as effectors. After 4h incubation,
supernatant was harvested with a frame (Molecular Devices, Sunnyvale, CA) and activity was measured in a gamma counter. The percentage cytotoxicity was calculated by the formula:

\[
\% \text{ cytotoxicity} = \frac{(\text{experimental} \ 51\text{Cr release} - \text{spontaneous} \ 51\text{Cr release}) \times 100}{(\text{total} \ 51\text{Cr release} - \text{spontaneous} \ 51\text{Cr release})}
\]

where spontaneous release values were obtained by RCC incubated in media, and total release values by RCC treated with 100\(\mu\)l of a 10% SDS solution. Cytotoxicity values were determined by averaging the \(51\text{Cr}\)-release from the triplicate wells. Lytic Units (LU) were calculated as previously described \(^{10}\). One LU was defined as the number of effector cells needed to lyse 20% of the 5000 target cells and expressed as the number of LU / 1.0 x 10\(^7\) effector cells.

**Statistical analysis**

Statistical analysis was performed with SPSS (paired samples t-test).

**RESULTS**

We initially performed screening experiments in which RCC were incubated with different concentrations of the drugs vinblastine, cisplatinum, gemcitabine and paclitaxel for 24 hours, prior to their use in cytotoxicity assays with LAK effector
cells to assess their capacity to produce a chemosensitization effect. Gemcitabine was found to have the greatest efficacy in increasing lytic sensitivity (data not shown) and was therefore selected for further investigations.

**Effect of various Gemcitabine concentrations on 769-P Renal cell carcinoma cell line**

We assessed the effect of different concentrations of gemcitabine on the RCC cell line using a 3d cell proliferation and viability assay.

Figure 1 shows that concentrations of gemcitabine ranging from 3.125µM to 100µM are cytostatic but not cytotoxic, i.e. they inhibit cell proliferation but leave nearly all cells alive (OD at time of plating (t₀) was 0.166, shown as red line). Significantly, this was observed with concentrations ranging from 60µM [18mg/L] to 100µM [30mg/L] which approximates in vitro the clinical situation, in which gemcitabine may control cancer growth but fails to induce apoptosis.

**Effect of Gemcitabine on RCC-sensitivity to LAK-mediated killing**

The effect of preincubation of RCC with gemcitabine on their lytic sensitivity to LAK cells was assessed (Figure 2). The results show that the lytic sensitivity of RCC cells following 24h incubation with 20µM gemcitabine, increased by 24 to 48% in comparison to RCC cells cultured for an equivalent period in medium alone (p=0.008).
Out next studies were designed to evaluate this effect under conditions that are comparable to the clinical use of gemcitabine in terms of infusion time and serum pharmacokinetics. These conditions are depicted in Figure 3 wherein 20µM gemcitabine was used to treat RCC cells for 4h, which represents the period of gemcitabine clearance subsequent to a 30 minute infusion of 1 gm/M^2. It can be appreciated that the chemosensitizing effects of gemcitabine treatment were preserved.

**Effect of Gemcitabine on immune cells**

One of the major drawbacks of chemotherapy is the detrimental effects produced on normal tissues. Therefore it was important for this study to investigate the effects of gemcitabine treatment on the activation of immune cells to assess whether enhanced tumor lytic sensitivity to LAK cells might be offset by deleterious effects on the activation of immune cells with IL-2.

To address this issue, we performed experiments in which gemcitabine was added to PBMC during stimulation with IL-2. Initial experiments involved activating PBMC with IL-2 for 3d in which 20µM gemcitabine was added during the final 24h of the activation period prior to testing the lytic activity of the elicited LAK cells.

As shown in Figure 4, most of the lytic activity gained by lymphocytes activated with IL-2 was lost when gemcitabine was added to LAK cells during the final 24h of the 3d activation culture. The value exhibited was comparable to that exhibited
by a lymphocyte sample that has been incubated in media in the absence of IL-2 for 3d (right bar).

