Inhibition of the PI3K/AKT signaling pathway increases efficacy of doxorubicin and its derivative AD198 in bladder and oral cancers

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DEDICATION

I dedicate my work to the one person who not only made my life possible but made the success in my life possible. None of this would be possible without my mother, Yelena Smolenskaya.

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ABSTRACT

Doxorubicin (Dox) is a successful chemotherapy to treat various cancers, including bladder and oral cancers. Many patients initially respond to Dox-based regimens, however often cancers become resistant. A novel derivatives of Dox, e.g. N-benzyladriamycin-14-valerate (AD198), have been developed to overcome Dox-induced drug resistance and cardiotoxicity. The purpose of this thesis was to determine the efficacy of AD198 and Dox in bladder and oral cancers *in vitro.*

Part-I of this dissertation focuses on the bladder cancer, including discussing risk factors, diagnosis, staging, and current treatment options, following by a description of altered molecular mechanisms responsible cancer progression. This section also focuses on alternative experimental drugs and current clinical trials designed to target specific molecular markers of bladder cancer.

Part-II of this dissertation compares the efficacy of AD198 and Dox and its molecular mechanisms of action in human T24 and UMUC3 bladder cells *in vitro*. AD198 was more effective than Dox in inhibition of cell viability of T24 and UMUC3 cells. Both Dox and AD198 significantly induced apoptosis in caspase-dependent and -independent manners. Dox and AD198 activated the pro-apoptotic p38 MAPK pathway; however, they also increased phosphorylation of AKT, a pro-survival signaling pathway, in T24 and UMUC3 cells. Combined treatment of PI3K inhibitor (LY294002) with Dox or AD198 inhibited cell viability of T24 and UMUC3 cells more effectively than any drug treatment alone.

Part-III of this dissertation discusses oral cancer, with special focus on causes, diagnosis, treatment, molecular pathogenesis, and potential molecular targets for treatments.

Part IV of this dissertation focuses on evaluation of the efficacy of Dox and its novel derivative AD198 in human (SCC25 and 1483), canine K9OSCC-Abby, and feline (FeOSCC-Sidney) oral squamous cell carcinoma cells *in vitro*. Dox and AD198 had a better anti-proliferative effect than Dox in human and canine OSCC. Our results suggest that the combined therapy of an anthracycline compound with inhibitor of PI3K/AKT pathway is a more effective treatment.

Part V of this dissertation discusses the implications of these studies and examines current literature on the potential of targeting PI3K/AKT to increase the efficacy of anthracycline treatments in bladder and oral cancers.

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CHAPTER I

MOLECULAR TARGETS IN UROTHELIAL CANCER: DETECTION, TREATMENT, AND MODELS OF BLADDER CANCER

Literature review described in this chapter is a slightly modified version of an article that is prepared for submission to the D*rug Design, Development and Therapy* journal.

In this paper, “our” or “we” refers to me and my co-authors. My contribution to the manuscript includes: 1) compile literature, 2) provide structure to the review, 3) prepare graphs and figures, and 4) write the review.

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Abstract

Bladder transitional cell carcinoma (TCC) remains one of the most expensive cancers to treat in the United States due to the length of required treatment and degree of recurrence. In order to treat bladder TCC more effectively, targeted therapies are being investigated. In order to use targeted therapy in a patient, it is important to provide a genetic/expression background of each obtained biopsy sample. Recent advances in genome sequencing, as well as transcriptome analysis, have identified major pathway components altered in bladder TCC. The purpose of this review is to provide a broad background on bladder TCC, including its causes, diagnosis and stages, as well as signaling pathways in bladder TCC and drugs that are currently being studied to target specific pathways. The major focus is given to the PI3K/AKT pathway, p53/pRb signaling pathways, and the histone modification machinery. Because several promising immunological therapies are also emerging in the treatment of bladder cancer, focus will also be given on general activation of the immune system for treatment of bladder cancer.

Keywords: bladder cancer, transitional cell carcinoma, molecular targets, and clinical trials

Introduction

Bladder cancer is the 5th most common cancer in the United States and accounts for 4.5% of all new cancer cases [1]. In 2016, an estimated 76,960 new patients will be diagnosed with bladder cancer, while 16,390 will die from complications of this disease [2]. Bladder cancer is the 4th most common cancer diagnosed in males and is three times less common in females [3]. The most common type of bladder cancer is transitional cell carcinoma (TCC), also known

as urothelial cancer (UC), and accounts for over 90% of all bladder cancer cases in the United States [4-6]. Transitional cells are specialized epithelial cells that line the inside of the bladder and some other organs; unlike normal epithelial cells, transitional cells can contract or expand. Less common types of bladder cancer include squamous cell carcinoma and adenocarcinoma [7]. Even rarer are sarcomas, which account for less than 1% of bladder cancers; sarcomas do not arise from the urothelial layer, but from the stroma layers of the bladder [8]. Because of the rarity of other types of bladder cancers, TCC is the most studied of the bladder cancers and is the focus of this review.

This review summarizes the risk factors for developing bladder TCC, molecular markers for diagnosis and personalized targeted therapies of TCC, and summarizes the outcomes of current clinical trials and studies using animal models to advance knowledge in managing bladder cancer.

Risk Factors for Bladder Cancer

The non-environmental risk factors for bladder cancer include age, sex, ethnicity, body weight, lifestyle, and familial history. With increasing age, the risk of developing bladder cancer increases. Currently, the median age of patients diagnosed with TCC is between 65–70 years old [9]. For unknown reasons, bladder cancer is three to four times more likely to occur in men than in women [6]. While the exact mechanism to account for the difference in risk of developing TCC as it relates to sex is unknown, in a study using a nude mice transplant model, it was determined that bladders of mice injected with male androgen hormones progress more often to carcinogenesis than bladders of mice treated with female estrogenic compounds [9, 10]. While race seems to be a contributing factor in the male population, with white males having twice the

incidence of Asian, black, or Hispanic males, the difference in incidence due to race in females is far less pronounced [9]. One interesting finding in the difference between black and white males who develop bladder cancer is that white males in the United States are more likely to develop non-invasive bladder cancer, while black males are more likely to develop invasive bladder cancer, leading to a worse survival rate in the black male population [9, 11, 12].

Lifestyle choices linked to cancer risks have been documented in many studies, and there is overwhelming evidence that obesity, poor diet, and physical inactivity are linked to increased risk of developing several types of cancers [13]. A strong correlation exists between obesity and an increased risk for development of bladder cancer [14]. It was recently shown that the combination of smoking and obesity increased not only the risk of developing bladder cancer, but also significantly increased the risk of bladder cancer reoccurrence and mortality of patients that were already successfully treated for non-invasive bladder cancer [15]. Because many toxins are expelled through excretion via the urinary system, those toxins can accumulate in the bladder and promote the initiation of bladder cancer. Some of the major environmental factors of developing TCC include smoking (tobacco products), occupational carcinogens (e.g. arsenic), and prior chemotherapeutic drug exposure. Tobacco use is perhaps the best documented risk factor for developing TCC [16]. A recent study has shown that cigarette smoking accounts for more than 50% of all bladder cancer diagnoses in the United States [17]. Cessation of smoking reduces the risk of recurrence of bladder cancer even if the initial diagnosis occurred while the patient was an active smoker [18]. These findings suggest that the continuation of smoking increases the risk of bladder cancer recurrence.

There are many occupational hazards that increase the risk of developing bladder TCC, as well. These risk factors include, but are not limited to, exposure to diesel exhaust, polycyclic aromatic hydrocarbons (PAHs), and certain pesticides and herbicides [19-21]. It has been

reported that patients treated with cyclophosphamide (Cytoxan) can develop renal or bladder cancer as one of the possible adverse events of chemotherapy [16, 22]. Therefore, lifestyle intervention would greatly benefit prevention and management, as well as decrease recurrence, of bladder cancer.

While familial bladder cancer seems to be rare, it has been determined that the risk of developing bladder cancer increases two-fold when another close family member has already been diagnosed with bladder cancer [16]. It has been suggested that familial mutations of the retinoblastoma protein (pRb) may contribute to the risk of developing bladder cancer [23]. The p53/pRb pathway is also often altered in bladder cancer and will be covered in detail in the molecular targets of bladder cancer section in this review. In addition, some evidence suggests that individuals, especially smokers with genetically overactive cytochrome-P450-1A2 (CYP1A2), may be at greater risk for developing bladder cancer [24]. Specific mutations in the *CYP1A2* gene can be activated by carcinogens present in cigarette smoke, including 4-aminobiphenyl (4-ABP), which can form DNA adducts and cause mutations of other genes [24, 25].

Diagnosis and Staging of Bladder Cancer

The most common signs and symptoms of bladder cancer include blood in the urine and pain during urination [6]. Several invasive and non-invasive techniques exist to diagnose bladder cancer. One of the primary non-invasive techniques is urine cytology evaluation, in which cells that are shed can be observed for any abnormalities or malignancies [26]. A urine culture may be inoculated in order to differentiate the diagnosis from an infection [26]. While a positive result for cancer using urine cytology can be used as a diagnosis for presence of

cancer, a negative result does not always indicate absence of cancer.[26] The urine sample is used for detection of bladder cancer biomarkers. One of the most common biomarker tests is the bladder tumor antigen test; however, the test's specificity and sensitivity can vary greatly, with a high incidence of false positives [27]. Several biomarkers that are used in combination to diagnose bladder cancer are reviewed by Tilki et al., 2011 [27].

Cystoscopy followed by biopsy is the gold standard for diagnosis of bladder cancer [26]. Currently, two forms of cystoscopy are available: white light cystoscopy and fluorescence cystoscopy. While papillary tumors can almost always be seen using white light cystoscopy, it becomes much more difficult to detect carcinoma in situ using white light cystoscopy alone. In a study by Fradet et al.,[28] only 62% of tumors were detected during white light cystoscopy; however, 92% of carcinoma-in-situ were detected when fluorescent cystoscopy was applied. The ability to differentiate tumor tissue from surrounding normal tissue using targeted fluorescence imaging will help to improve diagnosis, as well as outcome of image-guided surgeries in patients diagnosed with bladder cancer. Non-targeted fluorescent imaging agents, such as hexaminolevulinate or 5-amino levulinic acid, accumulate in cancer tissue, providing an increased signal when compared to normal epithelium. Additionally, porphyrins emit red light when excited with blue light for detection [29]. Fluorescence cystoscopy detects up to 15% more tumors than white light cystoscopy. Patients diagnosed who test positive via urine cytology tests but negative via white light cystoscopy are excellent candidates for fluorescence cystoscopy [29-31]. Targeting specific markers that are overexpressed in tumors by imaging agents is a key strategy for detection of tumor versus normal tissue. Development and synthesis of new imaging agents that specifically target tumor tissue is currently under intensive investigation (reviewed in Kim et al., 2015[32] and deBoer et al., 2015[33]). One example of such an agent is fluorocoxib A, a novel derivative of indomethacin that specifically binds to cyclooxygenase-2

(COX-2)-expressing bladder cancers [34-36]. Fluorocoxib A has shown promise in detection of bladder TCC using mouse and canine bladder cancer models [35].

Other imaging modalities used for the diagnosis of bladder cancers are computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound [37]. CT has been successful in imaging of bladder cancer and has advantages of being less invasive than cystoscopy. While sensitivity of CT was found to be as high as 95–99%, it fell short in specificity (~83%), with false positive results in detection of bladder cancer [38]. A combination of CT with cystoscopy improves diagnosis of bladder cancer to 100% with 94% specificity [38]. MRI, while not often used for diagnosis of bladder cancer, is an excellent imaging method to stage bladder cancer.[39] Staging accuracy for differentiation between invasive versus superficial bladder cancers was improved to 85% [40]. Sensitivity of ultrasound is around 72%, and that can be further improved by contrast-enhanced ultrasound with sensitivity of 88%; however, detection of tumors less than 5 mm diameter is only 20% [41].

After cystoscopy, the obtained biopsy sample is histologically evaluated for confirmation, grading, and staging of bladder cancer (reviewed in by Sul et al.) [42]. The classic tumor/node/metastasis (TNM) staging method (Table 1.1)[9, 43] involves evaluating the condition of the tumor and if it has invaded surrounding tissue (T), lymph node involvement (N), and metastasis (M) [43]. When the tumor is present on the epithelial layer and has not breached the basement membrane into the surrounding muscle tissue, it is referred to as a non-invasive superficial tumor, or carcinoma-in-situ [6, 44]. When tumor cells breach the basement membrane and invade the muscle tissue surrounding the bladder and other organs, it is referred to as invasive TCC[6, 45] and is associated with a poor prognosis [6]. While the five-year survival rate of patients diagnosed with the early stages of bladder cancer is 69.2%, the survival rate drops drastically to only 5.5% for patients diagnosed with metastatic bladder cancer [46].

Current Treatment Options for Bladder Cancer

Treatment of bladder cancer depends on the level of invasion and metastasis of the tumor and is divided into two distinct categories: superficial bladder cancer and invasive bladder cancer.

Superficial bladder cancer is well managed by transurethral resection (TUR), followed by intravenous or intravesical (directly into the bladder) administration of chemotherapeutic treatment, such as mitomycin, epirubicin, or doxorubicin [47-49]. This combination therapy is extremely important due to the high rates of bladder cancer recurrence [6]. The intravesical injection of bacillus Calmette-Guérin (BCG), as adjuvant immunotherapy, activates the immune system in the patient and greatly increases progression-free survival rates [49, 50]. Management and treatment for patients with muscle invasive bladder cancer is usually a radical cystectomy (removal of whole bladder) and possibly removal of surrounding organs, like lymph nodes; prostate and seminal vesicles in men; and the uterus, ovaries, and part of the vagina in women.[6] Radical cystectomy is usually followed by adjuvant therapy, such as chemotherapy and radiation therapy. Chemotherapy protocols without radiation include several options, such as cisplatin alone, cisplatin with 5-flourouracil, or mitomycin with 5-flourouracil [51]. Chemotherapy protocols in conjunction with radiation include gemcitabine with cisplatin; the MVAC protocol, which includes methotrexate, vinblastine, doxorubicin (Adriamycin), and cisplatin; or a combination of carboplatin with either paclitaxel or docetaxel [51]. Recently, Kanatani and colleagues have shown that cisplatin-based adjuvant therapy, including MVAC, greatly increases median survival time (MST) in patients with node-positive bladder cancer, while increasing body mass index (BMI), a marker of health. On the other hand, it was noted that cisplatin-based therapy had poor tolerance, and the dose must be lowered for many

patients who experienced side effects [52]. Because side effects of therapy can be intolerable for some patients, research and development into targeted therapies that have fewer side effects and will increase survival is warranted. More information on treatment options of bladder cancer can be found in a review by Carballidoet al., 2014 [53].

Molecular Targets of Bladder Cancer

In order to develop proper targeted therapy for any cancer, the molecular targets that drive the cancers need be well understood. Like other types of cancers, bladder cancer development is a multistage process beginning with initiation, promotion, and progression [54, 55]. In colorectal cancer progression, the loss of tumor suppressor APC is common in the early stages of cancer (initiation/promotion), while the loss of tumor suppressor BRCA1 or BRCA2 is common in ovarian and breast cancers [56, 57]. The multistage process of carcinogenesis is no different in bladder TCC, but TCC has its own unique pathways/genes that are commonly altered [58-60]. In 2014, the Genome Atlas Research Network (TCGA) published a study that not only outlined genome, transcriptome, and mutational data, but also correlated many molecular events to specific stages and prognosis of patients in 131 urothelial carcinomas [59].

p53 and pRb Pathways in Regulation of Cell Cycle of Bladder Cancer

As the cell undergoes stress with induction of DNA damage, the p53 protein is activated and localizes to the nucleus, where it functions as a transcription factor. The p53 protein controls cell cycle arrest genes, such as p21 and p16, as well as pro-apoptotic proteins, such as Bax.[61] The p21 and p16 proteins are cyclin-dependent kinase (CDK) inhibitors that prevent the downstream phosphorylation of retinoblastoma protein (pRb). The un-phosphorylated pRb

inhibits progression from the G1 to S phase of the cell cycle [62]. The p53 protein is a tumor suppressor, and the gene coding for *p53* is mutated in over 50% of all cancers [63]. The p53 pathway is disrupted in invasive bladder cancer and has been correlated to poor clinical outcome, progression to invasive from non-invasive bladder cancer, and resistance to radiation therapy [64-67]. The TCGA network found that the p53/pRb pathway is altered in 93% of patients whose genome was sequenced [59]. In many aggressive bladder cancers, the p53 gene is mutated, overexpressed, and highly localized to the nucleus, where it is rapidly degraded [64]. Further progression indicates loss of function of pRb as well as loss of expression of tumor suppressor genes p21 and p16 [67, 68]. While p16 and p21 are within the p53 pathway, their expression can be dependent or independent on p53 [67, 69]. The most interesting feature of this pathway in bladder cancer is that loss of function of expression of p53, p21, pRb, and p16 proteins appear to have an additive negative prognostic effect, suggesting that more than one linear pathway is responsible [67]. Other pathway genes that underwent alterations include ATM (activator of p53), MDM2 (inhibitor of p53), EF2A (target of pRb), and FBXW7 (ubiquitin kinase of cyclin E) [59].

While the alterations in the p53/pRb pathway have been clearly characterized, new sequencing data reveal other possible drug targets. For example, in one study, the p16 coding gene had an altered copy number in 46% of tested tumor samples [59]. Many possible drug targets have been identified within the pRb pathway, but because multiple alterations have an additive effect in one pathway, it is vital to study and develop drugs that act independent of the p53 pathway. This approach, if successful, will allow better treatment of patients with more invasive bladder cancer and perhaps circumvent it altogether. It is also important to study the in vitro effects of drugs on UC cell lines that have mutated p53 and altered expression of p21, p16, and pRb. Both T24 cell lines and UMUC3 cell lines contain mutated p53 genes [65]. T24 cells

have an in-frame deletion of Y126, while UMUC3 cells have two mutations consisting of R72P and F113C [65, 70]. It is important to note that the T24 and UMUC3 cell lines each have a mutation that is within the DNA binding domain of p53 protein, which has been shown to be a mutational hot spot in many cancers [71]. The 126 and 113 residues of p53 are both close to the K120 residue, which has been shown to be a contact site interacting with the major groove of specific DNA sequences [72]. suggesting that the mutant p53 protein of T24 and UMUC3 cell lines have a similar level of dysfunction. Furthermore, the UMUC3 cell line has an additional mutation that is within the proline-rich domain, which lies between the transactivation domain and the DNA binding domain [73]. At the same time, both T24 and UMUC3 cell lines have been shown to be more resistant to radiation therapy when compared to cell lines containing a wild type version of the p53 gene [70]. Recently, Zhu and colleagues have shown that silencing of mutant p53 in T24 cell lines inhibited cell growth, induced apoptosis through caspase activation, and lowered the expression of cyclins A and B1. Lowering the expression of mutant p53 also sensitized bladder cancer cells to chemotherapeutic drugs [66]. Another study showed that a mutant p53 protein can activate oncogenic proteins such as GEF-H1 at the transcription level in osteosarcoma cell lines and increase cell proliferation [74]. On the other hand, mutant p53 proteins have been shown to be activated by small molecule drugs such as PRIMA-1 and are able to induce apoptosis in bladder cancer cells including T24 similar to the wild type protein [75].

While circumventing the p53 pathway has proven difficult, drugs like doxorubicin have been shown to function in both a p53-dependent and -independent manner, which warrants further study on the efficacy of Dox and p53 pathway status [76]. Because p53 is often accumulated inside neoplastic cells, it undergoes proteolysis and is processed to be expressed on the cell surface with human major histocompatibility complex HLA-A2.[77] ALT-801, a drug

currently in clinical studies, targets this unique surface representation of p53 peptide 264-272 with the HLA-A2 complex by an antibody linked to interleukin-2, which is capable of recruiting cytotoxic T-lymphocytes selectively to tumors[78] (Table 1.2). Another method of targeting cell cycle abnormalities in bladder cancer is to use a nonspecific, anti-mitotic or anti DNA-synthesis chemotherapeutic agent. Cisplatin-based therapies, including MVAC, have been extremely successful in the past [79]. Several anti-cell cycle drugs, such as amrubicin–a derivative of the popular drug doxorubicin, are being researched to treat bladder cancer (Table 1.2).

Receptor Tyrosine Kinase Signaling Pathways in Bladder Cancer

Receptor tyrosine kinase (RTK) pathways are commonly activated in earlier stage bladder cancer. RTKs are often deregulated in various types of cancers, and one major hallmark of malignancy is RTK independence from growth factors by amplification or an activating mutation [80]. In bladder cancer, the altered RTKs include, but are not limited to, EGFR, ERbB2, ERbB3 and FGFR3 [59]. The downstream activators of RTKs are MAPKs and PI3K/AKT, which lead to the activation of many downstream products that induce cell proliferation.

In non-invasive bladder cancer, it is common to find a mutation of the fibroblast growth factor receptor 3 (FGFR3) gene, or less commonly, a direct mutation of RAS itself [69, 81, 82]. While mutation of the p53 tumor suppressor gene is very common in invasive bladder cancer, mutated FGFR3 and p53 are rarely found together [69, 81]. This may indicate two different models of initiation of bladder cancer, one leading to a far less aggressive cancer than the other. In another scenario, mTOR, a downstream target of AKT, has been linked to poor prognosis in bladder cancer patients with increased mortality [83]. One major obstacle is to

determine which gene alterations in the tyrosine kinase pathway are associated with transition to a more invasive bladder cancer and select better drug targets for patients with recurrent or invasive bladder cancer. Another important consideration is the ability to select good candidate patients for therapies targeting various parts of the RTK pathways. As FGFR3 alterations are often associated with non-invasive and non-recurrent bladder cancer, HER2 and EGFR alterations are associated with poor prognosis and more invasive bladder cancers [84-86]. While these findings suggest that certain tyrosine kinase receptors may prove to be a valuable target for cancer therapy, each of them are over-expressed in a small subset of cancers. For example, a meta-analysis of 2,242 patients in nine separate studies showed that the incidence of ERbB2-positive (over-expressing) cancers ranged from 27.8 to 85.2%, with the pooled average of ERbB2-positive cancers at 41.2% [86].

Another important RTK family protein is VEGFR and its ligands, which play an important role in angiogenesis, as well as cell survival and proliferation. Both VEGFR1 and VEGFR2 are over-expressed in bladder cancers, and bladder cancers express the VEGF ligand for new blood vessel formation [87]. Currently, there are a number of ongoing studies sponsored by the National Cancer Institute (NCI) targeting TKRs in bladder cancer (Table 1.2). Receptor tyrosine kinase inhibitors function by inhibiting the receptor, as in the case of afatinib, which targets both EGFR and ERbB2 and has shown much promise by sensitizing murine bladder cancers to radiation [88]. Bevacizumab, an antibody that binds to VEGF-A and inhibits its interaction with VEGFR, is currently under investigation as novel therapy for bladder cancer and renal cancers [89].