Since many studies report dependence of LAK induction on the duration of IL-2 activation, we hypothesized that a fully activated LAK cell or a LAK cell subjected to an extended activation period might be less susceptible to the inhibitory effects of gemcitabine as compared to cells activated with IL-2 for only 3d. We therefore incubated PBMC with IL-2 for 3, 4 and 6d prior to the addition of 20µM gemcitabine during the final 4h of the incubation period. The selection of a 4h treatment with gemcitabine was designed to replicate the in vivo exposure period produced by a typical gemcitabine treatment regimen. Furthermore, the experimental design eliminated variability in all factors other than the presence or absence of gemcitabine by employing a single activation culture in which IL-2 was present for the indicated period of time, prior to division of the induced LAK cells into two flasks and addition of gemcitabine for 4h in the test flask with addition of the equivalent amount of drug vehicle without gemcitabine in the other.

It can be appreciated from the results depicted in Figure 5 that it is possible to preserve the level of lytic activity induced by IL-2 in human PBMC by extending the IL-2 activation period up to 6d prior to gemcitabine treatment. That this result may also be obtained in vivo is suggested by results depicted in Table 1 which show equivalent numbers of viable cells recovered from media-treated and gemcitabine-treated samples under these conditions.
DISCUSSION

Our results show that gemcitabine, a cytotoxic agent that is not considered clinically useful for the treatment of RCC, possesses the ability to sensitize the RCC cell line 769-P to LAK-cell mediated killing elicited by IL-2 in human PBMC. Furthermore, potential inhibitory effects of gemcitabine on the response of immune cells to IL-2 activation can be attenuated by extending the period of IL-2 activation prior to the addition of gemcitabine. The dosage and schedule of gemcitabine and IL-2 elucidated by this study is achievable in patients. Taken together, these results suggest that gemcitabine may be useful in a combination treatment regimen with IL-2 for the treatment of patients with RCC.

In order to establish a concentration that can be used to guide subsequent in vivo investigations we focused on the average pharmacokinetic profile of gemcitabine when used in a standard dosing regimen of 1g/m² administered as an iv infusion over 30mins. The approaches included both calculation using a simple pharmacokinetic model and review of reported data from phase I/II clinical trials conducted with gemcitabine. The simple pharmacokinetic model calculated a peak plasma concentration of 20mg/l; this represented a very good estimate based on studies from Cattel et. al and Yilmaz et al. which reported measured peak plasma concentrations of 19-38mg/l and 24.5mg/l, respectively 11, 12. Notably, Cattel used a 1h infusion regimen instead of 30mins. Plasma half-lives in these studies ranged from 0.3 to 0.5h. Allerheiligen et al. estimated plasma
half-lives of gemcitabine to range from 0.7-1.5h, depending on infusion length and the patient’s age and gender. Our preliminary dose seeking experiments employing 20µM gemcitabine in vitro showed a reproducible tumor chemosensitization effect over 24h. A 20µM concentration equals 6mg/L which is below the measured peak plasma concentrations determined in the studies cited above.

Gemcitabine belongs to the group of antimetabolites that act as nucleoside analogues. It causes chain termination during DNA- and RNA-synthesis and depletes nucleotide pools by inhibition of ribonucleotide reductase. It is one of the more versatile drugs used both as single agent primary therapy for treatment of nonresectable stage II/III and metastatic adenocarcinoma of the pancreas; in combination regimens with cisplatin in inoperable stage IIIA/IIIB or metastatic non-small cell lung cancer; and as salvage therapy in relapsed ovarian cancer and in combination with paclitaxel in metastatic breast cancer. Its comparably mild toxicity profile is an ideal characteristic that supports its addition to standard-of-care and experimental immunotherapy regimens. The fact that its chemosensitization activity can be achieved with drug concentrations lower than what is produced with current treatment regimens is especially attractive for this purpose.

The LAK phenomenon describes the phenotypic shift of lymphocytes to lymphokine-activated killer cells induced by IL-2. First described by Grimm et al.
in 1982, the characterization of effector cells revealed their ability to lyse tumor cells without prior antigen priming pointing at NK cells being the precursor population. Grimm et al. reported that the differentiation phase required for LAK cell expression involves proliferation, DNA-, RNA- and protein-synthesis and is irradiation-sensitive.\textsuperscript{17}

Alvino et al.\textsuperscript{18} reported an immunotoxic effect of gemcitabine on natural and antigen-mediated immunity based on 16h incubation experiments of different immune cells, including LAK cells. Due to the fact that the clinical application of gemcitabine would never achieve an exposure of this duration we shortened the incubation time according to pharmacological considerations to 4h. Our data suggest that a 4h gemcitabine exposure of lymphocytes during the process of activation with IL-2 results neither in cell death nor in suppression of cellular proliferation. Thus, the reduction of lytic activity observed following gemcitabine addition to a standard 72h activation culture is likely to be related to functional impairment of LAK cells. That gemcitabine can also influence RNA-synthesis suggests that its capacity to attenuate the lytic activity of maximally-activated LAK cells may be due to reduction in the expression of cellular proteins critical for the expression of lytic function and not simply its development.

Nevertheless, our findings with LAK cells produced with extended IL-2 treatment periods show that gemcitabine cannot exert inhibitory effects at all phases of LAK cell differentiation. This emphasizes that the LAK-differentiation process can
reach a plateau phase \(^{17,18}\) during which the differentiated LAK cells are fully armed, capable of mediating tumor cell killing, and refractory to inhibition by gemcitabine.

The idea of using chemotherapeutic agents to modulate IT has been under investigation for some time. Many studies have assessed the influence that these agents exert directly on immune cells. For example, Ghiringelli et al. and Ercolini et al. showed that cyclophosphamide exhibits immunostimulatory effects by depleting CD4\(^+\)CD25\(^+\) regulatory T-Cells \(^{19,20}\), Mizumoto et al. reported an induction of dendritic cell maturation mediated by topoisomerase I inhibitors and microtubule depolymerizing drugs \(^{21}\) and Suzuki et al. found that gemcitabine selectively eliminates Gr-1\(^+\)/CD11b\(^+\) splenic suppressor cells in mice freeing cytotoxic CD8\(^+\) T-Cells from their inhibition \(^{22}\), to name a few.

Less well-studied are effects leading to improvement in IT due to actions of the drug on the tumor cells themselves. Where this has been investigated, one of the prominent mechanisms responsible for tumor chemosensitization against immune-mediated killing is through upregulation of the death receptor, Fas. Thus, Solary et al., Micheau et al. and Collins et al. reported that treatment of cancer cell lines with chemotherapeutic drugs caused an upregulation of Fas receptor and resulted in increased sensitivity to immune-cell mediated killing \(^{23-25}\). According to Uslu et al. cancer cell lines that are normally resistant to immune mediated killing can be transformed to a sensitive state by this mechanism \(^{26}\).
Increased tumor sensitivity to immune-mediated killing has also been shown to result from altered expression of surface-receptors involved in tumor recognition and immune cell inhibition/stimulation. For example, certain tumors can change the expression of NK-cell activation and/or inhibiting ligands \(^{27}\), particularly after experiencing genotoxic stress \(^{28}\). This may in fact contribute to the observed effects of gemcitabine shown in the present study since gemcitabine induces DNA-damage and thus, genotoxic stress by both chain termination through stalling of the polymerase, and inhibition of ribonucleotide reductase leading to depletion of substrate pools and subsequent DNA double strand breaks \(^{29}\). The damage is sensed by two kinases, ATM (double-strand breaks) and ATR (stalled DNA replication), which trigger a signal transduction pathway that ultimately results in cell-cycle arrest and upregulation of proapoptotic proteins, induction of DNA-repair mechanisms and expression of NKG2D ligands \(^{30}\). NKG2D ligands are grouped into the MIC and RAET1 gene families, relatives of MHC class I molecules, which can trigger lytic activity in cells from the innate immune response arm upon ligation with their NKG2D receptor \(^{28,31}\).

Upregulation of heat-shock proteins induced by cellular stress are also recognized as activating molecules by NK cells \(^{32,33}\) and may contribute to the effects reported herein. Still, other mechanisms that may be induced involve alteration of levels of various pro and anti-apoptotic proteins \(^{34}\), DNA-repair proteins, or other processes that modulate apoptotic and damage control.
pathways in cells. Such factors may include molecules that facilitate immune evasion or resistance by tumor cells such as NO \(^{35}\), TGF-\(\beta\), IL-10, VEGF or soluble ligands that block NK-cell receptors like NKG2D-L \(^{36}\).