Downstream of RTKs are cascades of signaling pathways, including the PI3K/AKT pathway. The PI3K/AKT pathway has been greatly implicated in the progression of bladder cancer. The ligand binds to RTK protein and is followed by self-phosphorylation of RTK and

downstream activation of PI3K through the phosphorylation of the p85 subunit. The PI3K complex is responsible for the conversion of phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3). PIP3 induces AKT activation by phosphorylation at the Tyr308 residue and in turn, AKT phosphorylates AMPK as well as several other target proteins. Downstream of MAPK, through the inhibition of TSC1/2 by AKT, mTOR is activated and induces cell growth, survival, and further resistance to apoptosis [90]. A negative regulator of the PI3K/AKT pathway is the tumor suppressor gene PTEN, which is responsible for reverting PIP3 to PIP2 [91]. The inactivation of PTEN carries a poor prognosis in bladder cancer patients and this poor prognosis is further increased with the loss of p53, this linking the p53, linking the p53 cell cycle pathway to the PI3K/AKT/mTOR pathway and suggesting the two pathways may work in combination to progress clinical bladder cancer [91, 92]. One interesting feature about the PI3K/AKT/mTOR pathway is that while it has been found to be altered in 72% of cancers, the alternations tend to be mutually exclusive, suggesting that altering only one gene in the pathway is enough to activate the downstream signaling cascade needed for enhanced tumorigenesis [59]. For example, mutations in PI3K subunit PI3KCA, AKT3, and TSC1 were almost never found in the same tumor sample [59]. Due to the variance and mutual exclusivity of these alterations, it will be extremely important to screen patients using genetic tests, as well as expression profiles, in order to better predict which patients are good candidates for the therapy targeting the specific proteins of the PI3K pathway. This makes the downstream target, mTOR, a valuable drug target for bladder cancer treatment. Drugs currently being researched by NCI-sponsored trials include rapamycin, albumin-bound rapamycin, and everolimus. One setback with using mTOR inhibitors such as rapamycin is that only the mTORC1 complex is sensitive to the drug, while the assembly of mTORC2 appears to be resistant. mTORC2 can phosphorylate AKT at the Ser473 residue and thus induce the AKT signaling cascade and still

increase mTOR activation; through this feedback loop, cancer cells can develop resistance to mTOR-targeting therapies [93]. New drugs like everolimus inhibit the assembly of both mTORC1 and mTORC2, thus providing a new tool to inhibit the PI3K/AKT/mTOR pathway [94].

As bladder cancer progresses, more pathways are activated in order to facilitate survival, invasion, and metastasis. In bladder cancer, angiogenesis, measured by micro vessel density (MVD), has proven to be an independent prognostic indicator when it comes to survival and, in some cases, staging [60, 95]. One of the main pathways activated in angiogenesis is the vascular endothelial growth factor (VEGF) pathway which is directly regulated by hypoxia [60, 95, 96]. When cells are depleted of oxygen, hypoxia-induced factor 1 and 2 (HIF-1 and HIF-2) are stabilized on the protein level [96]. HIF-1 and HIF-2 are transcription factors that directly upregulate VEGF expression [96]. Another way to increase VEGF expression is activation of the epidermal growth factor receptor (EGFR) [97]. Basic fibroblast growth factor (bFGF) is another angiogenic protein and has been shown to be an important prognostic marker in bladder cancer [98]. The regulation of bFGF expression is increased upon the activation of the protein kinase C (PKC) pathway, which is activated in most cancers by increasing cAMP concentration due to hypoxia and low energy levels [99]. Inflammation in the cancer site and release of inflammatory signals such as interleukin-8 (IL-8) work as chemo-attractants and also recruit blood vessel growth at the site inflammation [100, 101]. The p53 tumor suppressor, which is altered in many bladder cancers, has also been linked to angiogenesis [60].

Role of the Immune System in Bladder Cancer

Treatment of bladder cancer has had a long history with immunotherapy in order to activate the immune system to target cancer cells. The use of BCG, injected directly into the

bladder as an adjuvant to chemotherapeutic agents, has been used for over three decades [102]. Evasion of the immune system is a well-established hallmark of malignancy and thus, increasing the efficacy of the immune system in bladder cancer has been an active area of research [80]. There are two major approaches in targeting the immune system as an anti-cancer therapy. The first approach is to activate the immune system against the tumor by blocking or inhibiting negative regulators. The second approach is to increase the immune response by using agonist cytokines. Programmed death ligand 1 (PDL1), along with its receptor programmed death 1 (PD1), have been implicated as one of the mechanisms cancer cells use to suppress immune response. PD1 is expressed on T-cells and is a negative regulator of T-cell response, while PDL-1 is overexpressed by various types of cancer in order to suppress the immune response in the tumor environment. Tumor-infiltrating T-cells express high levels of PD1 and therefore are very sensitive to negative regulation by PDL-1 [103]. Several drugs target the PD1/PDL1 interaction, including MPDL3280A, pembrolizumab, and MEDI4736 (Table 1.2). For example, MK-3745, also known as pembrolizumab, is an antibody raised against the PD-1 receptor and blocks the PD1 to PDL1 interaction [104]. On the other hand, MEDI4736 and MPDL3280A are antibodies against the actual PDL1 ligand antibody which block the interaction between PD-1 and PDL1 [105, 106].

Another negative regulator of the immune system is the CTLA-4 antigen, which is highly expressed on regulatory T-cells and serves to disrupt the cytotoxic T-cell response [107]. Blocking antibodies of CTLA-4, including tremelimumab and ipilimumab, are currently under investigation to treat bladder cancer (Table 1.2). While studies using anti-CTLA-4 antibodies for bladder cancer are relatively new, both tremelimumab and ipilimumab have been effective in the treatment of lymphoma patients. Tremelimumab increases memory T-cell proliferation in lymphoma patients, thus potentiating a better immune response against cancer [108].

Ipilimumab in combination with GP100 has increased the mean survival time of melanoma patients from an average of 6.4 months to an average of 10 months (p value <0.001) [109, 110]. In renal cell convergence carcinoma, ipilimumab increased regression of the tumor in patients who previously did not respond to IL-2 immunotherapy; however, 14% of patients experienced very high toxicity [111].

Activation of the immune response against cancer in the form of immunization has been intensively studied. In animal models, such as dogs, cancer-specific antigens were targeted with promising results. An Ad/HER2/Neu dendritic vaccine, which targets ERbB2, is currently being studied for treatment of bladder cancer (Table 1.2.) It is vital to test for positive expression of ERbB2 prior to treatment because it has been shown that ERbB2 is altered in 12% of bladder cancer patients either by mutation or copy number amplification, making it a target for a very small subset of patients [59]. Another approach in activating the immune system against cancer is to use drugs such as ALT801, an IL-2-based immunotherapy, or ALT803, an IL-15-based immunotherapy (Table 1.2). These cytokines are agonists to immune response. IL-2 and IL-15 work by recruiting more cytotoxic T-lymphocytes to the tumor and helping convert naïve T-cells to effector cytotoxic T-lymphocytes [112, 113]. As mentioned earlier, ALT801 targets the p53-HLA-A2 complex, thus both targeting a defective p53 pathway while activating the immune response to the tumor [78].

Role of the Other Molecular Targets and Signaling Pathways

Heat shock proteins (HSP) have been shown to affect cancer by stabilizing oncogenic proteins as well as eliciting self-recognition (negative regulatory immune response) and have been observed to be overexpressed in various cancers [114, 115]. For example, HSP90 has been shown to stabilize RAF-mutated protein, which is downstream of RAS, activates the RAS-

ERK pathway in cancer cells, and can also activate the PI3K pathway mentioned earlier [116]. HSP27 has been shown to modulate p53 signaling by inhibiting the induction of p21, causing resistance to doxorubicin therapy in human breast cancer cells [117]. Ganetespib and OGX-427 are being researched to better treat bladder cancer. Ganetespib, an HSP90 inhibitor, has shown much promise in previous studies in lung cancer and has increased efficacy of other therapies while being well tolerated and showing low toxicity in cancer patients [118, 119]. OGX-427, an antisense oligonucleotide-based therapy against HSP27, has been shown to be effective against the bladder cancer cell line UMUC-3 by increasing activation of the caspase cascade, increasing efficacy of paclitaxel, and slowing tumor growth in a xenograft model [120].

Epigenetics have been known to play an important role in cancer for the past two decades. Hypermethylation of the promoter regions coding tumor suppressor genes, such as p14, p16, and APC, are often detected in bladder cancers [121]. Genetic profiling recently revealed that 89% of bladder cancers contain altered histone modification pathways and 64% of cancers contain alterations in the SWI/SNF complex, which is responsible for chromatin remodeling in order to turn on or turn off transcription [59]. Romidepsin, a histone deacetylase inhibitor, as well as 5-fluoro-2-deoxycytidine in combination with tetrahydrouridine, are being studied for bladder cancer to inhibit DNA methylation and deamination [122, 123]. Recently, the histone deacetylase inhibitor AR-42 has shown promise in combination with cisplatin in treating bladder cancer in the mouse model. It was also shown that AR-42, when combined with cisplatin, can be an effective treatment on stem cell populations in vitro [124].

Epithelial to mesenchymal transition (EMT) has been shown to play an important role in invasion and metastasis. In order for cells to migrate from the primary site to a secondary site, they must exhibit plasticity to adapt to new environments, excrete more extra-cellular matrix, and acquire further drug resistance [125]. Altered integrin expression can facilitate EMT by

increasing the expression of mesenchymal genes, while decreasing the expression of epithelial genes [126]. Integrin proteins not only change how cells interact with the ECM, but integrins can also trigger signal transduction pathways such as the AKT signaling pathway [127]. It has been shown that the αV group of integrins are expressed in metastatic bladder cancer in a stage/grade-dependent manner [128]. Several approaches to target integrins are under development in treatments for bladder cancer. Because integrins interact with specific ECM components with different affinity, an Arg-Gly-Asp peptide was synthesized to bind αVB3 and αVB5 integrins and was inserted into the fiber protein to facilitate adenovirus infection. The Arg-Gly-Asp motif increased transfection efficiency of bladder cancer cells with the adenovirus and will perhaps lead to further advances in oncolytic viral research for bladder cancer [129]. GLPG0187, a small molecule integrin agonist, has been shown to decrease migration and invasion in bladder cancer cells and has also been shown to decrease tumor burden in the mouse xenograft model using UMUC3 bladder cancer cells [130]. Change in integrin structure may also play a role in EMT. Integrin α3β1 has been shown to be abnormally glycosylated in bladder cancer cells, thus increasing its interaction with CD9 [131]. A recently developed antibody against integrin α3β1, BCMab1, has been shown to play a prognostic role in immunohistochemistry of bladder cancer patients: patients with weaker staining by BCMab1 exhibited longer survival than the patients with stronger staining. At the same time, BCMab1 has shown anti-tumor activity through natural killer T-cell and macrophage recruitment *in vitro* and reduced tumor burden in a mouse xenograft model *in vivo* [132].

In recent years, inflammation has become a popular target in the treatment of bladder cancer, as have anti-inflammatory drugs such as piroxicam to increase the efficacy of chemotherapy [101]. COX-2 has been shown to be overexpressed in many cancers, including TCC [133]. In a dog model, the inflammatory protein COX-2 is being actively studied to detect

bladder tumors in vivo during cystoscopy. Inhibition of the COX-2 signaling pathway may increase the efficacy of other treatments, including masitinib (AB1010, Paris, France) [35, 101, 134]. Perinuclear localization of COX-2 has been associated with bladder cancer cells expressing stem-cell like markers, including OCT 3/4 and CD44v6. COX-2-driven inflammation helps to drive proliferation of cancer stem cells [135].

Cancer Stem Cells of Bladder Cancer

Other studies of bladder cancer have suggested a new approach in treatment by targeting tumor initiating cells (TIC) or cancer stem cells (CSC). CSCs are cancer cells with unique properties such as self-renewal, tumor regenerating properties, and drug-resistance [136]. A subpopulation of CSCs have been isolated from various cancers, including bladder cancer tumors [137]. It has been reported that STAT3 activation is required for the acquisition of CSC-like properties in breast cancer [138]. It has also been reported that bladder cancer basal cells, which exhibit CSC-like properties, closely resemble breast cancer basal cells, which also exhibit CSC-like properties [139]. A novel small molecule inhibitor of STAT3, BBI608, is currently being studied in human bladder cancer patients in an NCI-sponsored study. Chemotherapy can actually cause a selective increase of CSC in some tumors. In a pancreatic cancer xenograft model, gemcitabine, while inducing an anti-cancer response initially, attributed to an increase in CSCs and induced a larger tumor load in the animal than the control 15 days after the drug was discontinued. On the other hand, BBI608 showed a lower tumor load when compared to both control and gemcitabine 15 days after drug treatment was discontinued [140]. Gemcitabine and cisplatin induce COX-2 expression in bladder cancer cells and increase downstream expression of prostaglandin 2 (PGE-2). PGE-2 from apoptotic cells can induce

CSC-like characteristics in neighboring cells. Inhibition of COX-2 by celecoxib inhibited repopulation of bladder cells after several gemcitabine and cisplatin treatments and reduced CSC-like characteristics in neighboring cells [141],[142].

Animal Models of Bladder Cancer

Rodent models of bladder cancer

Numerous experimental rodent models of bladder cancer have been established and characterized to study epidemiology and carcinogenesis of bladder cancer [143]. Bladder cancer models in rodents can be chemically-induced, genetically engineered, or transplantable [143].

The most commonly used carcinogens to induce bladder cancer in mice are N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT), and N-methyl-N-nitosurea (MNU).[144] BBN via drinking water, diet, or gastric intubation induces bladder tumors in mice [145, 146]. Mice exposed to BNN develop nodular invasive carcinoma preceded by carcinoma-in-situ, and rats develop polypoid exophytic cancers with late muscle invasion [146]. Rodent bladder tumors induced by BBN mirror their human counterparts histologically and genetically [147]. In a study comparing mRNA and protein levels of the rodent bladder cancer model with human bladder cancer, there were concordant changes in several genes/proteins, demonstrating that the bladder cancer model induced by BBN is a powerfully reliable study tool [148]. These rodent models provide useful information concerning the risk of chemical exposure and bladder cancer; however, they have limitations due to low-grade tumors and low rates of metastasis [149]. Also, tumor induction and progression takes time and is dependent on carcinogen and dosage.

Transgenic mice or genetically engineered mice (GEM) are generated by cloning oncogenes or by deletion of tumor-suppressing genes, individually or in combination [143]. GEM models have provided increasing insight on the role of numerous genes like HRAS,[150] p53,[92] pRB,[151] and PTEN,[92] and receptors such as FGFR[82] and epidermal growth factor receptor[152] in the development of bladder cancer. With target genes switched on or off, GEM are ideal for studying single or multiple gene functions, but this model may not fully reflect the genetic alterations in natural human tumorigenesis as it involves the deregulation of multiple signaling pathways [143]. Cancer cells in these models are less heterogeneous than human bladder cancer,[153] and GEM are usually not used to test the efficacy of novel therapeutic or preventive agents [143].

Xenogeneic models involve the implantation of human bladder cancer cells into an immune-deficient mouse. Various commercially available TCC cell line, like KU7, KU-19–19, T24, UM-UC1, UM-UC3 and UM-UC13, have been used to developed tumors in immunodeficient mice [153]. A major disadvantage of this technique is that the immune response, which is an important factor regulating tumor growth, cannot be assessed because of the immunodeficient nature of the host [144]. Syngeneic models, in contrast to xenogeneic models, are established by inoculating rodent bladder cancer to syngeneic, immunocompetent animals [143]. The commonly used rodent bladder cancer cell lines for syngeneic modeling are AY-27, MBT-2, and MB49 [143]. Tumors induced by this model are of rodent and not human origin, and therefore various characteristics, including tumor growth, latency, growth rate, invasion, and metastasis, may be different from their human counterparts [154] [155].

Based on whether the inoculation site is in the target organ, xenogeneic and syngeneic models could be further divided into orthotopic and heterotopic models. In orthotopic models, inoculation is done at the primary site from which the tumor lines were derived [156]. These

tumors mimic human bladder cancer behavior more closely, since the microenvironment is closer to natural conditions [143]. The disadvantages of the orthotopic human tumor xenograft model are that the surgeries are often complex, leading to the use of low numbers of mice per study [153].

In heterotopic models, the graft is not transplanted at the original site, but is usually subcutaneously placed in the flank or hind leg of the animal. This process is technically simple, and the tumor can be easily and non-invasively detected; subcutaneous bladder tumor models have been widely used in assessing the efficacy of novel therapeutic agents [156]. However, as the inoculation site is different from the original tumor site, the alteration of the tumor microenvironment may significantly affect the biological behavior of tumor growth and metastasis, genetic expression, or the efficacy of anti-proliferative agents [157].

A mouse model has several advantages, including small size, short gestation, inexpensive maintenance, and easy manipulation of gene expression [158]. However, the average rate of successful translation from mouse model to clinical cancer trials is less than 8% [159]. Also, a mouse model can tolerate higher drug concentrations than human patients. Considering the vast species differences between mice and humans, it is important to use other animal models, such as companion dogs with naturally occurring bladder cancer, to study human disease [158].

Canine Models of Bladder cancer

Urinary bladder cancer is an uncommon type of cancer in dogs (less than 2% of all canine malignancies);[160] however, 97% of diagnosed bladder tumors in dogs are malignant [35]. Bladder canine TCC is the most common neoplasm affecting the urinary tract of dogs

[161]. Risk factors that have been identified include exposure to insecticides,[162] and cyclophosphamides [163]. The male-to-female ratio of dogs with TCC has been reported to range from 1.71:1 to 1.95:1, with increased risk after spaying and neutering [164, 165]. Scottish terriers have a strong breed-associated risk factor for the disease [166]. In addition to spontaneous TCC, bladder tumors can be experimentally induced in dogs in a laboratory setting with chemical carcinogens such as N-butyl-n-(4-hydroxybutyl)-nitrosamine [167].

TCC typically occurs in older dogs ranging from 9 to 11 years of age [168]. Clinical staging of canine bladder cancer is performed with complete physical examination, radiography of the thorax and abdomen, and imaging of the bladder using contrast cystography, ultrasonography, or computed tomography [161]. The TNM classification scheme for canine urinary bladder cancer has been defined by the World Health Organization and is much like the staging system used for human cancers [169]. Each TNM stage in further divided into substages, as shown in Table 1.1 [169].

Treatment options on TCC in dogs include surgery, radiation therapy, chemotherapy and other drugs, and combinations of these treatments. The surgical complete cystectomy, although it may be routine in human bladder cancer patients, has not been attempted to any extent in the dog. Canine TCC is difficult to remove surgically because of the trigonal location of the tumor, frequency of urethral involvement, and metastases in 20% or more of dogs at the time of diagnosis [161, 168]. Radiation therapy is not routinely used to treat canine TCC due to various side effects, including pollakiuria, urinary incontinence, cystitis, stranguria, and hydronephrosis [170]. Chemotherapy drugs used in canine TCC include cisplatin, carboplatin, mitoxantrone, adriamycin, and actinomycin D as single agents.[166] Various combination therapies have also been used. Other treatment options include non-steroidal anti-inflammatory drugs (NSAIDs) like piroxicam as a single agent [171] or in combination with chemotherapy drugs [172, 173].

Naturally occurring bladder cancer in dogs very closely mimics human invasive bladder cancer, specifically high-grade invasive TCC, in cellular and molecular features; biological behavior, including sites and frequency of metastasis; and response to therapy. Incidence of TCC in both humans and dogs is 2% of all cancers [174]. TCC occurs in older dogs at average age of 11 years, which is equivalent to 60 years in humans [175]. Both human and canine TCCs have similar risk factors, including exposure to various chemicals, such as insecticides and aromatic hydrocarbons [162, 176].

Histopathology of canine TCC is similar to human bladder cancer, with invasive TCC of intermediate to high grade existing in both species [161, 176]. Distant metastasis have been reported in 15–20% of dogs diagnosed with TCC,[165] which is similar to humans, in which metastasis occurs in 5–20% of patients [176]. The sites involved in metastasis are also similar between dogs and humans and include lymph nodes, lungs, bones, liver, and kidneys [168]. Various similarities in cellular and molecular levels in canine and human TCC have been studied so far, including similar lipidomic profiling in both species [177]. Both human and canine TCC have shown over-expression of COX-2 in tumor tissue [178, 179]. Platinum-based chemotherapies are considered the most active agent in treatment of TCC in both species [165, 176]. The main difference between TCC in dogs and humans is sex predilection: in humans, TCC is twice as common in males than in females,[180] whereas in dogs, it is less common in males, with a 2:1 ratio in favor of female dogs [164]. The location distribution of TCC in humans is more balanced across areas of the bladder,[181] but dogs have trigonal disease with extension down the urethra [165].

Dogs diagnosed with spontaneous tumors offer a unique model to study bladder cancer development and detection, as well as evaluation of new therapies [158]. Dogs offer an exceptional opportunity to study potential genetic and environmental risk factors for TCC and

develop early detection and intervention strategies. Development of new treatment options and their study in the dog model can provide translational value to ultimately help develop better drugs for people with TCC. Single agent NSAIDs like piroxicam, deracoxib, and firocoxib have shown positive results in treating dog TCC;[165] further translation of this treatment option to humans is an obvious next step. A pilot study has shown positive results in treatment of human TCC using the NSAID celecoxib [182]. Folate-targeted therapy has been used for treatment of several forms of human cancers, including ovarian and lung cancer [183]. Recently, a dog study was conducted to determine the potential role of folate-targeted therapy in treatment of canine TCC [184]. Further epigenetic-based therapy using 5-azacitidine has been tested to treat canine TCC [185]. Metronomic chemotherapy, based on frequent and repetitive treatment with low-dose chemotherapeutic drugs to delay the progression of cancer,[186] has been recently used to treat canine TCC [187]. The positive outcome of this trial can help inform future investigations into new treatment options for human TCCs. Dogs have also been recently used in molecular imaging of TCC. Fluorocoxib A, a COX-2-specific inhibitor conjugated with rhodamine,[188] has shown to specifically detect COX-2-expressing TCC cells in vitro and in dogs during cystoscopy in vivo, but was not detected in normal urothelium [35, 189].

Spontaneously occurring TCC in dogs share molecular and clinical characteristics with human cancers [158]. Use of canine models can lead to better understanding and new therapeutic development for treatment of human TCC. Primary K9TCC cell lines are currently available and can also help in the study of various drugs in vitro before clinical trials. Currently, only limited canine TCC cell lines are available for research use [190, 191]. Therefore, utilizing the dog model in TCC research can benefit animal and human disease.

Conclusions

With increasing knowledge of specific pathways activated or altered in bladder cancer, an increasing number of new, promising therapies are on the horizon. In the future, it will be extremely important to test patients for personalized therapies because these therapies target only a small subset of patients. This pathway knowledge will also increase the knowledge base of potential drug targets for new and exciting drug development.

Abbreviations

4-ABP: 4-aminobiphenyl; Ad/Her2/Neu: protein encoded by ERBB2; AKT: v-akt murine thymoma viral oncogene homolog 1; AMPK: 5' adenosine monophosphate-activated protein kinase; APC: adenomatous polyposis coli protein; ATM: ataxia telangiectasia mutated; BCG: Bacillus Calmette–Guérin; BRCA1/2: breast cancer type 1/2 susceptibility protein; CDK: cyclin dependent kinase; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CYP1A2: cytochrome P450, Family 1, Subfamily A, Polypeptide 2; EF2A: eukaryotic elongation factor 2; EGFR: epidermal growth factor receptor; ERbB: family of proteins including EGFR, HER2, HER3 and HER4; ERK: extracellular signal regulated kinases; FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; GEF-H1: ARHGEF2 - Rho guanine nucleotide exchange factor 2; HER2: receptor tyrosine-protein kinase ERbB-2; HIF: hypoxia induced factor; HLA-A2: Major Histocompatibility Complex, Class I, A 2; HSP27: heat shock protein 27; IL: interleukin; MAPK: mitogen activated protein kinase; MDM2: E3 Ubiquitin Protein Ligase oncoprotein; MST: median survival time; mTORC: mTOR complex; MVAC: chemotherapy combination including

methotrexate, vinblastine, doxorubicin (Adriamycin) and cisplatin; MVD: micro vessel density; PAH: poly-aromatic hydrocarbons; PD1: programmed death 1 receptor; PDL1: programmed death ligand 1; PI3K: Phosphoinositide 3-kinase; mTOR: mechanistic target of rapamycin; PIP: phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C; pRB: retinoblastoma protein; PTEN: Phosphatase and tensin homolog; RAF: rapidly accelerated fibrosarcoma (a family of serine/threonine kinases); RAS: rat sarcoma viral oncogene homolog (a family GTPase proteins); TCC: transitional cell carcinoma; TCGA: the cancer genome atlas; TKR: tyrosine kinase receptor; TNM: a method of tumor staging (Tumor, Node and Metastasis); TSC1/2: tuberous sclerosis 1/2 tumor suppressor proteins; TUR: transurothelial resection; UC: urothelial cancer; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

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Author contributions

DS: Wrote, and approved final manuscript; designed tables; and agreed to be accountable for all aspects of the work. MC: Designed, wrote, and approved final manuscript; designed tables; and agreed to be accountable for all aspects of the work. KR wrote and approved final manuscript; and agreed to be accountable for all aspects of the work.

Disclosure

The author reports no conflicts of interest in this work.

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Appendix

Table 1.1 Clinical Staging of TCC

Stage	Tumor (T)	Lymph Node Involvment (N)	Metastisis (M)
Stage 0a	**Ta** - Non-invasive papillary carcinoma	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
Stage 0is	Tis- carcinoma in situ	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
Stage I	**T1** - Tumor has grown into connective tissue	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
Stage II	**T2a**- Tumor has grown into inner half of muscle layer	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
	T2b- Has has grown into outer half of muscle layer	**N0 -** No lymph node involvement	**M0** - No signs of metastasis

Table 1.1 Continued

Stage	Tumor (T)	Lymph Node Involvment (N)	Metastisis (M)
Stage III	**T3a** - Microscopic invasion of surrounding fatty tissue	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
	T3b - Macroscopically detectable invasion of surrounding fatty tissue	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
	T4a- Tumor spread into prostate (men) and uterus (women)	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
Stage IV	**T4b -** Tumor has grown into pelvic or abdominal wall	**N0 -** No lymph node involvement	**M0** - No signs of metastasis
	Any T	**N1-3 -** Lymph node involvement in proximal or distal lymph nodes	**M0** - No signs of metastasis
	Any T	**Any N-** Any lymph node involvement	**M1** - Metastasis is present

Notes: According to AJCC

Table 1.2: Overview of clinical trials for bladder TCC.

Target Pathway	Drug	Mechanism/Target	Trial Phase	Reference
Tyrosine Kinase	Bevacizumab	VEGF-A binding/inhibition	III	[1]
	Ziv-Aflibercept [2]	VEGF binding/inhibiting agent	I	[2]
	Cabozantinib	VEGFR-2 Inhibitor	II	[3]
	Pazopanib	Inhibitor of several tyrosine kinases	II	[4]
	Tamoxifen	Anatagonist of Estrogen Receptors	II	[5]
	Buparlisib	PI3K inhibitor	II	[6]
	Dovitinib	FGFR and VEGFR Inhibitor	II	[7]
	MEK162	MEK Inhibitor	II	[8]
	MGAH22	HER-2 Targetting antibody	I	[9]

Table 1.2 Continued

Target Pathway	Drug	Mechanism/Target	Trial Phase	Reference
	Afatinib	EGFR and HER2 inhibitor	II	[10]
	AZD5312	Angrogen receptor anti-sense inhibitor	I	[11]
PI3K/AKT/mTOR	Everolimus	m-TOR inhibitor (MTORC1 and MTORC2)	I, II	[12]
	Rapamycin	mTOR inhibitor	I, II	[13]
	ABI-009 (Albumin Bound Rapamycin)	mTOR inhibitor	I, II	[14]
Immunotherapy	ALT-801	p53/HLA-A2 expressing tumor cells	I, II	[15]
	HS-410	Immune Activator along with BCG	I, II	[16]
	ALT-803	Immune activator through IL-15	I, II	[17]

Table 1.2 Continued

Target Pathway	Drug	Mechanism/Target	Trial Phase	Reference
Immunotherapy (continued)	Ipilimumab	CTLA-4 antibody	II	[18]
	MEDI4736	PDL1 antibody antagonist	I	[19]
	Tremelimumab	CTLA-4 antibody Downregulation of T-reg cells	I	[20]
	AGS15E	Slitrk6 targeting immunotherapy	I	[21]
	MK-3745 (Pembrolizumab)	programmed death ligand 1 (PDL1)	I, II	[22]
	Ad/HER2/Neu Vaccine	Vaccination/Immune Activation	I	[23]
	SAR566658	anti-CA6-DM4 immunotherapy	I	[24]
	Lenalidomide	immonumodulation	I	[25]
	MPDL3280A	Anti-PDL1 immunotherapy	II	[26]

Table 1.2 Continued

Target Pathway	Drug	Mechanism/Target	Trial Phase	Reference
Mitosis/Cell Cycle	Eribulin Mesylate	Microtubule Formation/ Mitosis	I, II	[27]
	Abraxane	Protein bound Paclitaxel- Mitosis	I,II	[28]
	Tesetaxel	Tubulin stabilaztion - anti- mitotic	II	[29]
	ASG-22CE	Inhibition of tubulin formation in cancer cells by targeting cells expressing adhesion molecule nectin-4 with monomethyl auristatin E	I	[30]
	Amrubicin	Anthracycline targeting Topoisomerase-II	II	[31]
	Gemcitabine	Nucleoside analog targetting S phase	III	[32]

Table 1.2 Continued

Target Pathway	Drug	Mechanism/Target	Trial Phase	Reference
Epigenetic Changes	5-Fluoro-2-Deoxycytidine With Tetrahydrouridine	Inhibition of DNA Methylation/ Cytodine deamination	II	[33]
	Romidepsin	HDAC inhibitor	I	[34]
Other possible targets	BBI608	Cancer Cell Stemness	I, II	[35]
	Ganetespib	Inhibition of HSP90	I	[36]
	OGX-427	HSP27 inhibitor	II	[37]
	Veliparib	PARP inhibitor	I	[38]

Notes: In several studies, mentioned drugs are used in combination with other drugs as part of the traditional protocols for treatment of bladder TCC. This table does not represent all clinical trials sponsored by NCI, but a selected subset of trials with relevance to this review article.

CHAPTER II

PHOSPHATIDYLINOSITOL 3-KINASE INHIBITOR INDUCES CHEMOSENSITIVITY TO A NOVEL DERIVATIVE OF DOXORUBICIN, AD198 CHEMOTHERAPY IN HUMAN BLADDER CANCER CELLS *IN VITRO*

Research article described in this chapter is a slightly modified version of an article that was published in *BMC Cancer* 2015 by Dmitriy Smolensky, Kusum Rathore and Maria Cekanova.

Phosphatidylinositol 3-kinase inhibitor induces chemo-sensitivity to a novel derivative of doxorubicin, AD198, chemotherapy in human bladder cancer cells.

Dmitriy Smolensky, Kusum Rathore, and Maria Cekanova.

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In this paper, “our” or “we” refers to me and my co-authors. My contribution to the manuscript includes: 1) Compiling and interpretation of literature 2) Providing comprehensive structure to the paper 3) Preparation of graphs and figures 4) Writing and editing 5) Performing the experiments 6) Interpretation of results

Phosphatidylinositol 3-kinase inhibitor induces chemosensitivity to a novel derivative of doxorubicin, AD198 chemotherapy in human bladder cancer cells *in vitro*

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Abstract

Background: Doxorubicin (Dox) is widely used to treat progressed bladder cancer after transurethral resection. The Dox chemotherapy has been limited due to induced drug resistance and cumulative cardio-toxic effects. N-benzyladriamycin-14-valerate (AD198), a novel derivative of Dox, has a potential to become a more effective treatment than Dox by overcoming drug resistance and cardio-toxicity as shown in the rodent model of lymphoma. The purpose of this study was to compare the efficacy of Dox and AD198 on human bladder cancer and explore their mechanisms in inhibition of the human bladder cancer cells *in vitro*.

Methods: *In vitro* experiments were performed using human transitional cell carcinoma (TCC) cell lines, T24 and UMUC3. The effects of Dox and AD198 on cell viability of T24 and UMUC3 were analyzed by MTS assay. The effects of Dox and AD198 on cell apoptosis were determined by caspase 3/7 assay, generation of reactive oxygen species (ROS), and Western Blotting (WB) analysis.

Results: We found that AD198 was more effective than Dox in inhibition of cell viability of T24 and UMUC3 cells in dose-dependent manner. Both Dox and AD198 significantly increased the generation of ROS and induced apoptosis through caspase 3/7 activities in tested human TCC cells. AD 198 induced significantly higher production of ROS in dose-dependent manner as compared to Dox at same concentrations in human TCC cells. Dox and AD198 activated the pro-apoptotic p38 MAPK pathway; however, also increased phosphorylation of AKT, an anti-apoptotic signaling pathway in T24 and UMUC3 cells. Combined treatment of PI3K inhibitor, LY294002, with Dox or AD198 inhibited cell viability more effectively than any of drug treatments alone.

Conclusions: These data suggest that AD198 as novel derivative of Dox may be a valuable treatment option for human bladder cancer. Dox- and AD198-induced AKT phosphorylation that is an indicator of pro-survival and drug resistance mechanisms of chemotherapies in bladder cancer. Combined therapy of Dox or AD198 with inhibitors of PI3K/AKT pathway might lead to more effective treatment outcome for patients diagnosed with bladder cancer.

Key words: doxorubicin, AD198, bladder cancer, pro-survival signaling pathways

Background

Bladder cancer is the 6th most common cancer in the United States, with high rates of recurrence [39, 40]. While the exact reasons are unknown, bladder cancer presents itself four times more in males than females [39, 41]. Urothelial cancer, also known as transitional cell carcinoma (TCC), is the most common type of bladder cancer in the western world and accounts for over 90 % of all bladder cancer cases [40, 42]. The 5-year survival rates for patients diagnosed with the earlier stages of the bladder cancer are 69.2 %. However; the 5-year survival rates for patients diagnosed with invasive bladder cancer at stage IV are only 5.5 % [40]. The biggest challenges in treatment of bladder cancer are the high rates of reoccurrence and progression from non-invasive to invasive stages of bladder cancer. The invasion of bladder cancer into the muscle layer of the bladder serves as major prognostic marker for the development of the treatment plan [41]. Tobacco products have been determined to be the highest environmental risk factor for developing bladder cancer [40]. Other environmental risk

factors for bladder cancer include occupational exposure and chemotherapy drugs, while non-environmental risk factors include age, gender, race, obesity and family history [39, 40, 43].

Superficial bladder cancer is well managed by transurethral resection (TUR), followed by an intravenous or intravesical (directly into the bladder) administrations of chemotherapeutic treatment, such as mitomycin, epirubicin or doxorubicin (Dox) [44-46]. The intravesical injection of bacillus Calmette-Guérin (BCG), as adjuvant immunotherapy, activates immune system in the patient and greatly increases progression free survival rates [46, 47]. The management treatment for patients with muscle invasive bladder cancer is usually a radical cystectomy (removal of whole bladder) mostly followed by adjuvant therapy, such as chemotherapy and radiation therapy [41]. Chemotherapy protocols without radiation include: cisplatin alone, or cisplatin with 5-flourouracil or mitomycin with 5-flourouracil [48]. Chemotherapy protocols in conjunction with radiation include: gemcitabine with cisplatin, the MVAC protocol - methotrexate, vinblastine, Dox (Adriamycin), cisplatin or combination of carboplatin with either paclitaxel or docetaxel [48].

Dox is an anthracycline antibiotics and is one of the most widely used anti-cancer drugs [47, 49]. Dox interacts with topoisomerase II (TOPOII) [50-52] and induces apoptosis through production of reactive oxygen species (ROS) and by inducing DNA damage in bladder cells [53]. Dox induces ROS production through p53-dependent and p53-independent mechanisms [54, 55]. However, other mechanisms of Dox action remain unclear. While Dox has been successful in treating patients diagnosed with different cancers, long term use of Dox has two major setbacks. Firstly, Dox induces drug resistance through the upregulation of the multi-drug resistance 1 (*MDR1*) gene, also known as p-glycoprotein in the cell [56, 57]. Secondly, long term use of Dox has been linked to acute cardiotoxicity [58].

N-benzyladriamycin-14-valerate (AD198), one of the derivatives of Dox, which shows improvement in cardiotoxicity as compared to Dox [59]. The addition of an N-benzyl ring improves the lipophilic properties of AD198 and allowing rapid localization of AD198 in the cytoplasm of cells [59]. The structural similarity of a moiety of the AD198 molecule to diacylglycerol (DAG) allows AD198 to interact with the regulatory subunit of PKC-δ by releasing the catalytic subunit [60]. The released PKC-δ catalytic subunit attributes to its cytotoxic effects by inducing mitochondrial membrane depolarization and inducing ROS production [60]. In cardiomyocytes, AD198 activates PKC-ε, which attributes to cardio-protective effects by Dox-induced ROS production [61]. AD198 has been shown to be effective in inhibition of cell growth of mouse lymphoma and multiple-myeloma models [62]. In addition, AD198 is more effective in inhibition of cell proliferation and inducing apoptosis in canine TCC and osteosarcoma primary cell lines than Dox through the p38 MAPK signaling pathway [63]. Cardio-toxicity, which is a major concern for patients receiving Dox treatment, has not been detected when rats were treated with AD198 [64]. In rats, low dose administration of AD198 after Dox-induced cardiotoxicity, attenuated markers of cardiotoxicity, when compared to Dox alone [65]. This cardio-protective property of AD198 has been attributed to activation of PKC-ε, while PKC-ε knockout mice did not benefit from cardio protective effects of AD198 [61].

AD198 has not been evaluated for its potential use in bladder cancer treatment. So in this study, we evaluated the efficacy and mechanisms of Dox and its derivative, AD198 on cell proliferation and apoptosis in human UMUC3 and T24 TCC cell lines *in vitro*.

Methods

Reagents and Antibodies - Unless otherwise stated, all reagents and media were purchased from Fisher Scientific (Pittsburgh, PA.) Dox and LY294002 were purchased from Sigma-Aldrich,

St. Louis, MO. N-benzyladriamycin-14-valerate (AD198) was a kind gift from Dr. Leonard Lothstein [59]. The following antibodies were purchased from Santa Cruz Biotechechnology, (Santa Cruz, CA): Actin-HRP, p-ERK1/2, ERK 1/2, AKT, p38. The following antibodies were purchased from Cell Signaling (Boston, MA): PARP, p-AKT (serine 473 and threonine 308), p-GSK3β, p-p38.

Cell Culture – Human transitional cell carcinoma (T24 and UMUC3) cell lines were purchased from ATCC (Manassas, VA). The cells were grown in the following media: T24 in McCoy's media, UMUC3 in MEM media containing 10% FBS and penicillin/streptomycin mixture (Fisher Scientific, Pittsburgh, PA) in 37^{o}C and 5% CO_2.

Proliferation Assay - Cells were plated in 96-well plates at 5 x 10^3 cells per well and allowed to attach for 24 hrs. After seeding, cells were treated with AD198 or Dox in dose-dependent manner in complete media for additional 48h. DMSO was used as control. For treatment with PI3K inhibitor, LY294002, the cells were pretreated with 20μM LY294002 for 30 min prior to stated drug treatment and 20μM LY294002 was maintained for the rest of the 48 hour treatment. After treatment, the proliferation of cells was measured using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer's protocol. Briefly, 20 μL of the MTS reagent was added to each well and allowed to incubate at 37˚C for 1 h. Absorbance was measured at 490nM using a plate reader (Bio-Tek instruments, Winooski, VT). The treatment data were normalized to the DMSO control.

Reactive Oxygen Species (ROS) Assay by Flow Cytometry - For reactive oxygen species assay: The cells were incubated with 5 μM dihydrogen-dichlorodihydro-fluorescein-diacetate

(H_2DCF-DA) (Life Technologies, Grand Island, NY) for 1 hr. The cells were then washed with twice PBS and trypsinized. The trypsin was neutralized and the collected cells were centrifuged at 5,000 rpm for 5 min. The cell pellet was resuspended in 1 mL of PBS and the fluorescence was measured at 485 nM excitation and 530 nM emission using flow cytometer (BD Accuri® BD Sciences, San Jose, CA). The treatment results were normalized to the DMSO control.

Caspase-3/7 Assay - Cells were plated in 6-well plates at 5 x 10^5 cells per well. After 24 h, cells were treated with AD198 or Dox for 24 h. After treatment, cells were washed twice with PBS, and cell lysates were harvested using RIPA buffer. Protein concentration was measured using Bradford BCA assay. Forty micrograms of proteins were used for detection of caspases 3/7 following the Caspase Glo® 3/7 Substrate protocol (Promega). After 1 h incubation with reagents, the luminescence was measured using FLx800 plate reader (Bio-Tek instruments, Winooski, VT). The treatment data was normalized to the DMSO control.

Western Blot - Cells were plated at 1.5 X 10^6 cells per 10-cm plate. Twenty four hours after plating, the cells were treated with drugs for 24 hrs in dose-depend manner. For treatment with PI3K inhibitor, LY294002, the cells were pretreated with 20 μM LY294002 for 30 min prior to stated drug treatment and 20 μM LY294002 was maintained for the rest of the 24 hrs treatment. After treatment (unless otherwise stated), the cells were washed twice with PBS and lysed using cold RIPA buffer containing protease/phosphatase inhibitors. The cell lysates were kept at -80°C until further analysis. Protein concentration was measured using the BCA protein assay. Equal amount of proteins (60 μg) were loaded onto SDS-PAGE gels and transferred to a nitrocellulose membrane. Primary antibodies were hybridized according to the manufacturer's instructions overnight at 4°C. The secondary antibodies were hybridized for 1 hr at room temperature and

the immunoreactive bands were visualized using enhanced chemiluminescence system (Fisher) and acquired on ImageQuant LAS4000 (GE life sciences, Pittsburgh, PA.) The densitometry analysis were performed using ImageJ (NIH).

Statistical analysis - Statistics were analyzed using paired Student *t* test to established significance. Results were considered statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ when compared treatments to control group and $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ when compared Dox to AD198 at same doses, or when compared Dox to LY+Dox or AD198 to LY+AD198.

Results

DOX and AD198 inhibited cell viability of human TCC cells

Human TCC cell lines, T24 and UMUC3 were treated with 0.1, 0.5, 1, and 5 μM of Dox and AD198 for 48 hrs, as shown in Figure 2.1. Both, Dox and AD198, significantly reduced the proliferation of T24 (Figure 2.1a) and UMUC3 (Figure 2.1b) cells in dose-dependent manner. AD198 was significantly more effective in inhibition of cell viability of both T24 and UMUC3 cells as compared to Dox at the dose of 0.1 and 5 μM.

Dox and AD198 induced ROS production in human TCC cells

The effects of Dox and AD198 on generation of cellular ROS was evaluated using DCF assay. Dox and AD198 both significantly increased ROS in T24 and UMUC3 cells after 24 hrs treatment; and in addition, AD198 showed significantly higher activation of ROS production as

compared to DOX with 3-fold vs 2-fold increase in T24 cells and 6-fold vs 3-fold increase in UMUC3; respectively (*$p \leq 0.05$ in T24 and **$p \leq 0.01$ in UMUC3) as shown in Figure 2.2.

Dox and AD198 induced apoptosis in human TCC cells through activation of caspase cascade

The effects of Dox and AD198 on cell apoptosis were evaluated using the caspase-3/7 activities assay. Dox and AD198 both increased apoptosis in T24 and UMUC3 cells; however, Dox showed significantly higher caspase activation than AD198 in both TCC cell lines ($^{\#\#}p \leq 0.01$ in T24 and $^{\#\#\#}p \leq 0.001$ in UMUC3) as shown in Figure 2.3a.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in genomic stability and are downstream targets, which are cleaved by caspases to produce 89 and 24 kD fragments *[66, 67]*. The presence of degraded PARP is generally considered as a marker of apoptosis *[66]*. Dox and AD198 (1 μM) treatments increased a cleavage of PARP in both tested cells as confirmed by WB analysis (Figure 2.3b). Densitometry values of cleaved PARP protein after DOX and AD198 treatments were normalized to actin and then to control group as shown in Figure 2.3c. A statistically significant increase in PARP cleavage (***$p \leq 0.001$) by 15- and 12-fold was observed in Dox as compared to control treatment. Also Dox significantly increased PARP cleavage by 3- and 4-fold ($^{\#}p \leq 0.05$) as compared to AD198 treatment in T24 and UMUC3 cells, respectively (Figure 2.3c).

Dox and AD198 activated AKT signaling pathway in human TCC cells

To better understand the mechanisms of AD198 and Dox-induced cell growth inhibition in T24 and UMUC3 cells, we investigated role of MAPK signaling pathways. Dox increased the

phosphorylation of AKT protein at both Ser473 and Thr308 sites in T24 and UMUC3 cells in time- and dose-dependent manner (Figure 2.4). GSK-3β is a critical downstream element of the PI3K/AKT cell survival pathway, and when phosphorylated, its pro-apoptotic function is attenuated by AKT [68]. Dox increased phosphorylation of GSK-3β in dose-dependent manner as shown in Figure 2.4a. There was no significant increase in the phosphorylation of ERK in neither TCC cells when treated with Dox or AD198 (Figure 2.4a). MAPK p38 has been shown to be activated by ROS and plays a vital role in apoptosis [69, 70]. Both Dox and AD198 increased phosphorylation of MAPK p38 in a time-dependent manner with increased activation at 1-3 hrs after treatment in T24 cells (Figure 2.4b). In UMUC3 cells, the activation of p38 is was seen to be higher with AD198 than with Dox treatment, confirming previous results of higher ROS production by AD198 (Figure 2.4b).