While previous reports described beneficial effects of gemcitabine on antigen-dependent effector mechanisms\(^{22,37,38}\) no study to our knowledge has examined the influence of gemcitabine on tumor cell sensitivity to antigen-independent innate immune responses or more specifically, IL-2 induced killer cells. In the present study, the results show that gemcitabine affects both tumor and immune cells through multiple, and as yet, unknown molecular mechanisms. Elucidation of the mechanisms responsible for each kind of effect will enhance the ability to develop optimum combination treatment protocols.

Furthermore, these mechanistic studies are timely given recent anecdotal reports of clinical trials combining gemcitabine with immunotherapy in limited numbers of patients who failed first-line treatment. Neri et al.\(^{39}\) used gemcitabine with IFN-\(\alpha\) and low-dose subcutaneous IL-2 in patients with metastatic renal cell cancer and reported an overall response rate of 28\%. Zustovich et al.\(^{40}\) conducted a trial with multiple treatment regimens in metastatic renal cell carcinoma patients which included 12 of 25 patients who were treated with gemcitabine in combination with IL-2. While reporting an overall response rate of 17\%, the multiplicity of regimens makes analysis difficult. Inoue et al.\(^{41}\) conducted similar studies in metastatic renal cell carcinoma patients with some suggestion of activity of combinations of
IFN-α, IL-2 and gemcitabine. While it is impossible to draw conclusions from such anecdotal experiences, it is encouraging that combinations of gemcitabine and biologic agents appear to be tolerable in patients and may have activity in refractory disease. They are sufficiently provocative to warrant additional investigation. The result provided in the present study emphasize the need for utilizing doses and schedules which optimize the chemosensitizing effects of gemcitabine while sparing the immunomodulatory effects of IL-2.

In summary, this in-vitro model demonstrates both the efficacy and feasibility of combining gemcitabine with current immune modulating agents in RCC. The principles revealed herein should guide the development of preclinical and subsequent clinical in-vivo studies.
REFERENCES


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Figure 1

Effect of gemcitabine on RCC cell number in a 3d MTT assay. Concentrations of gemcitabine ranging from 3.125µM to 100µM inhibit cell proliferation but leave nearly all cells alive (optical density at time of plating [t₀] was 0.166).
Figure 2

Effect of gemcitabine pretreatment on RCC-sensitivity to LAK-mediated killing.

RCC were incubated with 20μM gemcitabine for 24h and subsequently used in a

$^{51}$Cr-release cytotoxicity assay. Gemcitabine pretreatment sensitizes RCC cells

as depicted by the increase in lytic units compared to media control.
Figure 3

Effect of gemcitabine pretreatment on RCC-sensitivity to LAK-mediated killing.

Shortening of the incubation time from 24h to 4h preserves the chemosensitization effect.
Figure 4

Effect of gemcitabine on LAK cell function. Isolated lymphocytes were activated with IL-2 over a period of 3d and 20μM gemcitabine was added during the last 24h of activation. This resulted in a marked loss of lytic activity which became comparable to a lymphocyte sample that has not been activated with IL-2.
Figure 5
Effect of gemcitabine on LAK cell function. Shortening of gemcitabine exposure to 4h and using different IL-2 activation periods with gemcitabine addition 4h before the $^{51}$Cr-release experiment showed a decrease in LAK lytic activity that was inversely proportional to IL-2 incubation time. Y-Axis depicts the % recovery of lytic units with a lymphocyte control that received the same treatment except the gemcitabine exposure.
Table 1

Effect of gemcitabine on the number of recovered LAK cells. PBMC were harvested and activated with IL-2 for 3d/ 4d/ 6d. In the last 4h of the incubation, the contents of the tissue culture flask were equally divided into two flasks. Gemcitabine was added to one of the flasks to obtain a final concentration of 20µM. After 4h the cells were harvested and counted on a hematocytometer. Only cells excluding trypan blue were counted as being viable. Equivalent numbers of viable cells were recovered from media-treated and gemcitabine-treated samples under these conditions.

<table>
<thead>
<tr>
<th>IL-2 activation time</th>
<th>LAK-cells recovered after 4h incubation with</th>
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<tbody>
<tr>
<td></td>
<td>Media</td>
</tr>
<tr>
<td>3d</td>
<td>11,490,000</td>
</tr>
<tr>
<td>4d</td>
<td>10,770,000</td>
</tr>
<tr>
<td>6d</td>
<td>14,760,000</td>
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</tbody>
</table>
RESULTS

IC$_{50}$ Estimation with 3-day MTT Assays

An MTT assay using RCC and gemcitabine is described in manuscript 1. OVCAR3 cells were treated with vinblastine and paclitaxel for 3 days. We estimated the IC$_{50}$ visually from figure 2 (vinblastine, IC$_{50}$ ~0.9nM) and figure 3 (paclitaxel, IC$_{50}$ ~2.6nM).

Figure 2: IC$_{50}$ Determination – OVCAR3, Vinblastine
Figure 3: IC₅₀ Determination – OVCAR3, Paclitaxel

**5¹Cr-release Cytotoxicity Assay with OVCAR3 after Pretreatment with Vinblastine and Paclitaxel**

Cytotoxicity assays with RCC and gemcitabine are described in manuscript 1. For experiments with the OVCAR3 cell line we used concentrations in the range of the respective IC₅₀ values for 24h exposures prior to the ⁵¹Cr-release assay. Results are depicted in Figure 4 and 5.
Figure 4: $^{51}$Cr-release cytotoxicity assay – OVCAR3, Vinblastine

![Figure 4: $^{51}$Cr-release cytotoxicity assay – OVCAR3, Vinblastine](image)

Figure 5: $^{51}$Cr-release cytotoxicity assay – OVCAR3, Paclitaxel

![Figure 5: $^{51}$Cr-release cytotoxicity assay – OVCAR3, Paclitaxel](image)
Characterization of new Cell Population that Arises after Treatment of RCC with Gemcitabine

Figure 6 and 7 show cytospin images of RCC incubated for 24h in media and media with 20µM gemcitabine, respectively.

Figure 6: Cytospin Image, H&E Staining – RCC, Media (24h)

![Cytospin Image, H&E Staining – RCC, Media (24h)](image)

Figure 7: Cytospin Image, H&E Staining – RCC, Gemcitabine (24h)

![Cytospin Image, H&E Staining – RCC, Gemcitabine (24h)](image)
Cell measurements using the total cell area reveal a shift to a larger cell size observed in the gemcitabine-treated sample (Figure 8 and 9).

Figure 8: Cell Size Measurements – RCC, Media (24h)

![Figure 8](image)

Figure 9: Cell Size Measurements – RCC, Gemcitabine (24h)

![Figure 9](image)
RCC were sorted with a flow cytometer. Using the parameter of forward scatter and gating on approximately 20% of cells in the low and high forward scatter plot area, we obtained small and large cell samples from RCC incubated for 24h in 20µM gemcitabine. Subsequent use of those cells in cytotoxicity assays revealed differences in lytic activity as shown in figure 10. To examine whether this effect can also be observed in RCC cultured in media only we used the same flow setup, sorted the cells and performed a cytotoxicity assay using the same conditions as in the previous one. Figure 11 shows no differences in lytic activity for the different cell populations.

Figure 10: $^{51}$Cr-release Cytotoxicity Assay – RCC, Gemcitabine (24h), After Sorting by Flow Cytometry According to Forward Scatter
Flow Cytometry Expression Analysis of Fas Receptor and MHC ABC

Prior to flow analysis, RCC were grown in culture. 20μM gemcitabine was added 24h and 4h before analysis. The expression levels of Fas and MHC ABC in the RCC samples treated with media, 24h of 20μM gemcitabine and 4h of 20μM gemcitabine are depicted in Figures 12 and 13. Fas receptor was expressed in media-treated samples and was upregulated after 24h treatment with gemcitabine but not in the 4h treated samples. MHC ABC expression was detected in RCC but remained unchanged over all treatments.
Figure 12: Flow Cytometry, MHC ABC Expression – RCC, Media, Gemcitabine (4h and 24h)