Inhibition of PI3K/AKT signaling pathway sensitizing the cytotoxic effects of Dox and AD198 in human TCC cells

Dox and AD198 activated AKT pro-survival signaling pathway that is an indicator of resistance of cells to chemotherapy. To confirm our hypothesis, we tested the effects of PI3K inhibitor, LY294002, in combination with Dox or AD198 on cell growth of TCC cells. Co-treatment with LY294002, increased the anti-proliferative effects of both Dox and AD198 in T24 and UMUC3 cells. The combination of Dox and LY294002 suppressed most effectively cell viability of both cell lines (Figure 2.5a). In order to further investigate the PI3K/AKT inhibitor's chemosensitizing effect to Dox and AD198 chemotherapy, we measured caspase-3/7 activities and PARP cleavage. Indeed, co-treatment of Dox and AD198 with PI3K inhibitor, LY294002, increased caspase-3/7 activation and PARP cleavage in both T24 and UMUC3 cells as shown

in Figure 2.5b and Figure 2.5c. LY294002 inhibited the AD198- and Dox-induced phosphorylation of AKT at Thr308 and Ser473 sites as shown in Figure 2.5d. In addition, higher levels of active (unphosphorylated) GSK3β were present when T24 and UMUC3 cells were co-treated with Dox or AD198 and LY294002.

Discussion

Dox has been used to treat human bladder cancer for over three decades and continues to be one of the most common chemotherapeutic agent [71]. Dox is not as effective alone as it is in combination with other drugs; however, Dox increases side effects and decreases completion of regimen due to intolerances by the patients [72]. Another setback of Dox in the treatment of bladder cancer is the development of drug resistance by up-regulation of p-glycoprotein efflux transporter protein expressions. The established Dox-resistant bladder cancer cell lines, KK47/ADM, shows that complete reversal of resistance was not possible even when Dox was used in combination with a sensitizing agent, verapamil [73]. The development of novel derivatives of Dox may overcome those Dox adverse events, and even exceed its anti-cancer effects [74].

A novel derivative of Dox, AD198 has been developed by Dr. Lothstein's group [59]. AD198 is a highly lipophilic drug, which rapidly localizes to the cellular cytoplasm and it has been shown to circumvent efflux transport by p-glycoprotein in Dox-resistant macrophage cells [59, 75]. AD198 has been shown to overcome Bcr-Abl pro-survival signaling pathway in human leukemia cells through the activation of ERK1/2 and STAT-5 followed by cytochrome C release and apoptosis [76]. Breast and ovarian cancer cell lines, which are resistant to Dox due to p-glycoprotein expression, have been shown to rapidly accumulate AD198 in the cytoplasm. The

efficacy of AD198 to inhibit cell growth is comparable to Dox treatment in non-resistant cells [77]. Our results are in agreement with this study and confirmed that AD198 anti-proliferative effect was similar and compared to Dox in T24 and UMUC3 cells. This might be relevant for the intravesicular treatment of bladder cancer, where a high dose of the Dox is used (~200 mM) [46].

It has been shown that AD198 and Dox have a similar effect in generating ROS in murine cardiomyocytes [61]. However; ROS production was induced by AD198 more than by Dox in both tested human bladder cell lines (Fig. 2.2). In addition to ROS production in the cytoplasm, Dox induces DNA damage via Topoisomerase II, while AD198 mainly functions in the cytoplasm by increasing ROS and activating PKC-δ [59]. In mouse myeloid cells, AD198 induces apoptosis through activation of PKC-δ and is not effected by the expression of Bcl-2 [60]. AD198 acts through PKC-δ-independent manner in TRAF-3 deficient mouse B-lymphoma cells through the suppression of oncogenic protein c-Myc [62]. AD198 might be beneficial for treatment of c-Myc overexpressing cancer cells. While AD198 had an equal or greater anti-proliferative and ROS generating effects than Dox in TCC, it showed significantly less caspase activation and PARP cleavage in both T24 and UMUC3 (Fig. 2.3a-c). It has been previously shown that AD198 induces cytochrome-C release and initiate mitochondrial-activated apoptosis, even when caspase activation is blocked by a pan-caspase inhibitor, Z-VAD-FMK [60]. In contrast, Dox has been shown to function in a caspase-dependent manner in T-leukemia cells and apoptosis was inhibited when Jurkat cells were treated with Z-VAD-FMK [78]. Dox has been shown to induce apoptosis in ROS-independent manner in cardiomyocytes [79]. Our data provides evidence that AD198 induced apoptosis in caspase-dependent and -independent pathways. Dox, on the other hand, induce apoptosis primarily through caspase-dependent pathway.

To further investigate the differences in mechanisms between Dox and AD198 action in TCC cells, we investigated PI3K/AKT and MAPK signaling pathways. AKT has been shown to increase drug resistance in other cancers and PI3K itself can contribute to expression of multidrug resistance protein 1 (MDR1) to induce drug resistance [68,80,81]. Dox activates the PI3K/AKT pathway in several cancers including ovarian, hepatic and breast cancer cells [82-84]. Activation of AKT by Dox has been linked to the presence of human epidermal growth factor receptor 3 (HER3, ERBB3) in ovarian cancer and was attenuated by the addition of tyrosine kinase inhibitors lapatinib and/or erlotinib [82]. In our study, Dox more efficiently phosphorylated AKT (Ser473 and Thr308) and its downstream target GSK3β than AD198 in dose- and time-dependent manner (Fig. 2.4a and 2.4b). PI3K/AKT pathway has been greatly implicated in the progression and prognosis in bladder cancer patients [85]. The growth factor receptors including ERBB2, ERBB3 and EGFR have been found to be altered or amplified in bladder cancer and have the potential to activate PI3K/AKT signaling pathway [86]. PI3K mutation is inversely associated with later stages, indicating that mutation of PI3K is not crucial to bladder cancer progression [87, 88]. AKT after activation by PIP3, has a wide range of downstream targets including activation of anti-apoptotic targets such MDM2 and mTOR as well as deactivation of apoptotic targets such as BAD, GSK3β and TSC1 [89, 90]. It has been shown that inhibition of PI3K sensitizes TCC cells to Dox chemotherapy and lowers the IC50 of Dox when used in combination with LY294002 [91]. We confirmed that co-treatment of PI3K/AKT inhibitor with Dox or AD198 reduced cell proliferation more efficiently than Dox or AD198 treatments alone in tested human bladder cells (Fig.2.5a). Furthermore, co-treatment of LY294002 with either Dox or AD198 induced an activation of caspase-3/7 activity and cleavage of PARP than AD198 or Dox treatments alone (Fig. 2.5b and 2.5c). In T24 and UMUC3 cells, phosphorylation of GSK3β was decreased by co-treatments, indicating that its pro-apoptotic function was restored (Fig.

2.5d), while either Dox or AD198 alone increase phosphorylation of GSK3β indicating an anti-apoptotic resistance activated by AKT signaling. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) antagonizes the PI3K/AKT signaling pathway and mutation or decrease of *PTEN* expression has been shown to be as a poor prognostic marker in breast cancer patients [92]. The *Pten* gene therapy in mice increases tumor sensitivity to Dox therapy *in vivo* [93]. T24 cells have mutation in *PTEN* gene, but cells express PTEN protein, while intragenic deletion of *PTEN* gene in exons1-8 in UMUC3 cells results in no PTEN protein expression [94]. We have observed that phosphorylation of AKT levels were much higher in UMUC3 cells than T24 probably due to absence of PTEN (data not shown).

Conclusion

In conclusion, we have shown that AD198 has comparable anti-proliferative efficacy as Dox in tested human TCC cell lines *in vitro*. AD198 was more effective in induction of ROS production. AD198 induced apoptosis in caspase-dependent and -independent pathways. Dox, on the other hand, induced apoptosis primarily through caspase-dependent pathway. Both drugs activated PI3K/AKT signaling pathway, which may explain a common mechanisms of bladder cancer to acquire a drug resistance. The inhibition of the PI3K/AKT pathway plays an important role in increasing the effectiveness of Dox and AD198 treatments in human bladder cancer cells in vitro. AD198 a novel derivative of Dox, with no cardio-toxic effects as has been shown in mice *in vivo* model, may be a new candidate for the replacement of Dox treatment in bladder cancer. Further investigations using rodent animal model of bladder cancer *in vivo* are required to support these *in vitro* findings.

List of abbreviations

AD198: N-benzyladriamycin-14-valerate; AKT: V-akt murine thymoma viral oncogene homolog 1; BCG: bacillus calmette-guerin; Dox: Doxorubicin; ERBB: human epidermal growth factor receptor; ERK: extracellular signal regulated kinases; GSK3β: Glycogen synthase kinase 3 beta; H2DCF-DA: Dihydrogen-dichlorodihydro-fluorescein-diacetate; LY: LY294002; *MDR1:* multidrug resistance protein 1; MVAC: Methotrexate, vinblastine, Dox and cisplatin chemotherapy protocol; PARP: Poly (ADP-ribose) polymerase; *PI3K:* phosphatidylinositol-3-kinase; *PTEN:* phosphatase and tensin homolog; ROS: Reactive oxygen species; TCC: Transitional cell carcinoma; TOPOII: Topoisomerase II; TUR: Trans-urothelial resection.

The authors declare that they have no competing interests.

Author's contributions: DS has made substantial contributions to acquisition and analysis of data for in vitro assays; performed the statistical analysis;has been involved in drafting the manuscript; and has given a final approval of the version to be published. **KR** has made substantial contributions to acquisition and analysis of data for in vitro assays; has been involved in drafting the manuscript; and has given a final approval of the version to be published. **MC** made substantial contributions to conception and design of experiments, analysis and interpretation of data; has been involved in drafting the manuscript and revising it critically for important intellectual content; has given a final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Appendix

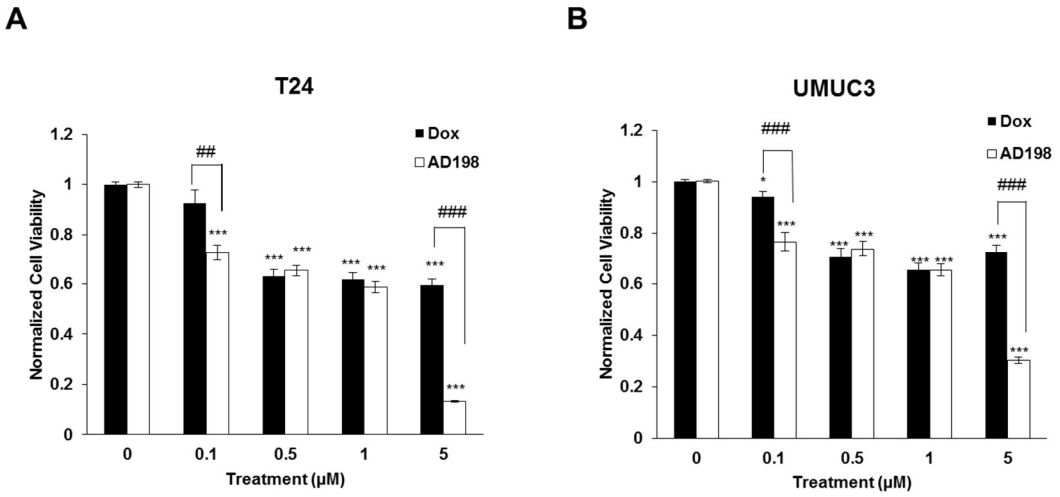


Figure 2.1 DOX and AD198 inhibited cell viability of human TCC cells.

(**a**) Human urinary bladder transitional cell carcinoma (TCC) cells T24 and (**b**) UMUC3 cells were treated with Dox (black bars) and AD198 (white bars) at 0.1, 0.5, 1 and 5 μM for 48 h and compared to control groups. Cell proliferation was determined by MTS assay and relative cell growth rate was normalized to control counterpart. Values represent mean ± S.E. of four replicates from three independent experiments. Paired Student *t*-tests were used to compare Dox and AD198 treatment to control; * $p \leq 0.05$ and *** $p \leq 0.001$. Paired Student *t*-tests were used to compare among Dox and AD198 group at the same dose treatment; $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001 \leq 0.001$ comparing Dox and AD198 treatment to control. Paired Student t test $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ comparing among Dox and AD198 group at the same dose treatment.

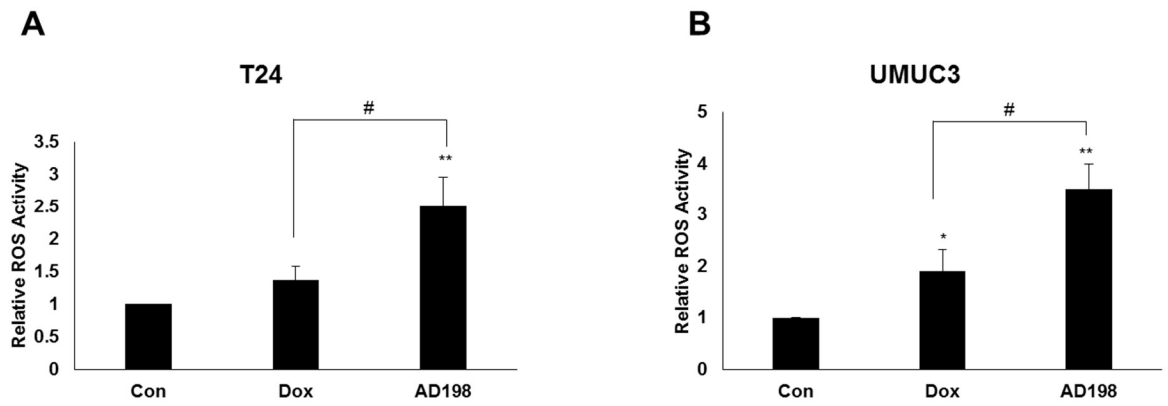


Figure 2.2: Dox and AD198 induced ROS in human TCC cells.

(**a**) T24 and (**b**) UMUC3 cells were treated with 1μM Dox and AD198 for 24 h and ROS levels were measured with dihydrogen-dichlorodihydro-fluoresceindiacetate assay; percent of ROS positive cells were measured and normalized to the control. Values represent mean ± S.E. of three independent experiments. Paired Student t- tests were used to compare Doxand AD198 treatments to controls, $*p \leq 0.05$, and $**p \leq 0.01$. Paired Student t-tests were used to compare among Dox and AD198 group at the same dose treatment; $^{\#}p \leq 0.05$.

Figure 2.3: Dox and AD198 induced apoptosis in human TCC cells.

Dox and AD198 induced apoptosis in human TCC cells. **(a)** T24 and UMUC3 cells were treated with 1 μM Dox and AD198 for 24 h and caspase-3/7 activities were measured using the Caspase-Glo 3/7 luminescence assay. Relative caspase-3/7 activities were normalized to control. Values represent mean ± SE of three independent experiments. Paired Student *t*-test were used to compare treatment to control $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Student *t*-tests were used to compare among Dox and AD198 treatments $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$. **(b)** T24 and UMUC3 cells were treated with 1 μM Dox and AD198 for 24 h. The expression of PARP (cleaved fragment) was evaluated by WB analysis. Actin was used as a loading control. **(c)** Densitometry evaluation of cleaved PARP protein bands from WB analysis was done using ImageJ software. Values represent mean ± S.E. of measured densitometry of each band from three independent experiments. Paired Student *t*-tests were used to compare controls to Dox and AD198 treatments, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Paired Student *t*- tests were used to compare Dox to AD198 treatment, $^{\#}p \leq 0.05$

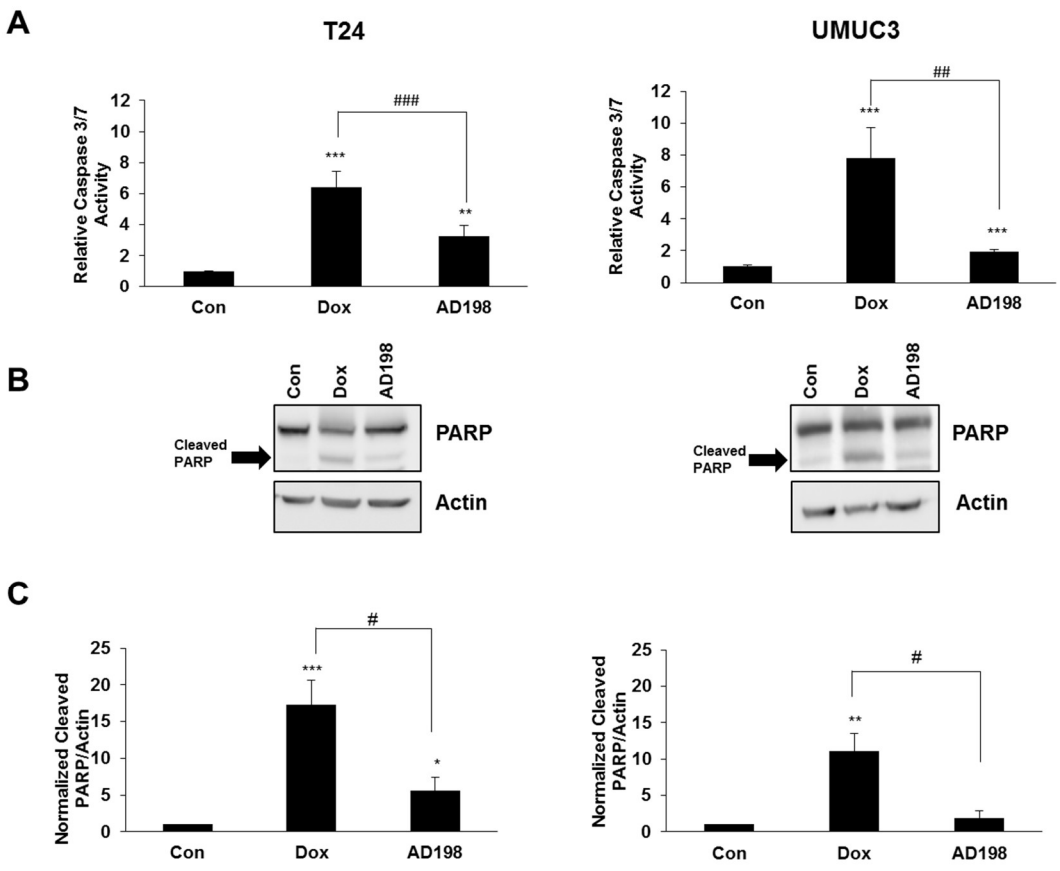


Figure 2.3 Continued

Figure 2.4: Dox activated AKT signaling pathway in human TCC cells in dose- and time-dependent manner.

(a) T24 and UMUC3 cells were treated with 0.1, 0.5 and 1 μM Dox and AD198 for 24 h. Protein levels of p-AKT (T308), p-AKT (S473), AKT, p-GSK-3β, p-ERK1/2 and ERK1/2 were detected by WB. Actin was used as a loading control. **(b)** T24 and UMUC3 cells were treated with 1 μM Dox and AD198 for 0, 0.5, 1, 3, 6 and 24 h. Protein levels of p-AKT (T308), p-AKT (S473), AKT, p-p38, p38, p-ERK1/2 and ERK1/2 were detected by WB. Actin was used as a loading control

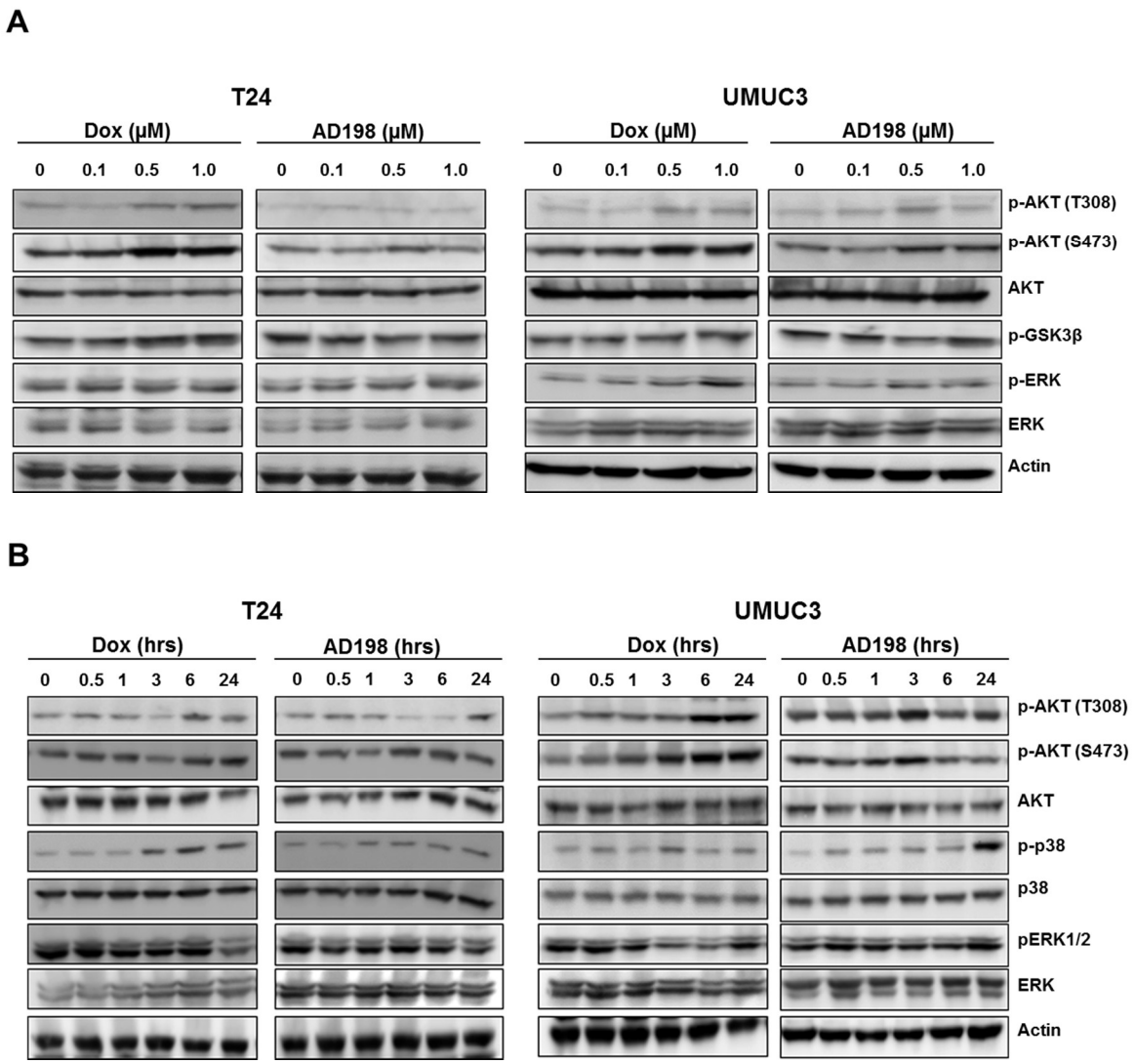


Figure 2.4 Continued

Figure 2.5: Inhibition of AKT signaling pathway sensitizing the cytotoxic effects of Dox and AD198 in human TCC cells.