Figure 13: Flow Cytometry, Fas Receptor (CD95) Expression – RCC, Media, Gemcitabine (4h and 24h)
Fas Receptor Expression Differences on Small and Large RCC Cells after Treatment with Gemcitabine

By gating on cells in the low and high FS areas during an experiment using Fas receptor antibody in gemcitabine treated RCC samples we examined the level of Fas expression of those particular subsets. By calculating the relative fluorescence value for each condition by using values obtained from gating on the low and high FS subsets of RCC stained with an isotype antibody the level of receptor expression was corrected for cell size and surface area. The resulting values showed no significant difference. Results are depicted in Figure 14.

**Figure 14: Flow Cytometry, Fas Receptor (CD95) Expression, gated on Low and High Forward Scatter – RCC, Media, Gemcitabine (4h and 24h)**
Gene Expression Microarray

The data analysis is still ongoing. We identified various clusters showing differentially regulated genes amongst the four groups and focused our attention on clusters showing a congruent positive or negative correlation between the media + high FS samples (lower sensitivity to LAK-mediated killing) and the GEM20 + low FS samples (higher sensitivity to LAK-mediated killing). All genes were evaluated for a possible effect on sensitivity or resistance and candidates fitting into categories like cell surface receptor expression, pro-/anti-apoptotic effects, cell cycle regulation, DNA damage repair pathways and signaling pathways were isolated. The next step will be the verification of differential expression by polymerase chain reaction (PCR).

In vivo Mouse Model

A preliminary study showed that subcutaneous implantation of the mouse model renal cell carcinoma cell line RENCA resulted in reliable tumor growth over a time period of 18 days. The study also showed that combinatorial treatment with IL-2 and gemcitabine in total doses of 100,000 IU (four injections) and 150mg/kg (one injection), respectively, was tolerated by mice. A second study with minor modifications as described in the “Materials and Methods” section is ongoing at the time of submission.
SUMMARY AND DISCUSSION

Immunotherapy is currently the best and often the only option for treatment of chemoresistant tumors but suffers from comparably low response rates and lacks the power to achieve long-term disease control. Therefore, alternatives and options to increase efficacy are highly sought after. An even worse situation is found in chemoresistant tumors that have become resistant to immunotherapy.

Our group focuses on ways to augment immunotherapy. It has long been hypothesized and even proven in basic science and few clinical studies that the addition of chemotherapeutic drugs confers beneficial effects on the overall efficacy of immunotherapy. The majority of studies focused on modulatory effects exerted on immune cells: Ghiringhelli et al. and Ercolini et al. found that application of cyclophosphamide acted as an immunostimulant by selectively depleting CD4+CD25+ regulatory T cells which resulted in restoration of immune cell function (Ercolini et al., 2005; Ghiringhelli et al., 2006). Mizumoto et al. observed induction of dendritic cell maturation mediated by topoisomerase I inhibitors and microtubule depolymerizing drugs with concomitant upregulation of stimulatory- and antigen-presenting molecules (Mizumoto et al., 2005). Gemcitabine was shown to selectively eliminate Gr-1+/CD11b+ suppressor cells located in the spleen of mice and thereby abolishing their suppressive action on CD8+ cytotoxic T cells and NK cells (Suzuki et al., 2005). Bang et al. reported that in patients with advanced pancreatic cancer and severely impaired cell-mediated immunity the combined use of gemcitabine and cisplatin was able to
restore the impaired immune functions (Bang et al., 2006). Studies of Singh et al., Sodhi et al. and Ranjan et al. found that exposure of macrophages to cisplatin induced expression of membrane-associated IL-1 and TNF and stimulated production of nitric oxide resulting in increased killing of tumor cell lines (Ranjan et al., 1997; Singh et al., 1991; Sodhi and Suresh, 1992).