(a) T24 and UMUC3 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (LY, 20 μM) for 48 h and compared to control groups. Cell proliferation was determined by MTS assay and relative cell growth rate was normalized to control counterpart. Values represent mean ± SE of four replicates from three independent experiments. Paired Student *t*-tests were used to compare DOX and AD198 treatments to control; ***$p \leq 0.001$. Paired Student *t*-tests were used to compare Dox to Dox + Ly and AD198 to AD198 + LY treatment, $^{###}$ $p \leq 0.001$. **(b)** T24 and UMUC3 cells were treated with DOX and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h and caspase activities were measured using the Caspase-Glo 3/7 luminescence assay. Relative caspase activities were normalized to control. Values represent mean ± SE of three independent experiments. Paired Student *t*-tests were used to compare treatment to control **$p \leq 0.01$, ***$p \leq 0.001$. Paired Student *t*-tests were used to compare Dox to Dox + Ly and AD198 to AD198 + LY treatments, $^{###}$ $p \leq 0.001$. **(c)** T24 and UMUC3 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h. The expression of PARP (cleaved fragment) were evaluated by WB analysis. Actin was used as a loading control. Densitometry evaluation of PARP protein bands from WB analysis was done using ImageJ software. Values represent mean ± S.E. of measured densitometry of each protein's band from three independent experiments. Paired Student *t*-tests were used to compare controls to Dox and AD198 treatments, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. Paired Student *t*-tests were used to compare Dox to AD198 treatment, $^{#}$ $p \leq 0.05$, $^{##}$ $p \leq 0.05$, and $^{###}$ $p \leq 0.001$ **(d)** T24 and UMUC3 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h. The expression of p-AKT (T308), p-AKT (S473), AKT and p-GSK-

3β proteins were evaluated by WB analysis. Actin was used as a loading control. Densitometry evaluation of p-AKT (T308), p-AKT (S473) protein bands from WB analysis was done using ImageJ software. Values represent mean ± S.E. of measured densitometry of each band from three independent experiments. Paired Student *t*-tests were used to compare controls to Dox and AD198 treatments, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Paired Student *t*-tests were used to compare Dox to Dox + LY or AD198 to AD198 + LY treatments, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$

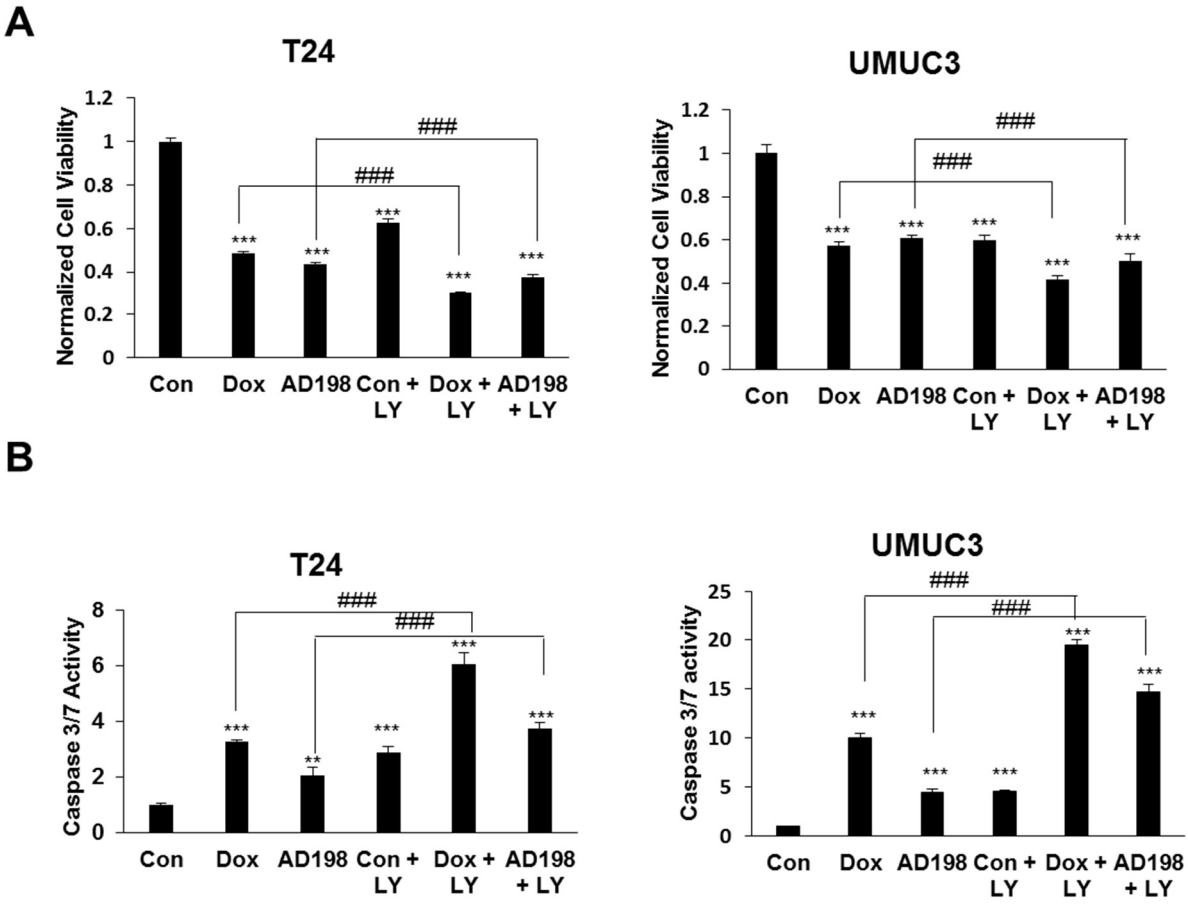


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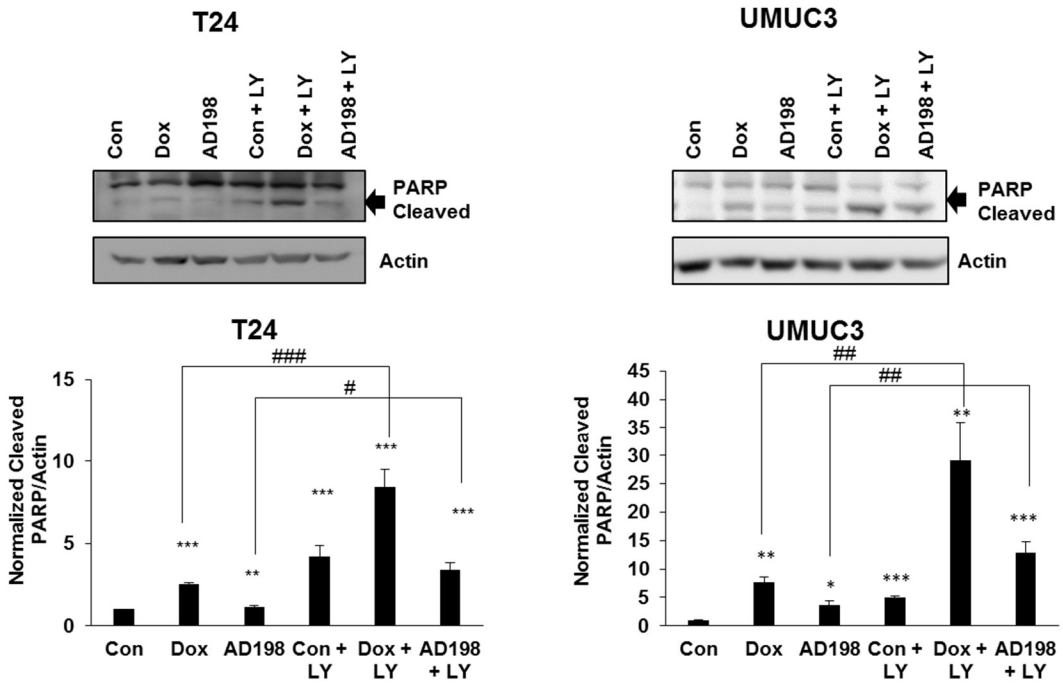


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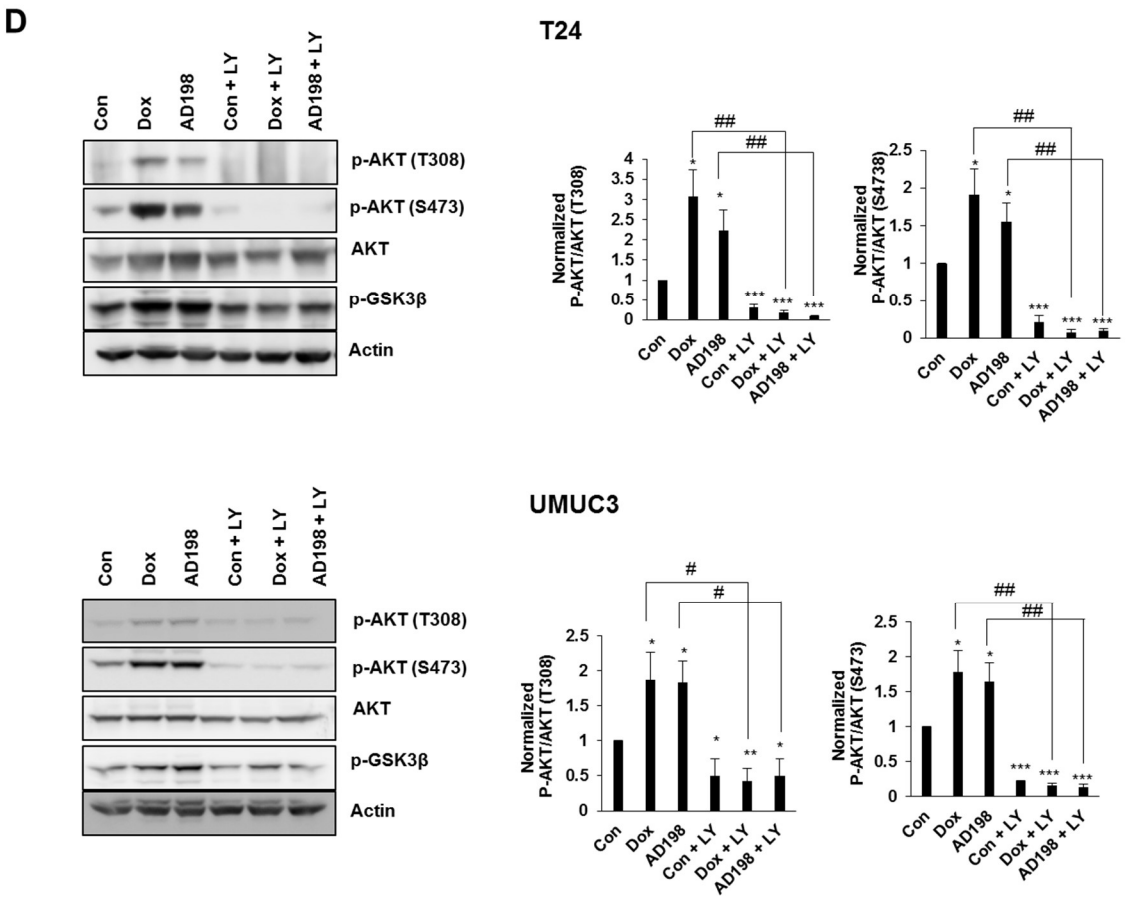


Figure 2.5 Continued

CHAPTER III

INTRODUCTION TO ORAL CANCER TARGETED THERAPY

Background

In 2015, an estimated 39,500 people were diagnosed with oral cancers resulting in an estimated 7,500 deaths [1, 2]. About 1.1% of men and women in the US will be diagnosed with oral and/or pharyngeal cancer within their lifetime [3]. When compared to other cancers, cancers of the oral cavity and pharynx have a relatively low five-year survival rate of 63.2% across all stages [3]. The five-year survival rate in the later stages can be as low as 20% for oral cancers [4]. Approximately 31% of oral cancers are diagnosed in the earlier stages prior to lymph node metastasis, while 69% are diagnosed after lymph node metastasis [3]. While some progress in the treatment of oral cancer has been made – shown by the increase of the five-year survival rate from 52.7% in 1975 to currently 66.3% – there is vast room for improvement [3].

Oral squamous cell carcinoma (OSCC) comprises 90% of oral malignancies and arises from the epithelial layer of cells lining the oral cavity. Other cancers found in the oral cavity include: salivary gland cancer, lymphoma, and melanoma [5]. Because OSCC comprises 90% of all oral malignancies, this review will focus primarily on OSCC as well as head and neck squamous cell carcinoma (HNSCC), a subset of OSCC. Firstly, this review will cover the risk factors, diagnosis, staging, and conventional treatment options for OSCC. Secondly, this review will look at the molecular mechanisms behind OSCC and the potential drug targets these mechanisms present along with current drugs being researched for targeted OSCC therapy.

Risk Factors

Risk factors for developing OSCC include, but are not limited to: tobacco use, consumption of alcohol, exposure to oral human papillomavirus (HPV), age, and genetic

disorders including Fanconi anemia and Dyskeratosis congenital [6]. In the United States the primary cause of OSCC is tobacco use followed by exposure to HPV.

Consumption of any tobacco products, which include smoking, chewing and snuff, has been shown to increase the incidence of oral cancer by 85% (in current tobacco users) when compared to people who have never smoked [7]. The risk of developing oral cancer increases with the length of tobacco use in years and total consumption of tobacco over a lifetime. People who have consumed tobacco products for over 45 years have a 93% chance increase to develop oral cancer compared to non-smokers [7]. On the other hand, people who have used tobacco for less than 30 years only have a 13% chance increase of developing oral cancer [7]. The consumption of alcohol, when used with tobacco, greatly increases the risk of developing oral cancer. One meta-analysis study found that people who consumed 1-2 drinks per day and smoked had an increased incidence of oral cancer 184% compared to non-smokers/non-drinkers, while smokers who did not drink increased incidence of oral cancer 52% when compared to non-smokers/non-drinkers [8]. Consumption of 1-2 drinks a day did increase the incidence of oral cancer 44% compared to non-smokers/non-drinkers [8]. HPV increased the risk of developing oral cancer in the younger population [9]. The molecular mechanisms of carcinogenesis associated with oral cancer caused by tobacco and HPV have distinct differences and will be covered in greater detail later in this review.

There are observed gender differences in the incidence of oral cancer and a contributing factor may be that more males use tobacco products than females, putting males at higher risk of getting oral cancers [10].

Excessive exposure to UV radiation is associated with increased incidence of skin cancer in the lip region. Specifically, prolonged occupational exposure to UV light has been shown to increase the incidence of lip cancer in outside workers [11, 12].

Fanconi anemia is a rare genetic autosomal recessive disorder where one or more genes in the Fanconi anemia/BRCA (FA/BRCA) pathway are mutated. This renders DNA to be extremely sensitive to crosslinking agents and leads to genomic instability [13]. Previous studies have shown that individuals who have Fanconi anemia are 500% more likely to develop head and neck cancer than the general population. Unfortunately, for these patients, chemotherapy and radiation are not viable options due to a high risk of death [14, 15]. For patients with genetic disorders who do not tolerate chemotherapy or radiation well, targeted therapy may become a more reasonable option in the future. Another genetic disorder associated with increased oral cancer incidence is Dyskeratosis congenita, which is characterized by a mutation in one of the genes maintaining telomere maintenance and function. The disorder is associated with bone marrow failure and increased incidence of a wide variety of cancers, including oral cancer [16, 17].

Diagnosis

The proper diagnosis for oral cancer begins with a consultation with a health care professional who will review family history, risk factors, and conduct a visual examination for the detection of oral cancer. If oral cancer is detected in the visual examination, the exam is followed with further testing as confirmation. The highest standard for a definitive diagnosis of oral cancer is a biopsy followed by histological evaluation of the tissue by a pathologist [18]; however, the ability to detect earlier stages of cancer or pre-cancerous lesions are limited by this approach [19, 20].

Scalpel biopsy is the gold standard for diagnosis of oral cancer and must be performed prior to planning treatment. An incisional biopsy is performed in the center of the lesion with high sensitivity and specificity. The biopsy is performed with the proper dimensions and depth so as

to prevent seeding of the cancer into surrounding tissues or the blood. If proper dimensions are not achieved cancer cells are disseminated into the peripheral blood in about 16-20% of the patients, which may lead to distal seeding of cancer cells and metastasis [21, 22]. Following biopsy, immunohistochemistry (IHC) can be performed to further evaluate the cancer. For example, HPV positive cancers overexpress p16 protein [23]. Performing IHC for the p21 protein following biopsy is a useful tool in differentiating HPV positive from HPV negative oral cancers [24].

An alternative method to biopsy and histological examination of tumors is exfoliative cytology. In exfoliative cytology, suspected cancer cells are scraped off and evaluated for the presence of malignant cells [19]. Studies have reported the sensitivity and specificity of exfoliative biopsy to be 100% and 92.6% respectively; however, other studies have reported the sensitivity and specificity to be as low as 71% and 32% respectively [19]. Due to conflicting results and a lack of strong supporting evidence, exfoliative cytology tests would not be warranted to replace biopsies at this time.

In order to visually screen for cancer and to better visualize a tumor for biopsy, methods beyond conventional oral examinations have been used. Toluidine blue staining was used in a clinical settings to screen for potential cancer within the oral cavity by staining the tumor to create better margins for biopsies [20]. Toluidine blue is sensitive to cancers and a recent study found the sensitivity to be as high as 94%. On the other hand, toluidine blue is prone false positive results. The same study reported specificity at only 39% [25]. Due to these findings, toluidine blue is not widely used anymore.

Another visual detection method uses the reflective property of cancer cells stained with acetic acid. Bright light is then focused on the oral cavity, where normal mucosa appear blueish in color and cancer cells appear more white [19]. These devices have reported very high

sensitivity, as high as 100% in two recent studies using light reflective devices from two different companies [26, 27]. Similar to toluidine blue staining, these methods suffer from a high false-positive rate of 34.2-37.5% when compared to biopsies [26, 27].

Tissue fluorescence may be another viable detection method for the screening and detection of oral cancer. One attempt was made to use tissue fluorescence by Visually Enhanced Lesion Scope (VELScope), which used a wavelength of 400-460 to excite endogenous fluorophores in oral mucosa. Cancer cells have been reported to lose the fluorescent ability of normal cells and do not emit a green color upon excitation [28]. A recent study found VELScope to be 30% sensitive and 63% specific in detecting oral cancer lesions [28]. These recent findings suggest this technology may not be a useful tool in screening and detecting oral cancer due to the very high rate of false negative results, which would be detrimental in patients with early cancers. For this reason, VELScope was never employed in a clinical setting.

Another attractive detection method is the use of biomarkers found in saliva to detect signatures of cancer presence. Two emerging biomarkers for the detection of oral cancer in the saliva are interleukin-8 (IL-8) and subcutaneous adipose tissue (SAT). A recent analysis of 395 subjects found high levels of IL-8 and SAT across many biomarkers and had a combined predictive value ranging from 74% to 85% [29]. While these results are promising, more studies need to be conducted in order to better evaluate the efficacy of using IL-8 and SAT in detection of early versus late OSCC and further validation is warranted.

Staging

Staging of oral cancer is done by conventional TNM staging in order to determine the clinical stage 0-IVC (Table 3.1) which was proposed by the American Joint Committee on Cancer Staging (AJCC) [30]. In summary, the tumor is evaluated and assigned a T stage, N indicates lymphatic, and metastasis is indicated by M0 or M1 [30]. Complete definitions of the T, N, and M stages can be found in Table 3.2 [30]. Early stage cancers are classified as stage I and II (T1N0, T2N0). Locally advanced cancers are classified as stage IVA and IVB (T3N0 and T4N0). Locally and regionally advanced cancers are classified as stages IVA to IVC (T3N1, T3N2, T4N1, and T4N2). The survival rate of oral cancer is highly dependent on the location, the stage, and the cability to surgically remove the tumor. Five-year survival of oral cancer patients diagnosed in the earlier stages (I-III) ranges from 73% to 93%, but depending on location, five-year survival in the last stage of IVC ranges from 20% to 52% (see Table 3.3) [4] .

Current Treatments

Treatment of OSCC is highly dependent on the stage of the cancer, location of the tumor and the patient's comorbidity. Surgery is always performed when possible. Early stage cancers can be treated with surgery alone, but later stages require a combination of chemotherapy or radiation as well as surgery. Complete removal of cancer tissue can include radical neck dissection, where a large portion of neck tissue is removed in order to remove involved/suspected lymph nodes [31]. If surgery cannot be performed, a combination of radiation and chemotherapy treatments is typically carried out [32]. In the case of metastasis, chemotherapy is used to treat systematic disease [32]. For more information on surgery for head and neck cancers see the review by Carlson and Oreadi 2012 [33].

The most common first line chemotherapy option for OSCC in stages III-IVC is a platinum based chemotherapeutic agent (carboplatin or cisplatin) in combination with 5-flourouracil or a taxane (typically paclitaxel or docetaxel) [34]. Cetuximab, an antibody against epidermal growth factor receptor (EGFR), has recently been approved by the Food and Drug Administration (FDA) and has shown an improved median survival of patients diagnosed with OSCC from 7.4 to 10.1 months when combined with platinum-based chemotherapy versus platinum-based chemotherapy alone [35]. In another recent study, cetuximab conferred an advantage in progression free survival to patients overexpressing p16 but not EGFR, when combined with radiation and chemotherapy [36]. Overexpression of p16 is associated with HPV-positive (HPV+) OSCC and recent findings would indicate that HPV+ OSCC patients may be good candidates for cetuximab targeted therapy [23].

Targeted Therapy

In order to better understand and devise better targeted therapy for OSCC, there have been many developments in discovering the aberrant molecular mechanisms in OSCC. The following sections discuss the molecular targets activated in OSCC and drugs in current clinical trials, which target the aberrant mechanisms within OSCC. Most of the molecular drug targets currently being researched in NCI supported clinical trials covered in this review fall into three major categories: receptor tyrosine kinases (RTKs), PI3K/AKT/mTOR pathway, and immunotherapy targets (Table 3.4).

Receptor tyrosine kinases (RTKs)

Epidermal growth factor receptor (EGFR) has been a molecular target of interest in the case of OSCC, and is currently approved for use in targeted therapy for OSCC by the FDA [36,

37]. Expression of EGFR has been found to be altered in 15% of HPV-negative (HPV-) and in 6% of HPV+ OSCCs [38]. Another study showed EGFR is amplified in 16% of OSCC patients [39]. These findings indicate only a small subset of patients may benefit from EGFR inhibitors. Other RTKs may also be a beneficial drug target either in combination with chemotherapy or in combination with other RTK inhibitors. The hepatocyte growth factor (HGF) and its receptor, the transmembrane tyrosine kinase (cMET), have been shown to play a vital role in proliferation, invasion, and metastasis [40]. In OSCC cells *in vitro,* HGF increased invasion, but invasion was reduced by the inhibition of Phosphoinositide 3-kinase (PI3K), linking the two pathways together [41]. Ficlatuzumab, a monoclonal antibody targeted against HGF being studied in OSCC patients, has already shown some efficacy in lung adenocarcinoma and multiple myeloma in human trials [42-44]. The receptor of HGF, cMET, can be targeted with INC280, which is a small molecule inhibitor of cMET [45]. GSK2849330, an antibody targeting HER3, is also being studied in OSCC patients [46]. It has been shown HER3 plays an important role in drug resistance to anti-EGFR therapy, making HER3 an attractive drug target that can be targeted in combination with EGFR [47]. Afatinib is an inhibitor of both EGFR and HER2 [48]. A recent phase II trial in human head and neck squamous cell carcinoma patients showed that afatinib had an objective response rate of 16.1% while cetuxamib had an objective response rate of 6.5% ($p < 0.09$) [49].