A smaller group assessed the reaction of tumor cells to insults with cytotoxic agents: Solary et al., Micheau et al., Uslu et al., Collins et al., Lee et al. and Mattarollo et al. conducted experiments with various cancer cell lines (colorectal, prostate, breast, lung, lymphoma) and discovered that treatment with chemotherapeutic drugs like cisplatin, doxorubicin, mitomycin C, fluorouracil, camptothecin or cyclophosphamide caused an upregulation of Fas receptor and thereby increased their sensitivity to Fas/FasL pathway-mediated killing by lymphocytes and NK cells. Uslu et al. and Collins et al. also observed that formerly resistant cell lines were converted to a susceptible state by the chemosensitization treatment. Besides Fas-upregulation, Mattarollo et al. detected drug-induced upregulation of TRAIL-R2 (DR5) showing a second, TRAIL-mediated pathway by which NK cells are able to kill tumor cells (Collins and Kao, 1989; Lee et al., 1997; Mattarollo et al., 2006; Micheau et al., 1997; Solary et al., 1998; Uslu et al., 1997). A very recent study conducted by Ewens et al. used a breast cancer model in mice and a treatment regimen combining doxorubicin and IL-2; the group found a significantly increased response rate and tumor-free long term survival in the group receiving both agents (Ewens et al., 2006).
If one evaluates the body of present literature it will become apparent that presumably many different mechanisms exist for which it is unclear whether they are linked at some molecular level or not. As a result, more research needs to be invested into that area to optimize results and our understanding.

Another important aspect that we investigated in our first draft is the role of the immune system. Even though beneficial effects of cytotoxic agents such as depletion of Tregs have been described it is important to acknowledge that those effects depend on drug dose and exposure time. Considering this area we have to deal with three questions: if we apply a certain dose of a drug over a certain time period, how will it affect a) the immune-cells that are responsible for the effector function that is modulated by immunotherapy, i.e. killer cells (NK cells, CD8+ T cells, LAK cells), b) the “supporting” cells of the immune system that modulate the function of effector immune cells or even the tumor itself (Tregs, CD4+ T cells, NKT cells, dendritic cells, macrophages/monocytes) and c) the tumor cells and its stromal support. A comprehensive and simultaneous evaluation of all questions within the same study is difficult to achieve and would only be feasible in an in vivo system.

We assessed whether the chemosensitizing effect of a cytotoxic drug given at a certain dose over a certain time is still present while the immunomodulatory effect of the concurrent IL-2 application can be preserved. Our results put an emphasis on the need for carefully developed treatment schedules.
We predominantly used the RCC cell line due to the fact that metastatic renal cell carcinomas encountered in oncology practice are chemoresistant and are often treated with immunotherapy. Gemcitabine was selected from screening studies which showed a high level of tumor cell sensitization. The inclusion of the OVCAR3 cell line and vinblastine and paclitaxel allowed us to assess whether the observed chemosensitization effect is limited to a certain tumor type (RCC) or if it can be extended to other tumor types. Our data favors the latter and is supported by other studies that use different combinations of tumor and chemotherapeutic drugs. Even though chemosensitization may possibly be a generalized effect our data points at differences in effectiveness depending on the drug and the targeted tumor.

In conclusion, our study supports the existence of a chemosensitization effect exhibited by cytotoxic drugs on tumor cells which increases their susceptibility to immune cell-mediated killing. Preliminary screening experiments looking at possible mechanisms yielded promising candidates like Fas receptor and intercellular adhesion molecule (ICAM) that are upregulated and might either be fully or in part responsible for the increased susceptibility of tumor cells.


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combined with transvaginal ultrasonography for ovarian cancer screening. In Vivo 18, 831-836.


ABSTRACT

Due to the inherent chemoresistance of some cancer types, for example renal cell carcinomas (RCC), treatment approaches using cytotoxic drugs ultimately fail. This necessitates the search for alternative therapy. Immunotherapy agents like Interferon and Interleukin-2 (IL-2) have activity in some patients but are not curative. Thus, strategies to improve immunotherapy (IT) are needed. One approach is to use chemotherapy (CTX) to enhance IT. This could come about by exploiting immuno-modulatory effects of specific cancer drugs. Alternatively, CTX could be used to “sensitize” tumor cells to lytic effects of immune cells. The current investigation tested the hypothesis that tumor cells exposed to CTX will exhibit increased sensitivity to lysis by lymphokine activated killer (LAK) cells and investigated possible candidate genes involved in the molecular mechanisms responsible for the observed effect.