PI3K/AKT/mTOR pathway

PI3K/AKT/mTOR pathway is one of the major pathways activated in a variety of cancers [50]. A simplified scheme version of the PI3K pathway action on cell survival is shown in Figure 3.1. PI3K can be activated by tyrosine kinase receptor signaling, RAS signaling, and through internal stress responses [50, 51]. PI3K is a kinase, which phosphorylates phosphatidylinositol

4,5-bisphosphate (PIP2) to form phosphatidylinositol (3,4,5)-trisphosphate. The protein PTEN that dephosphorylates PIP3 to form PIP2 antagonizes this action. AKT, also known as protein kinase B, is activated by PIP3 through PDK1 [50]. AKT is one of the central hubs of pro-survival signaling within cancer cells [52]. Some of the major targets of AKT include but are not limited to: mTOR, BAD, GLUT4, GSK3β and IKKα [50]. When AKT is activated, mTOR increases proteins and RNA synthesis, which in turn increases cell survival and proliferation [53, 54]. BAD is a pro-apoptotic member of the Bcl-2 family of proteins. Through phosphorylation by AKT, the function of BAD is inhibited allowing cancer cells to avoid apoptosis [55, 56]. GLUT4 is a glucose transporter that increases glucose uptake and has been shown to be upregulated in human cancers [57]. AKT induces the translocation of GLUT4 to the cell surface where it functions to increase glucose uptake and in turn increase cell proliferation through an increase in cellular metabolism and nutrient uptake [58]. GSK3β is a pro-apoptotic protein, which plays a major role in intrinsic apoptosis through downstream activation of caspase-2 and caspase-8 proteins [59, 60]. AKT has been shown to inhibit GSK3β through phosphorylation [59]. AKT phosphorylates IKKα, which in turn phosphorylates IκB and targets it to be degraded through ubiquitination. This allows nuclear factor kappa B to translocate to the nucleus and activate transcription of pro-survival and pro-inflammatory genes [61].

In OSCC, PI3K/AKT/mTOR signaling has been shown to be dysregulated. In OSCC, on the gene level, PI3K has been shown to be activated in 34% of HPV- patients and in 56% HPV+ patients either through amplification or mutation, while its antagonist PTEN has been shown to be dysregulated in 12% of HPV- patients and 6% of HPV+ patients either through mutation or deletion [38]. Reduced PTEN function has been shown to increase the incidence of head and neck cancer in the mouse model [62]. In human OSCC, PTEN was under expressed in 61% of tissue samples which correlated with the activation of AKT in 68.5% of tissue samples [63].

Aberrant PTEN expression was not correlated to gender, age, or race; however, low PTEN expression was well-correlated with the more advanced stages of OSCC (stages I,II vs stages II,IV; p= 0.002) and increased pAKT expression was correlated the later stages (p= 0.006) [63]. Inhibition of PTEN in OSCC cell lines decreased the expression of E-Cadherin, decreased cell adhesion and increased the epithelial to mesenchymal phenotype of the cells indicating the loss of PTEN may lead to a more invasive cancer [64]. Downstream of PI3K/AKT, the overexpression of mTOR increased the recurrence risk ratio of head and neck cancer in patients treated with surgery and radiation by 3.25 and decreased patient survival (p = 0.013 and p= 0.029 respectively) [65].

Due to the fact PI3K/AKT/mTOR pathway is highly activated in many OSCC patients it presents a potentially effective drug target. Buparlisib is an inhibitor of PI3K and is being studied for several cancers including OSCC, non-small cell lung carcinoma, advanced breast cancer, and glioblastomas [66-69]. Everolimus, currently studied for advanced OSCC, is an analogue of rapamycin and inhibits the formation of function mTOR complex 1 (mTORC1) and has already received FDA approval to treat pancreatic, renal and breast cancers [70, 71]. Another potent inhibitor of the PI3K/AKT/mTOR pathway is PF-05212384. PF-05212384 is a small molecule inhibitor against both PI3K and mTOR. In a recent clinical I phase study, PF-05212384 was shown to be well-tolerated by patients at 8mg daily dose and inhibited the activity of AKT, but in the same study PF-05212384 has not shown to provide a measurable antitumor response in any of the 23 patients [72]. Another clinical study showed treatment with PF-05212384 achieved stable disease survival in 12 of 47 patients [73].

Immunotherapy targets

In 2011, Robert Weinberg proposed cancer's avoidance of the immune system is a major emerging hallmark in the characteristics of cancer [74]. Immunologists have hypothesized there are three distinct stages of carcinogenesis and its escape from immune-surveillance. The first stage is elimination, where cancer cells are actively destroyed by the immune system through various mechanisms to be discussed later in this section. The second stage is equilibrium. In equilibrium the cancer is held at bay by the immune system but is not fully eliminated. It is during equilibrium where most immune-editing occurs and the tumor mass acquires new mechanisms to further evade the immune system. The third and final stage is immune-evasion, where the tumor cells acquire enough advantages to overcome the immune system, and in many cases use the immune system to promote a pro-carcinogenic environment [75, 76].

The immune system employs three major mechanisms to eliminate cancer cells from the body and protect it from clinical cancer:

1) The immune system is capable of detecting viral components and proteins, which result in eliminating virus-induced cancers before they start [75].

2) The immune system can resolve infections and eliminate pathogens before a pro-tumorigenic environment can form [75].

3) The immune system can detect and eliminate cancer cells through the recognition of new tumor associated antigens and through sensing cellular distress within the tumor environment [75].

A major strategy to inhibit cancer cells' immune response is to express inhibitory ligands on the surface of cancer cells and tumor associated cells, such as dendritic cells. Effector T-

cells function through a combination of inhibitory and activating signals. This checkpoint system is important to prevent autoimmune disease in peripheral tissues. Cancer cells take advantage of this check and balance system by expressing inhibitory ligands on their cellular surface, or through cytokines such as IL-10 and TGFβ. This can force other cells – such as dendritic cells – to express inhibitory ligands and limit T-Cell activation [77]. Two major checkpoint interactions have been the primary focus of immunologists and have provided viable drug targets, which are being validated in multiple clinical trials.

First, the CTLA4 ligand binds to CD80 or CD86 receptors. CD80 or CD86 are normally present on effector T-cells and interact with CD28 on antigen presenting cells. This interaction activates T-cells by providing signal 2 activation. CTLA4 binds to the same CD80 or CD86 receptors; instead of activating, the T-cell inhibits the activation through a signaling cascade. At the same time, CTLA4 has a higher affinity for CD80 or CD86 than CD28 and can easily displace CD28, inhibiting the activating signal. The tumor microenvironment, through cytokines on tumors, and actions of MDSCs can induce dendritic cells to express CTLA4, travel to the lymphoid organ, and inhibit T-cell activation [77-79].

Another ligand family, which has received a lot of attention in research as of late, is the programmed death ligand (PDL), including PDL1 and PDL2. Unlike CTLA4 which acts within the lymphoid organ, PDL1/2 are inhibitory ligands expressed directly on tumor cells, and inhibit T-cell function as well as T-cell proliferation within the tumor environment [80]. Unlike CTLA4, PDL1/2 are actually expressed on tumor cells, while programmed death receptor (PD1), is highly expressed on effector T-cells and NK cells [80]. Even more detrimental to tumor elimination, when Th1 CD4 helper cells interact with tumor cells through PDL1 to PD1 interaction, the Th1 cells convert to T-regs cells, further suppressing immune elimination within the tumor environment [81].

Perhaps the most successful immunotherapy class that has been developed to date is checkpoint inhibitors. While many checkpoint inhibitors are under development, CTLA4 inhibitors ipilimumab and tremelimumab have already been approved by the FDA for several cancers and are expected to become the favored therapy in the near future; meanwhile, PD1 inhibitor pembrolizumab has received FDA approval to treat melanoma and is expected to receive approval for several other cancers [77, 82, 83]. In a phase III trial, the CTLA4 antibody tremelimumab induced slightly longer overall survival, and had longer response duration than the standard chemotherapy regimen for high grade metastatic melanoma [84]. Autoimmunity is a major setback with CTLA4 inhibition. While autoimmunity can be managed with corticosteroids and the discontinuation of anti-body treatment, side effects can be severe and include endocrine damage, even death [85-87]. The reason CTLA4 inhibitors induce severe side effects is due to the function of these antibodies in the lymphoid organs. Because PD1 to PDL1/2 interaction happens in the peripheral tissue, PD1 inhibition does not cause severe side effects like CTLA4 inhibition. In a phase II trial, pembrolizumab was matched against chemotherapy (chosen by the provider) in refractory melanoma. Remarkably, the average 6-month survival rate of chemotherapy group was 16% and the average 6-month survival of the pembrolizumab group 32% and 36% (two different doses were used). At the same time, adverse effects were noticed in 11% and 14% of the pembrolizumab group while adverse effects in the chemotherapy group was much higher at 26% [88]. In advanced melanoma, pembrolizumab provides a longer relapse, exceeding 14 months for patients who responded to the therapy [89]. While these checkpoint inhibitors have shown tremendous results in other cancers, the results of clinical trials in OSCC remain to be seen. Avelumab is another PD-1 antibody under clinical investigation in OSCC [90].

Another potent strategy in targeting the immune response toward cancer is activation of the immune system. CD27 is a costimulatory receptor found on the surface of lymphocytes. When CD27 on the surface of T-cells binds to CD70 on the surface of antigen presenting cells it activates T-cell expansion, effector (and survival), and memory T-cells [91]. Varlilumab, an agonist antibody for the CD27 receptor, helps activate T-cells within the tumor microenvironment [92]. While most clinical studies have yet to be completed, in a recent study, varlilumab showed encouraging results by increasing the ratio of effector T-cells to T-regs within the tumor microenvironment when used in combination with a PDL1 antibody [93].

A third strategy for targeting cancer cells by immunotherapy is to present new antigens to immune cells that are only present on cancer cells. Adoptive T-cell transfer allows an injection of activated T-cells with engineered T-cell receptors (TCR) directly into a patient. Adoptive transfer of T-cells would be a very viable option if very specific tumor antigens are being targeted by these T-cells. In engineered TCRs, a gene coding for the TCR can be isolated from a patient, which has extremely high anti-tumor response or can be isolated *in vitro* after a specific tumor antigen screen screens for many potential T-cells [94, 95]. In the case of HPV+ cancers, the E6 viral protein expressed by HPV+ cells targets the tumor suppressor p53 protein [96]. E6 overexpression and p53 suppression has been found to be very common in OSCC cells [97]. Recently, a gene coding for the TCR against HLA-A*02:01-restricted epitope of HPV16 E6 was isolated from a stable disease undergoing patient and is currently being studied in adoptive T-cell transfer in other patients using their own T-cells [98].

Conclusion

While the five-year survival rates of OSCC remain relatively low, new targeted therapy is being developed for more hopeful outcomes. The potential success of targeted therapy in

treating OSCC has already been validated in the case of EGFR targeting cetuximab by providing increased survival to patients already receiving traditional platinum based regimens [99, 100]. The potential of new drugs and drug targets still remain to be seen. In the future, it will be important to move toward personalized targeted therapy. In the case of EGFR, only a small subset of patients (~16%) have altered EGFR levels [38]. While studies still need to determine if this small subset of cancer patients would benefit more from anti-EGFR therapy than others, it is important to conduct this research as soon as possible. As Schmitz et al. have noted, cancers are quick to develop resistance to primary therapies, and in the case of targeted therapies, a combination of targeted therapy may be more beneficial to the patient than single agent targeted therapy [99]. With many new drugs and therapies undergoing clinical trials, the future of OSCC patients is finally beginning to look brighter and more significant improvements in five-year survival may be developed within the next few years.

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Appendix

Table 3.1 Clinical Staging of Oral Cancer

Clinical Stage	Tumor Stage	Lymph Node Involvement	Metastasis
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	ANY	M0
	ANY	N3	M0

Table 3.1 Continued

Clinical Stage	Tumor Stage	Lymph Node Involvement	Metastasis
IVC	Any	Any	M1

Table adapted from AJCC Cancer Staging Manual, Sixth Edition (2002) [1].

Table 3.2 Oral Cancer Staging Definitions

Tumor	
Tis	Carcinoma *in situ*
T1	Tumor is 2cm or less in greatest dimension
T2	Tumor is greater than 2cm but less than 4cm in greatest dimension
T3	Tumor is more than 4cm and/or with extraparenchymal extension (salivary gland)
T4a	*Salivary Gland:* Tumor invaded skin, mandible, ear canal, and/or facial nerve
	Lip: Tumor invaded cortical bone, inferior alveolar nerve, floor on mouth, or skin of face
	Oral Cavity: Tumor invaded cortical bone, into deep muscle tissue of tongue, maxillary sinus, or skin of the face
T4b	Tumor has invaded skull base and/or pterygoid plates and/or encases carotid artery
Nodal Involvement	
N0	No regional lymph node metastasis present
N1	Metastasis is present in a single ipsilateral lymph node, 3cm or less in greatest dimension
N2a	Metastasis is present in a single ipsilateral lymph node, more than 3cm but less than 6cm in greatest dimension
N2b	Metastasis is present in multiple ipsilateral lymph nodes, more than 3cm by less than 6cm in greatest dimension
N2c	Metastasis is present in bilateral or contralateral lymph nodes, no more than 6cm in greatest dimension
N3	Metastasis is present in bilateral or contralateral lymph nodes, more than 6cm in the greatest dimension
Metastasis	
M0	No distant metastasis present
M1	Distant metastasis is present

Table adapted from AJCC Cancer Staging Manual, Sixth Edition (2002) [1].

Table 3.3 Oral Cancer Five Year Survival Rates by Stage and Location

	5 year Survival Rate by location		
Stage	Lip	Tongue	Floor of Mouth
0-III	93%	73%	75%
IVA-IVB	43%	63%	38%
IVC	52%	36%	20%

Table compiled based on information provided by American cancer society [2]

Table 3.4 Selected NCI sponsored trials in oropharyngeal cancer

Pathway Targeted	Drug	Target Mechanism	Trial Phase	Source(s)
Tyrosine Kinase Receptors	Ficlatuzumab	anti-hepatocyte growth factor antibody	I	[3, 4]
	GSK2849330	anti-HER3 antibody	I	[5]
	Afatinib	EGFR and HER2 inhibitor	III	[6]
	Cetuximab	EGFR inhibitor	III, II	[7]
	INC280	c-met inhibitor	II, I	[8]
PI3K/mTOR	PF-05212384	PI3K and mTOR inhibitor	I	
	Buparlisib	PI3K/AKT pathway	II, I	[9, 10]
	Everolimus	mTOR inhibitor	I	[11, 12]
Immunotherapy	Nivolumab	Anti PD-1 antibody	III	[13]
	Pembrolizumab	Anti PD-1 antibody	III	[14]
	Tremelimumab	CTLA-4 antibody	III	[15]
	Avelumab	Anti PD-1 antibody	I	[16]
	Varlilumab	CD-27 Antibody	II, I	[17]
	TCR engineered T-Cells	E6 protein of HPV	II, I	[18]

Note: Source(s) in the table are for the mechanistic discussion of the drugs. Source of trials: National cancer institute (NCI)

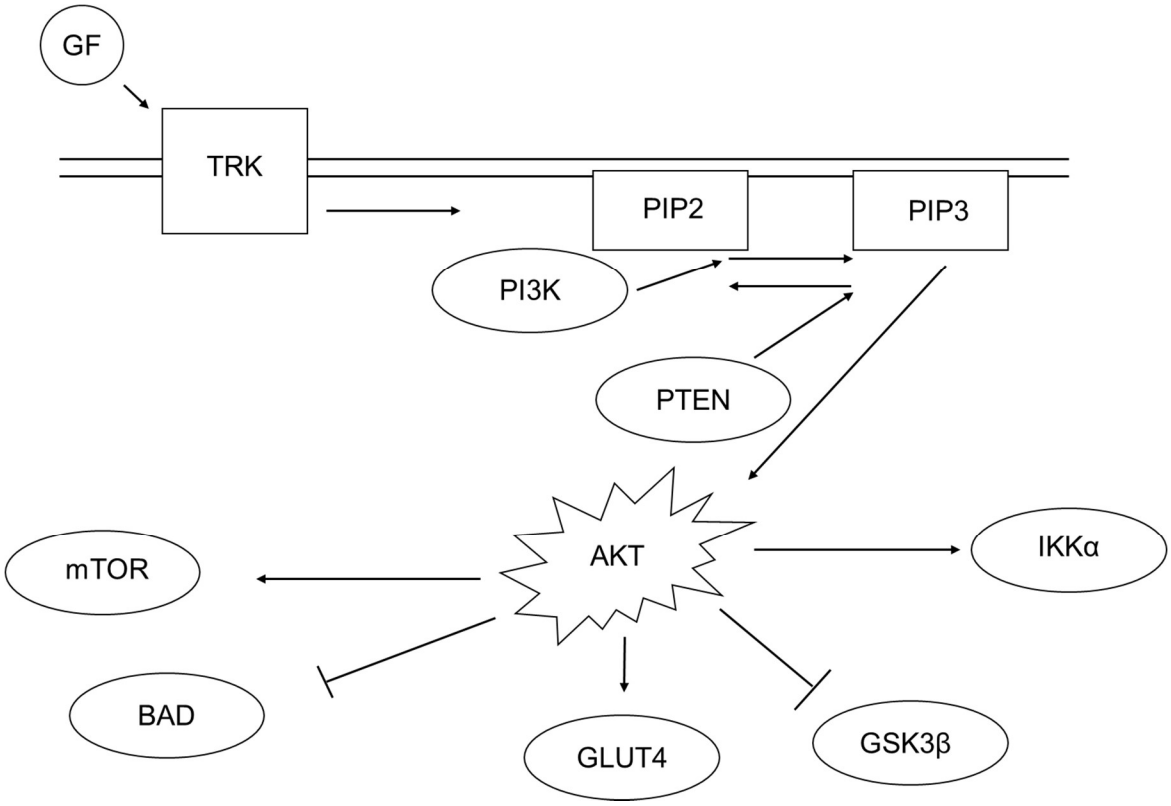


Figure 3.1: PI3K/AKT/mTOR signaling

Schematic representation of the PI3K/AKT signaling pathway GF= Growth Factor; TRK = Tyrosine Kinase Receptor; PIP2= Phosphatidylinositol 4,5-bisphosphate; PIP3= phosphatidylinositol 3,4,5 trisphosphate; PI3K= Phosphatidylinositol 3 Kinase; PTEN= Phosphatase and tensin homolog; AKT= Protein Kinase B; mTOR= Mechanistic Target of Rapamycin; BAD= Bcl-2-Associated Death Promoter; GLUT4= Glucose Transporter Type 4; GSK3β= Glycogen Synthase Kinase 3 Beta; IKKα= IκB kinase Subunit Alpha

CHAPTER IV

INHIBITION OF THE PI3K/AKT PATHWAY SENSITIZES OSCC CELLS TO ANTHRACYCLINE DRUGS, DOXORUBICIN AND AD198

Research article described in this chapter a is slightly modified version of an article that was prepared for submission to the *American Physiological Society Journal of Cell Physiology* by Dmitriy Smolensky, Kusum Rathore and Maria Cekanova in February 2016.

Inhibition of the PI3K/AKT pathway sensitizes OSCC cells to anthracycline drugs doxorubicin and AD198 *in vitro*

Dmitriy Smolensky, Kusum Rathore, and Maria Cekanova.

American Physiological Society Journal of Cell Physiology. (Manuscript in Progress)

In this paper, “our” or “we” refers to me and my co-authors. My contribution to the manuscript includes: 1) Compiling and interpretation of literature 2) Providing comprehensive structure to the paper 3) Preparation of graphs and figures 4) Writing and editing 5) Performing the experiments 6) Interpretation of results

Inhibition of the PI3K/AKT pathway sensitizes oral squamous cell carcinoma cells to anthracycline drugs doxorubicin and AD198 in vitro

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Abstract

Background Patients with oropharyngeal cancer have a low survival rate of 57%; new chemotherapy and targeted drug therapy approaches are needed in order to improve patient outcome. Anthracycline-based chemotherapy, such as doxorubicin (Dox), while effective against many solid tumors, is not widely used for oral cavity cancers. In this study, we evaluated the efficacy of Dox and its novel derivative AD198 against human, canine, and feline head and neck cancer cell lines. In order to increase the efficacy of anthracycline-based chemotherapy in oral cancers, we evaluated the effects of Dox or AD198 combined with PI3K/AKT inhibitor - LY294002. *Results* Dox and AD198 had an anti-proliferative effect on human, canine, and feline head and neck cancer cell lines. AD198 had a better anti-proliferative effect than Dox in human and canine head and neck. In the human oral squamous cell carcinoma cell line SCC25, Dox and AD198 increased the production of reactive oxygen species and subsequently increased apoptosis through activation of caspases. It was observed that both Dox and AD198 increased activation of AKT, ERK 1/2, and p38 MAPK signaling pathways. Our results showed that the efficacy of Dox and AD198 is increased when PI3K/AKT is inhibited with LY294002. *Conclusion* AD198 may be more effective that Dox in treating oropharyngeal cancers. Our results suggest that an anthracycline therapy, such as Dox or AD198, can be made more effective when combined with inhibitors of the PI3K/AKT pathway.

Keywords

Oral cancer, PI3K/AKT signaling pathway, doxorubicin, and AD198

Introduction

Oral squamous cell carcinoma (OSCC) is a major subset of head and neck cancers (HNC), which contribute to ~46,000 new cases a year in the United States, with a relatively low 5-year survival rate of only 57% [19, 20]. The broad definition of oral cancer encompasses cancers both in the mouth and in the throat or pharynx (oropharyngeal cancer). OSCC arises from squamous epithelial cells. It occurs most commonly in older individuals with the average age of diagnosis being 62 years old [21]. The most common risk factors of oral cancer are exposure to carcinogens (mainly tobacco use), alcohol consumption, and human papilloma virus (HPV), which is found in 40–60% of oral cancer patients [19, 21, 22]. Studies indicate that HPV-positive OSCC is more responsive to treatments and carries a better prognosis than HPV-negative OSCC [23, 24] [25-27]. Importantly, while survival for patients with HPV-positive cancer has increased, the survival for patients with HPV-negative cancers has not changed [28]. HPV-negative cancers are usually associated with p53 mutations and dysregulation of the p53/pRb cell cycle arrest pathway [24]. Other pathways that have been observed to be altered in HNC are the p63/NOTCH, TGFβ, and PI3K/AKT signaling pathways [23].

While the standard of care for patients with oral cancer is surgery, followed by radiation and chemotherapy, patients diagnosed with late stages very often cannot have a surgery due to the cancer's intensive spread [22]. Because chemotherapy for patients with unresectable tumors may prolong survival by 10 to 22%, there exists a great need for more effective protocols and new drugs to treat oral cancers [22]. Most commonly chemotherapy agents used for treatment of oral cancers include cisplatin, carboplatin, cetuximab, 5-flourouracil, docetaxel, paclitaxel, bleomycin, vinblastine, vincristine, and methotrexate [22, 29].

Doxorubicin (Dox) has been used over the past three decades to treat solid metastatic tumors and is one of the most successful chemotherapeutic agents [30]. Dox inhibits growth of cancerous cells by inducing DNA damage through type II topoisomerases and other mechanisms, including generation of reactive oxygen species within the cytoplasm [31, 32]. Although cisplatin and 5-fluorouracil-based chemotherapy is usually the first line of treatment for HNC, new formulations of Dox are being researched in order to target HNC [33-35]. While Dox has been shown to be effective against a wide range of cancers, its long-term use comes with the drawbacks of drug resistance and cumulative cardio-toxicity [36, 37]. A novel derivative of Dox, N-benzyladriamycin-14-valerate (AD198), has been specifically designed to address drug-resistance and cardio-toxicity in anthracycline-based therapies [38]. AD198 readily diffuses into the cytoplasm of the cell and is less susceptible to efflux transport due to its lipophilic structure [39]. Unlike Dox, which is cardio-toxic, AD198 has not only been shown to have no detectable cardio-toxicity in the mouse and rat models, but AD198 has also been shown to have a cardio-protective effect through the activation of PKC-ε [40].

In order to better evaluate experimental treatments for OSCC, well characterized animal models are needed. Dogs and cats are both more closely related to humans genetically than mice, and both cats and dogs develop spontaneous cancers, which make them a valuable model of human spontaneous carcinogenesis [41]. In addition, both canine and feline populations are affected by OSCC. Specifically, HNC cancer accounts for 6% of all canine cancers and 10% of feline cancers [41]. Tobacco exposure has been linked to increased incidence of HNC in both human and pet populations, with cats having an increased incidence of OSCC and dogs having an increased incidence of nasal cancers [42-44]. As with humans, both canine and feline OSCC is locally invasive, and the same difficulties in complete resection are encountered [41]. In order to better understand how Dox and AD198 effect feline and canine

cancers, we evaluated the anti-proliferative effect of both drugs on primary canine and feline OSCC cell lines that were isolated and established in our laboratory [45].

The efficacy of AD198 in inhibiting growth of OSCC cells has not yet been studied. Additionally, because Dox is not the first line of treatment in HNC cancers, strategies on how to increase its efficacy in these cancers are not well understood. Our study aimed to evaluate the efficacy of AD198 as compared to Dox in multiple HNC cell lines and to elucidate a possible combination therapy approach that would make anthracycline therapy more effective in HNC.

Methods

Reagents and antibodies

Unless otherwise stated, all reagents and media were purchased from Fisher Scientific (Pittsburgh, PA, USA) Dox and LY294002 (LY) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-benzyladriamycin-14-valerate (AD198) was a kind gift from Dr. Leonard Lothstein, University of Tennessee Health Science Center in Memphis, TN, USA [38]. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): Actin-HRP, p-ERK1/2, ERK 1/2, AKT1, and p38. The following antibodies were purchased from Cell Signaling (Boston, MA, USA): PARP, p-AKT1 (Ser473 and Thr308), p-GSK3β, and p-p38.

Cell culture

Human oral squamous cell carcinoma (SCC25) cells were purchased from ATCC (Manassas, VA). The 1483 cell line was a kind gift from Dr. Lawrence Marnett at Vanderbilt University, Nashville, TN, USA, and was developed by the Parsons lab [46, 47]. Canine and feline oral squamous cell carcinoma cell lines (K9OSCC-Abby and FeOSCC-Sidney;

respectively) were established and characterized previously in our laboratory [45]. The SCC25 and 1483 cells were grown in the following media: DMEMF12 containing 10% FBS penicillin/streptomycin mixture at 37°C and 5% CO_2; feline and canine OSCC cells were grown in RPMI media containing 10% FBS and penicillin/streptomycin mixture (Fisher Scientific) at 37°C and 5% CO_2.

Proliferation assay

Cells were plated in 96-well plates at 5×10^3 cells/well and allowed to attach for 24 h. After seeding, cells were treated with AD198 or Dox in a dose-dependent manner in complete media for an additional 48 h. DMSO was used as a control. For treatment with PI3K inhibitor (LY), cells were pretreated with 20 μM LY for 30 min prior to stated drug treatment, and 20 μM LY was maintained for the rest of the 48 h treatment. After treatment, cell proliferation was measured using CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, 20 μL MTS reagent was added to each well and allowed to incubate at 37°C for 1 h. Absorbance was measured at 490 nM using a plate reader (Bio-Tek instruments, Winooski, VT). The treatment data were normalized to the DMSO control.

Reactive oxygen species (ROS) assay by flow cytometry

For the ROS assay, the cells were incubated with 5 μM **dihydrogen-dichlorodihydro-fluorescein-diacetate** (H_2DCF-DA) (Life Technologies, Grand Island, NY) for 1 h. Cells were then washed with twice PBS and trypsinized. The trypsin was neutralized and the collected cells were centrifuged at 5,000 rpm for 5 min. The cell pellet was resuspended in 1 ml PBS and

fluorescence measured at 485 nM excitation and 530 nM emission using a flow cytometer (BD Accuri BD Sciences, San Jose, CA). Treatment results were normalized to the DMSO control.

Caspase-3/7 assay

Cells were plated in 6-well plates at 5×10^5 cells per well. After 24 h, cells were treated with AD198 or Dox for 24 h. After treatment, cells were washed twice with PBS, and cell lysates were harvested using RIPA buffer. Protein concentration was measured using a Bradford BCA assay. For detection of caspases 3/7, 40 µg proteins were used following the Caspase Glo-3/7 Substrate protocol (Promega). After 1 h incubation with reagents, luminescence was measured using an FLx800 plate reader (Bio-Tek instruments, Winooski, VT). The treatment data was normalized to the DMSO control.

Western Blot

Cells were plated at 1.5×10^6 cells per 10-cm plate. Twenty four hours after plating, cells were treated with different doses of drugs for 24 h. For treatment with the PI3K inhibitor LY294002, the cells were pretreated with 20 µM LY294002 for 30 min prior to stated drug treatment, and 20 µM LY294002 was maintained for the rest of the 24 h treatment. After treatment (unless otherwise stated), the cells were washed twice with PBS and lysed using cold RIPA buffer containing protease/phosphatase inhibitors. The cell lysates were kept at –80°C until further analysis. Protein concentration was measured using the BCA protein assay. Equal amount of proteins (60 µg) were loaded onto SDS-PAGE gels and transferred to a nitrocellulose membrane. Primary antibodies were hybridized overnight at 4°C according to the manufacturer's instructions. The secondary antibodies were hybridized for 1 h at room temperature and the immunoreactive bands were visualized using enhanced

chemiluminescence system (Fisher) and acquired on ImageQuant LAS4000 (GE Life Sciences, Pittsburgh, PA.) The densitometry analysis were performed using ImageJ (NIH, Bethesda, Maryland).

Statistical analysis

Statistics were performed using a paired Student *t* test to established significance. Results were considered statistically significant at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ when treatments were compared to the control group and $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ when comparing Dox to AD198, Dox to Dox + LY294002, or AD198 to AD198 + LY294002 at the same doses.

Results

DOX and AD198 inhibited cell viability of human, canine and feline OSCC cells

The human OSCC cell lines, SCC25 and 1483, as well as FeOSCC-Sidney and K9OSCC-Abby cell lines, were treated with 0.1, 0.5, and 1 μM Dox and AD198 for 48 h, as shown in Fig. 4.1. Both Dox and AD198 significantly reduced the proliferation of SCC25 (Fig. 4.1a) and 1483 (Fig. 1b) cells in a dose-dependent manner. AD198 was more effective at reducing cell viability at all doses when compared to Dox in human OSCC cells. Both Dox and AD198 inhibited viability of FeOSCC-Sidney (Fig. 4.1c) and K9OSCC-Abby (Fig. 1d) cells in a dose-dependent manner. AD198 was significantly more effective in inhibition of cell viability compared to Dox in FeOSCC-Sidney at 0.1 μM and significantly more effective than Dox in inhibition of cell viability in K9OSCC-Abby at 0.5 and 1 μM.

Dox and AD198 induced ROS production and induced apoptosis through caspase activation in human OSCC cells

To evaluate the mechanisms of the anti-proliferative effects of Dox and AD198 in OSCC cells, we have used representative human SCC25 cell line for further experiments in this study.

Both Dox and AD198 significantly increased ROS production in SCC25 cells after 24 h treatment with a 2.8-fold and 1.6-fold increase in SCC25 cells, respectively (***$p \leq 0.001$), compared to control (Fig. 4.2a). In addition, AD198 showed significantly higher activation of ROS production as compared to Dox in SCC25 cells ($^{\#\#}p \leq 0.01$).

The effects of Dox and AD198 on induced apoptosis were evaluated using the caspase-3/7 activity assay. Both Dox and AD198 increased caspase activity in SCC25 cells. Dox increased caspase 3/7 activity by a 2.4-fold, and AD198 increased caspase activity by a 2.8-fold when compared to control (***$p \leq 0.001$), but there was no significant difference between Dox and AD198 treatments (Fig. 4.2b). Poly (ADP-ribose) polymerase (PARP) is a downstream target of caspases cascade. PARP protein is cleaved by caspases and the presence of cleaved fragments indicates apoptosis [48]. Dox and AD198 (1 μM) treatments increased the cleavage of PARP in SCC25 cells as confirmed by WB analysis (Fig. 4.2c). Densitometry values of cleaved PARP proteins after Dox and AD198 treatments were normalized to actin and then to the control groups, as shown in Fig. 2c. According to densitometry analysis of three independent experiments, a statistically significant increase in PARP cleavage by 6-fold (*$p \leq 0.05$) and by 5-fold (**$p \leq 0.01$) was observed in Dox and AD198, respectively, as compared to control treatment. There was no significant difference in cleaved PARP between Dox and AD198 treatments.

Dox and AD198 activated AKT1 signaling pathway in human OSCC cells

To better understand the mechanisms of AD198 and Dox action on cell proliferation and apoptosis in OSCC, we investigated the role of PI3K/AKT and MAPKs signaling pathways. The p38 and ERK1/2 MAPKs have been shown to be activated by ROS to play vital role in apoptosis [49, 50]. Both Dox and AD198 increased the phosphorylation of p38 and ERK1/2 MAPKs in a dose-dependent manner (Fig. 4.3a). Both Dox and AD198 increased phosphorylation of p38 MAPK in a time-dependent manner with the highest activation at 24 h after treatment (Fig. 4.3b). On the other hand, Dox and AD198 increased the pro-survival PI3K/AKT signaling pathway in SCC25 cells in dose- and time-dependent manners (Fig. 3a and 3b). Dox had the greatest effect of increasing phosphorylation of AKT protein at both Ser473 and Thr308 at the 1 μM dose, while AD198 had the greatest effect on the phosphorylation of AKT protein at 0.5 μM dose.

Inhibition of PI3K/AKT signaling pathway sensitizing the cytotoxic effects of Dox and AD198 in human OSCC cells

Dox and AD198 activated the pro-survival PI3K/AKT signaling pathway, which is one of the indicators of resistance of cells to chemotherapy. To confirm our hypothesis, we tested the effects of the PI3K inhibitor (LY294002) in combination with Dox or AD198 on growth of SCC25 cells. Co-treatment with LY294002 increased the anti-proliferative effects of both Dox and AD198 in SCC25 cells. Cell morphology changes were detected, when cells were pre-treated with LY294002 and followed by Dox and AD198 treatments as compared to either Dox or AD198 treatments alone. Co-treatment of Dox with LY294002 caused cells to shrink and detach from plate surface resulting in cellular death of SCC25 cells (Fig. 4a.) The combination of Dox or

AD198 and LY294002 more effectively suppressed cell viability of SCC25 cells as compared to either treatment alone (Fig. 4b).

In order to further investigate the PI3K/AKT inhibitor's chemosensitizing effect to Dox and AD198 chemotherapy, we measured caspase-3/7 activities and PARP cleavage. Co-treatment of Dox and AD198 with LY294002 increased caspase-3/7 activation and PARP cleavage in SCC25 cells, as shown in Fig. 4c and Fig. 4d. LY294002 inhibited the AD198- and Dox-induced phosphorylation of AKT at Thr308 and Ser473, as shown in Fig. 4e. In addition, higher levels of active (unphosphorylated) GSK-3β were present when SCC25 cells were co-treated with Dox or AD198 with LY294002. Inhibition of PI3K/AKT further increased the Dox- and AD198-induced phosphorylation of p38 MAPK, but decreased the Dox- and AD198-induced phosphorylation of ERK1/2.

Discussion

As published previously, AD198 is effective as novel derivative of Dox even for treatment of Dox-resistance leukemia and melanoma tumors in the mouse model [51]. The objectives of our study was to evaluate the efficacy of AD198 in human, canine, and feline OSCC cell lines *in vitro*. Our data showed that AD198 had a better inhibitory effects on cell proliferation than Dox in all tested human, canine and feline OSCC cell lines (Fig. 1). This data correlates with our previously obtained results that show that AD198 is more effective than Dox in primary canine transitional cell carcinoma and osteosarcoma cell lines [52]. We and others have shown that feline and canine OSCC models might be helpful for testing of novel therapeutics, including Dox and its derivatives, receptor tyrosine kinase inhibitors, and non-steroidal anti-inflammatory drugs [43, 45].

In order to elucidate mechanistic differences and potential ways to increase efficacy of Dox and AD198 chemotherapies, we studied the Dox and AD198-induced apoptosis in OSCC *in vitro*. While both Dox and AD198 increased ROS production when compared to control, AD198 increased ROS production by a 2.8-fold, Dox increased ROS production by only 1.6-fold ($^{\#\#}p \leq 0.01$ between Dox and AD198, Fig. 2a). AD198 has been shown to have comparable ROS production with that of Dox in cardiomyocytes [53]. We have shown that AD198 induces production of ROS more than Dox in human UMUC3 and T24 bladder cancer cells *in vitro* [54]. Caspase cascade and its downstream target PARP cleavage are common methods to detect apoptosis in cells [48, 55]. Both Dox and AD198 had similar effects in increasing caspase 3/7 activities and inducing downstream PARP cleavage (Fig. 2b and 2c). There was no significant difference in caspase 3/7 activities with regard to the amount of cleaved PARP between Dox and AD198. These data suggest that while AD198 has a greater ROS-generating effect, this effect may not contribute to an increase in caspase-dependent apoptosis, but instead contributed to caspase-independent pathways. Dox-induced apoptosis was caspase-dependent, and the effects of Dox diminish when caspases are inhibited in leukemia cell lines [56]. Another explanation for the greater efficacy of AD198 when compared to Dox, even though both drugs show similar caspase induction and PARP cleavage, is that ROS-induced apoptosis can function through caspase-independent pathways, as shown previously in cardiomyocytes [57]. This suggests that the greater increase in ROS production may still lead to greater apoptosis without triggering a great increase in caspase activity.

To further investigate the mechanism behind Dox and AD198 anti-tumor effects, we evaluated the involvement of signal transduction pathways in OSCC cells *in vitro*. Both Dox and AD198 increased phosphorylation of ERK1/2, and p38 MAPK, as well as AKT in time- and dose-dependent manner (Fig. 3a and 3b). The ERK1/2 signaling pathway plays an important

role in regulation of cell's survival and apoptosis [58, 59]. Activation of ERK1/2 generally promotes cell survival; but can also have pro-apoptotic functions [60, 61]. The inhibition of ERK2 activity has been reported to sensitize ovarian carcinoma cells to cisplatin-induced apoptosis [62, 63], but it has been also reported to induced drug resistance of various carcinomas to chemotherapy drugs [64, 65]. The p38 MAPK signaling pathway is also associated with apoptosis and is an important pathway that is activated with various chemotherapy drugs [66]. We have shown that Dox- and AD198-induced apoptosis is a p38 MAPK-dependent in canine transitional cell carcinoma and osteosarcoma cell lines [52]. On the other hand, the PI3K/AKT pathway has been shown to be dysregulated in many cancers, and its activation is responsible for increased cell survival, decreased apoptosis, and increased drug resistance [67-69]. In breast cancer, activation of AKT leads to multi drug resistance and increased expression of p-glycoprotein [70]. In contrast, inhibition of PI3K/AKT reduces drug resistance by decreasing transport activity of p-glycoprotein [71]. When the PI3K/AKT pathway was inhibited, chemosensitivity to Dox was increased in various cancers, including breast, bladder, and ovarian [72-74]. It has also been shown that Dox increases phosphorylation of AKT in various breast cancer cell lines [75], which correlates with our observations in SCC25 cells.

In order to better understand how PI3K/AKT pathway activation plays a role in antagonizing Dox and AD198-induced apoptosis in OSCC cells, we studied the effects of Dox and AD198 in combination with the PI3K inhibitor LY294002. Cell viability of tested OSCC was greatly reduced when Dox or AD198 was combined with LY294002 treatment when compared to either Dox or AD198 treatment alone (Fig. 4a and 4b). Apoptotic markers, including caspase 3/7 activity and PARP cleavage, increased significantly with the combination of Dox + LY294002 or AD198 + LY294002, as compared to either Dox or AD198 alone (Fig. 4c and 4d). These results confirmed by previous studies in other cancers, showing that inhibition of

PI3K/AKT increased chemosensitivity towards Dox. Our results also show that chemosensitivity to AD198 can also be increased through the inhibition of the PI3K/AKT pathway.

Inhibition of the PI3K/AKT signaling pathway decreased Dox and AD198-induced activation of p38 MAPK and reduced activation of the ERK1/2 signaling pathways (Fig. 4e). In cardiomyocytes, activation of AKT is correlated with deactivation of p38 MAPK [76]. AKT/PI3K signaling pathway has also been shown to downregulate p38 MAPK signaling in endothelial cells [77]. While the interplay between PI3K/AKT and ERK signaling pathways is poorly understood, it has been previously observed that inhibition of the ERK signaling pathway increases activity of the p38 MAPK pathway [78]. Our results indicate that LY294002 co-treatment with Dox or AD198 increased chemosensitivity through the inhibition of ERK and increased activation of p38 MAPK. In conclusions, our data show that the inhibition of the PI3K pathway may be an important for increasing the efficacy of anthracycline-based chemotherapy in HNC with agents such as Dox and AD198.

Conclusion

In this study, we have shown that AD198 is more effective at inhibiting cell proliferation than Dox in all tested OSCC cell lines. Both Dox and AD198 increased ROS production and activated the caspase-dependent apoptosis cascade. Phosphorylation of AKT, p38 MAPK, and ERK1/2 was increased by both Dox and AD198 treatments. Because AKT was shown to have an anti-apoptotic effect in previous studies, we evaluated the combination therapy of inhibiting PI3K/AKT along with Dox and AD198. Inhibition of PI3K/AKT further decreased cell proliferation and increased apoptosis in human OSCC cells that were treated with Dox or AD198. Furthermore, inhibition of PI3K/AKT increased the activation of p38 MAPK by Dox and AD198

while decreasing the activation of ERK 1/2. Results collected from this study show that AD198 may be an effective anthracycline treatment for HNC cancers, and the inhibition of PI3K/AKT can further increase the efficacy of Dox or AD198-based chemotherapy in HNC cancers in vitro.

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Conflict of interest

The authors declare no conflict of interest.

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Appendix

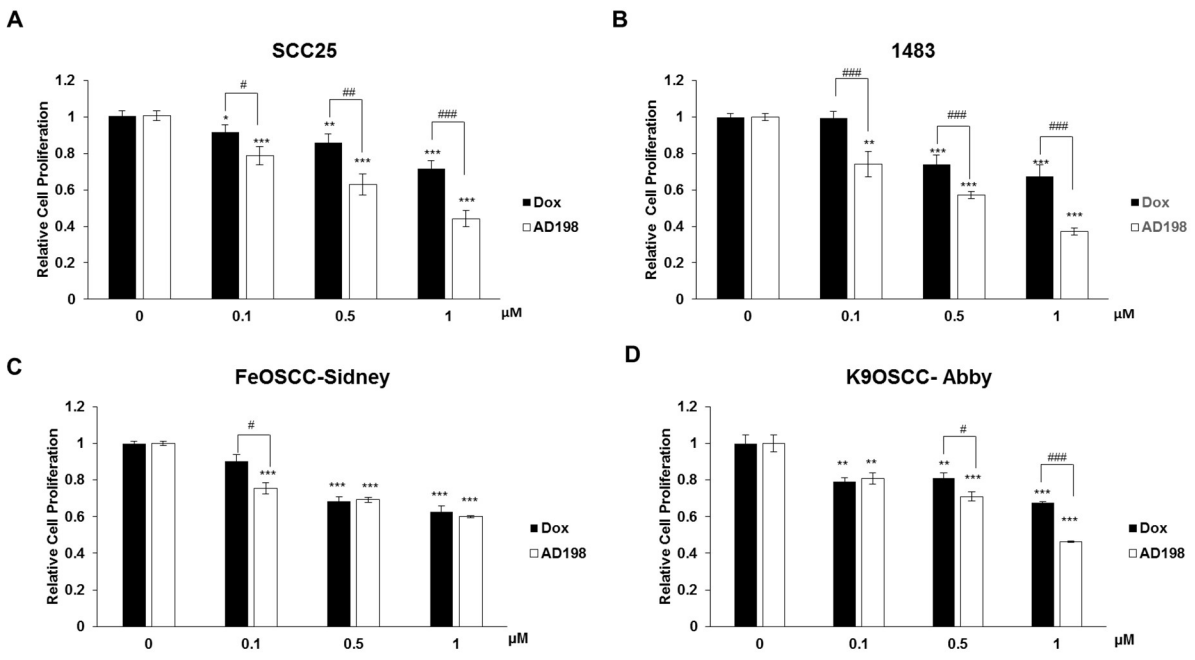


Figure 4.1: DOX and AD198 inhibit cell viability of human and animal OSCC cells.

(a) Human oral squamous cell carcinoma SCC25 cells, **(b)** 1483 cells, and **(c)** FeOSCC-Sidney and **(d)** K9OSCC-Abby cells were treated with Dox (black bars) and AD198 (white bars) at 0, 0.1, 0.5, and 1 μM for 48 h and compared to control groups. Cell proliferation was determined by MTS assay and relative cell growth rate was normalized to control, DMSO treated groups. The values are mean ± S.E. of four replicates from three independent experiments. Paired Student *t*-tests were used to compare Dox and AD198 treatment to control; $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$. Paired Student *t*-tests were used to compare among Dox and AD198 group at the same dose treatment; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, and $^{\#\#\#}p \leq 0.001$.

Figure 4.2 Dox and AD198 induced ROS in human OSCC cells and activate apoptotic caspase cascade in OSCC cells.

(a) SCC25 cells were treated with 1 μM Dox and 1 μM AD198 for 24 h, and ROS levels were measured with dihydrogen-dichlorodihydro-fluorescein-diacetate labeling flow cytometry; median fluorescence was measured and normalized to the control. These values are mean ± S.E. of four replicates performed in three independent experiments. Paired Student *t* test comparing Dox and AD198 treatment to control; $*p \leq 0.05$, $***p \leq 0.001$, and Dox and AD198 treatments; $^{\#\#}p \leq 0.01$, respectively. **(b)** SCC25 cells were treated with 1 μM Dox and 1 μM AD198 for 24 h, and caspase activity was measured using the Caspase-Glo 3/7 luminescence assay. Relative caspase activities were normalized to control. The values are mean ± S.E. of three independent experiments in two replicates. Paired Student *t* test compared treatment to control groups; $***p \leq 0.001$. There was no significant difference in caspase activity between Dox and AD198. **(c)** SCC25 cells were treated with 1 μM Dox and AD198 for 24 h. The expression of PARP (cleaved fragment) was evaluated by WB analysis. Actin was used as loading control. The right panel represents densitometry evaluation of three independent experiments. Cleaved PARP fragment was normalized to actin and these values were normalized to control. Values represent mean ± S.E. of three independent experiments. Paired Student t test comparing Dox and AD198 treatment to control groups; $*p \leq 0.05$ and $**p \leq 0.01$.

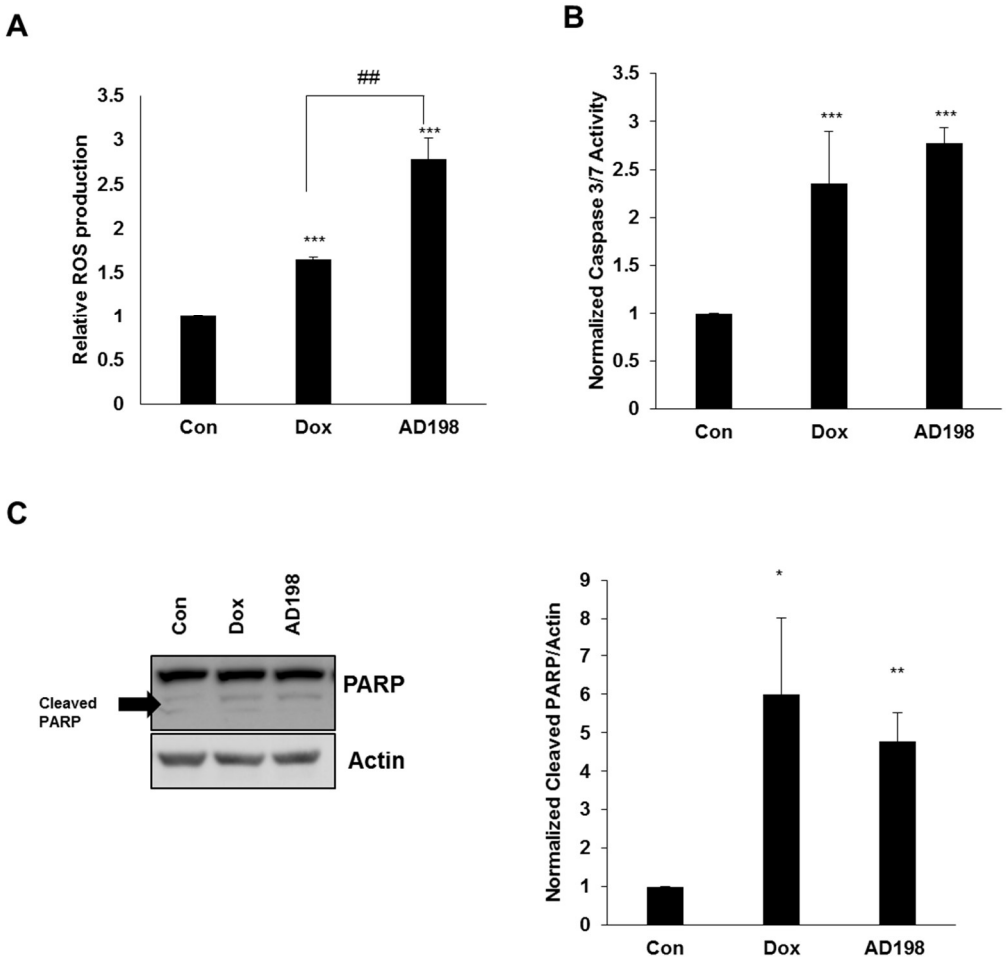


Figure 4.2 Continued

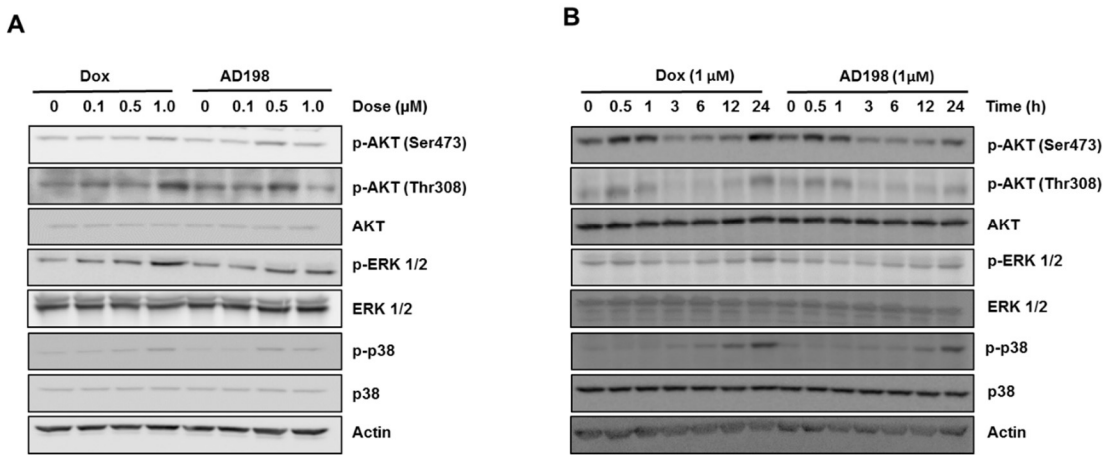


Figure 4.3: Dox activated AKT, p38 MAPK and ERK 1/2 signaling pathway in human OSCC cells in dose and time dependent manner.

(a) SCC25 cells were treated with 0, 0.1, 0.5, and 1 μM Dox and AD198 for 24 h. **(b)** SCC25 cells were treated with 1 μM Dox and AD198 for 0, 0.5, 1, 3, 6, and 24 h. The expression of p-AKT (Thr308), p-AKT (Ser473), AKT, p-ERK, ERK, p-p38, and p38 proteins was detected by WB. Actin was used as loading control.

Figure 4.4: Inhibition of AKT signaling pathway potentiates the cytotoxic effects of Dox in human SCC25 cells.

Figure 4.4: (a) SCC25 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (LY, 20 μM) for 24 h. Pictures were taken at 100× magnification in order to observe cell morphology and amount of cells. Scale bar represents 50 μm. (b) SCC25 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (20 μM) for 48 h, and cell viability was measured using an MTS assay. Relative cell viability was normalized to untreated counterpart. The values represent mean ± SE of three independent experiments performed in four replicates. Paired Student *t* tests compared DOX, AD198, Dox + LY294002 and AD198 + LY294002 treatments to control; *** $p \leq 0.001$. Paired Student *t* tests were used to compare Dox to Dox + LY294002 and AD198 to AD198 + LY294002 treatments, $^{###}p \leq 0.001$. (c) SCC25 cells were treated with DOX and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h, and caspase activities were measured using the Caspase-Glo 3/7 luminescence assay. Relative caspase activities were normalized to control. The values represent mean ± S.E. of three independent experiments performed in duplicates. A paired Student *t* test compared treatment to control; ***$p \leq 0.001$, as well as Dox to Dox + LY294002 and AD198 to AD198 + LY294002 treatments; $^{###}p \leq 0.001$. (d) SCC25 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h. The expression of PARP (cleaved fragment) was evaluated by WB analysis. Actin was used as loading control. Densitometry evaluation of cleaved PARP/actin protein bands from WB analysis was done using ImageJ software. Values are mean ± S.E. of measured densitometry of each protein's band from three independent experiments. Paired Student *t* tests were used to compare controls to Dox and AD198 treatments, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$, as well as Dox to Dox + LY294002 and AD198 to AD198 + LY294002

treatments; $^{###}p \leq 0.001$. (e) SCC25 cells were treated with Dox and AD198 (1 μM) with and without LY294002 (20 μM) for 24 h. The expression of p-AKT (T308), p-AKT (S473), AKT, p-p38, p38, p-ERK, ERK, and p-GSK-3β proteins was evaluated by WB analysis. Actin was used as loading control.

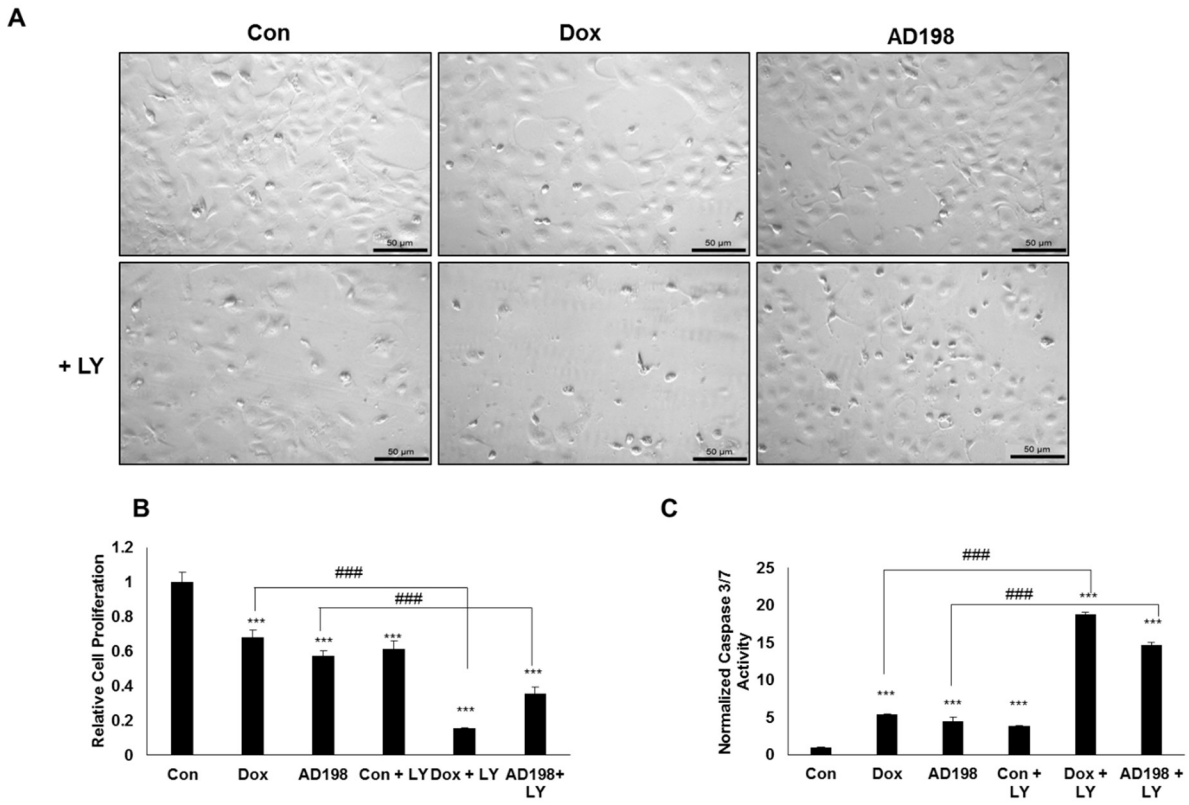


Figure 4.4 Continued

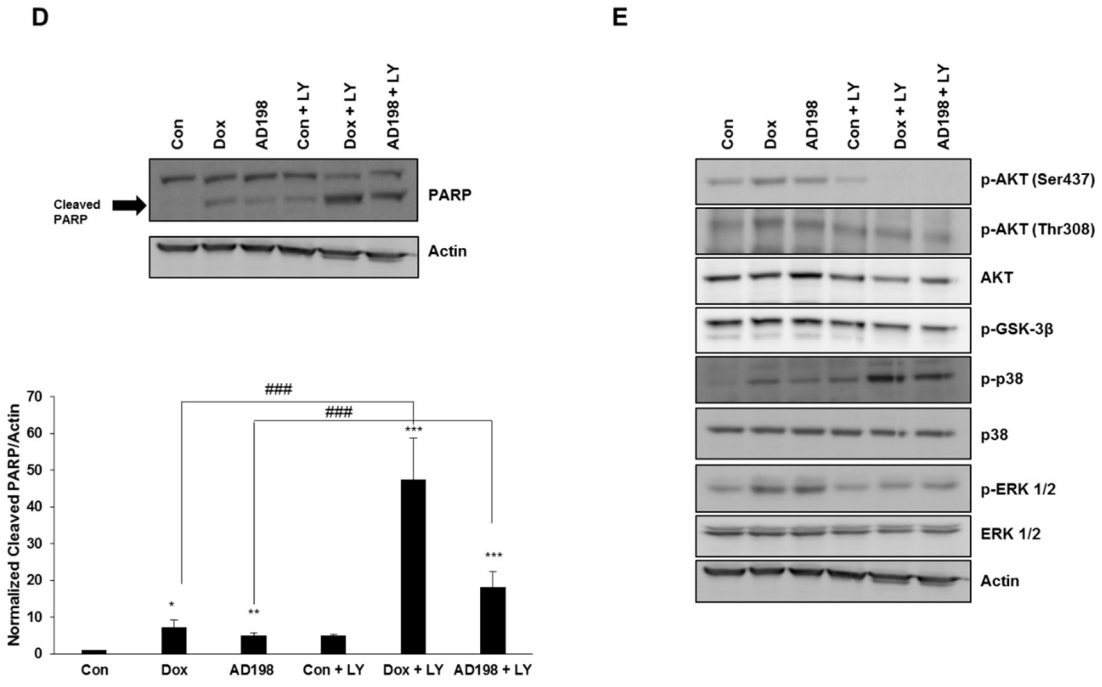


Figure 4.4 Continued

CHAPTER V

DISCUSSION

General Discussion

The studies presented in this dissertation were designed to 1) evaluate the anti-proliferative efficacy of AD198 compared to doxorubicin (Dox) in human transitional cell carcinoma (TCC) and human, canine, and feline oral squamous cell carcinoma (OSCC) cell lines *in vitro* and 2) study the mechanisms of Dox and AD198 proliferation inhibition and increase the efficacy of both Dox and AD198 through combination targeted therapy *in vitro*.

Summary of Results

Bladder cancer

The bladder cancer study shows the novel derivative of Dox, AD198, was effective in inhibiting proliferation of TCC cell lines *in vitro* in a dose dependent manner using the MTS assay (Figure 2.1). When compared to Dox, AD198 was more effective at 0.1μM and 5.0μM doses in both T24 and UMUC3 cell lines (Figures 2.1a and 2.1b).

To identify the mechanisms involved in the inhibitory effect of AD198 in TCC cell lines, we evaluated reactive oxygen species (ROS) generation by measuring DCF fluorescence using flow cytometry. We found AD198 increased ROS production more than Dox in both T24 and UMUC3 cell lines (Figure 2.2).

We next studied the ability of Dox and AD198 to induce apoptosis in human TCC cells through caspase-3/7 activity and the downstream cleavage of Poly ADP ribose polymerase (PARP) protein (Figure 2.3). Results showed that Dox was more effective than AD198 at increasing caspase-3/7 activity and inducing PARP cleavage in both T24 and UMUC3 cells. These findings indicated Dox and AD198 may inhibit cell proliferation through different

mechanisms with Dox relying more on caspase activity, and AD198 relying more on ROS generation.

In studying the effects of Dox and AD198 on signal transduction pathways, we observed that both Dox and AD198 increased the activation of the Phosphatidylinositol-3 kinase/anti-apoptotic receptor tyrosine kinase (PI3K/AKT) signaling pathway through phosphorylation of AKT at the T308 and S473 residues, in both time and dose dependent manners (Figure 2.4). Because PI3K/AKT is recognized as a pro-survival and pro-proliferation pathway, we hypothesized that the anti-cancer effects of Dox and AD198 can be increased through combined treatment with agents that inhibit the PI3K/AKT pathway [1]. Co-treatment with the PI3K inhibitor LY294002 (LY) increased the anti-proliferative efficacy of both Dox and AD198 (Figure 2.5) in T24 and UMUC3 cells. Co-treatment with LY and Dox or AD198 had a greater effect on increasing caspase-3/7 activity than either Dox or AD198 alone in both T24 and UMUC3 cells. To verify the increase in caspase-3/7 activity, we evaluated the cleavage of PARP in cells treated with LY and Dox or AD198. In both T24 and UMUC3 cells, the co-treatment of LY with either Dox or AD198 resulted in a greater increase in PARP cleavage compared to Dox or AD198 alone. To confirm the efficacy of the PI3K inhibition by the LY compound, we have shown the LY compound inhibits Dox- or AD198-induced phosphorylation of the AKT protein at the T308 and S473 residues.

Oral Cancer

AD198 was more effective at inhibiting cell proliferation than Dox at all the doses tested (at 0.1μM, 0.5μM and 1.0μM) in human OSCC cell lines, SCC25 and 1483. In the primary feline head and neck squamous cell carcinoma cell line (Sidney) both AD198 and Dox inhibited cell proliferation in a dose dependent manner. In the primary canine OSCC cell line (Abby) AD198

was more effective at inhibiting cell proliferation than Dox at 0.5μM, and 1.0μM doses. These results suggested AD198 may be a viable option to replace Dox for treatment of OSCC (Figure 4.1).

In order to study the mechanism of action of Dox and AD198, we compared the effectiveness of the two drugs in generating ROS and activating the caspase apoptotic pathway. Similar to previous results obtained in TCC cell lines, AD198 generated more ROS than Dox in SCC25 cells. However, unlike results obtained in TCC cell lines, both Dox and AD198 increased caspase-3/7 activity to similar levels in SCC25 cells (Figure 4.2).

Similarly to TCC cell lines, both Dox and AD198 increased activation of the PI3K/AKT pathway, as was observed by phosphorylation of AKT at T308 and S473 residues in both dose and time dependent manners in SCC25 cells. We also observed that both Dox and AD198 increased phosphorylation of Erk1/2 and p38 MAPK proteins in both time and dose dependent manners (Figure 4.3). Both PI3K/AKT and ERK pathways are recognized as pro-survival pathways, while p38 MAPK is generally recognized as a pro-apoptotic pathway [1-4]. We observed inhibition of PI3K by LY increased the ability of both Dox and AD198 to inhibit cell proliferation in SCC25 cells. These results were confirmed by increased caspase-3/7 activation and PARP cleavage induced by Dox + LY or AD198 + LY as compared to Dox or AD198 alone. On the protein level, inhibition of PI3K inhibited activation of the AKT and ERK pathways by Dox and AD198. Conversely, inhibition of PI3K further increased Dox- and AD198-induced activation of p38 MAPK. Previous studies in the laboratory showed that Dox- and AD198-induced p38 play important pro-apoptotic roles in canine TCC and osteosarcoma cell lines [5].

PI3K/AKT and its role in human cancers

PI3Ks are a downstream target of many pro-survival, pro-growth and RTKs including: EGFR, HER2, PDGFR, c-Kit, and Ras. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [6]. Downstream of PIP3 is the protein AKT, which initiates major pro-survival signaling pathways [6]. AKT can activate mechanistic target of rapamycin (mTOR) through the phosphorylation and inhibition of its negative regulator TSC2 [7, 8]. In turn, mTOR increases protein synthesis, glucose uptake, and proliferation in several cancers including oral and bladder cancers [9, 10]. To prevent apoptosis, AKT phosphorylates MDM2 causing it to translocate to the nucleus and induce degradation of tumor suppressor p53. AKT also phosphorylates BAD, modulating its pro-apoptotic functions [11-13]. Another downstream target of PI3K is the RAF/MEK/ERK pro-survival signaling pathway [14, 15]. These and other findings indicate that the PI3K/AKT pathway is a central convergence point for many signals that may increase cell proliferation and decrease susceptibility to anti-cancer therapy [16].

According to the cancer genome network, at least part of the RTK/PI3K/AKT pathway is altered in 72% bladder cancer patients and in 61-62% head and neck squamous cell carcinoma patients [17, 18]. Because PI3K/AKT is central to many signaling pathways, it can play a major role in resistance and susceptibility to targeted therapy [19]. PTEN serves as one of the major negative regulators of PI3K by dephosphorylating PIP3 back to PIP2 [20]. PTEN protein deficiencies and mutations have been identified in both bladder and oral cancers [21-24]. In bladder cancer, PTEN protein deficiency induces resistance to mTOR inhibition therapy and is also correlated with reduced survival [25, 26]. In oral cancer, PTEN protein deficiency leads to further progression of disease and is associated with reduced survival [27, 28].

Targeting the PI3K/AKT pathway to increase efficacy of Dox has been suggested in other cancers. Inhibition of PI3K by LY blocks the export of Dox in drug-resistant colon cancer cells which restores sensitivity to Dox therapy [29]. In ovarian cancer, Dox has been shown to activate PI3K/AKT through activation of the HER3 receptor and inhibition of the HER3/PI3K/AKT pathway increased Dox-induced apoptosis [30]. Another recent report showed that Dox activates the PI3K/AKT pathway and inhibition of this pathway by LY increased sensitivity to Dox in human bladder cancer cells [31].

The data obtained in these studies has shown that both Dox and AD198 activate the PI3K/AKT pathway in bladder and oral cancer cell lines. The fact that many patients already have an over-active PI3K/AKT pathway in oral and bladder cancer cell lines suggests the PI3K/AKT pathway is a valid target for co-treatments with anthracycline based chemotherapy.

Prospects and future directions

The results of our studies have demonstrated that: 1) AD198 is a viable treatment when compared to Dox in both TCC and OSCC cell lines *in vitro,* and 2) Both Dox and AD198 increase the activity of the PI3K pathway and show increased efficacy upon inhibition of PI3K *in vitro*.

In order to move forward, the efficacy of AD198 must be validated using *in vivo* models for both TCC bladder cancers and OSCC oral cancers. Previous studies have suggested AD198 may have certain advantages over Dox. AD198 has no known cardio-toxic effects and has been shown to have cardio-protective qualities in rat models [32, 33]. A xenograft model of human TCC or OSCC would be necessary to test the efficacy AD198 compared to Dox. Another viable option could use chemically induced tumors. Mice exposed to N-butyl-N-(4-hydroxybutyl)

nitrosamine (BBN) in drinking water will develop bladder cancer and can be used to study the effects of AD198 and Dox after the primary tumor is established [34]. To accomplish the same experiment for OSCC, 4-nitroquinoline-1-oxide (4NQO) could be used to induce OSCC in CBA mice when repeatedly applied to the palate [35]. Following success of mouse models, AD198 should be studied in the canine and feline models of TCC and OSCC.

The combination of a PI3K inhibitor with Dox or AD198 should be validated with a clinically relevant inhibitor of the PI3K/AKT pathway *in vitro*. There are currently several PI3K/AKT pathway-targeting drugs in the pipeline for both TCC and OSCC cancers [36, 37]. Buparlisib is a PI3K inhibitor currently being researched for treatment of several cancers in clinical trials [38-41] and has been shown to exhibit efficacy against OSCC cells *in vitro* [42]. In order to validate the results obtained in our studies, *in vitro* experiments using buparlisib combined with AD198 or Dox should be performed. Following positive *in vitro* results, combined treatment using buparlisib along with Dox or AD198 needs to be studied *in vivo* of TCC and OSCC and compared to Dox or AD198 alone.

Conclusion

These studies indicate that in both TCC and OSCC cancers, Dox and AD198 activate the pro-survival PI3K/AKT pathway while having anti-proliferative effects. Targeting the PI3K/AKT pathway along with Dox or AD198 treatment increases the efficacy of anthracycline therapy *in vitro* (Figure 5.1). These results can be used to study overcoming Dox-induced cardio-toxicity by replacing Dox with AD198, overcoming drug resistance in cancers with active PI3K/AKT pathways, and making the established use of Dox more effective in treating bladder and oral cancers.

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Appendix

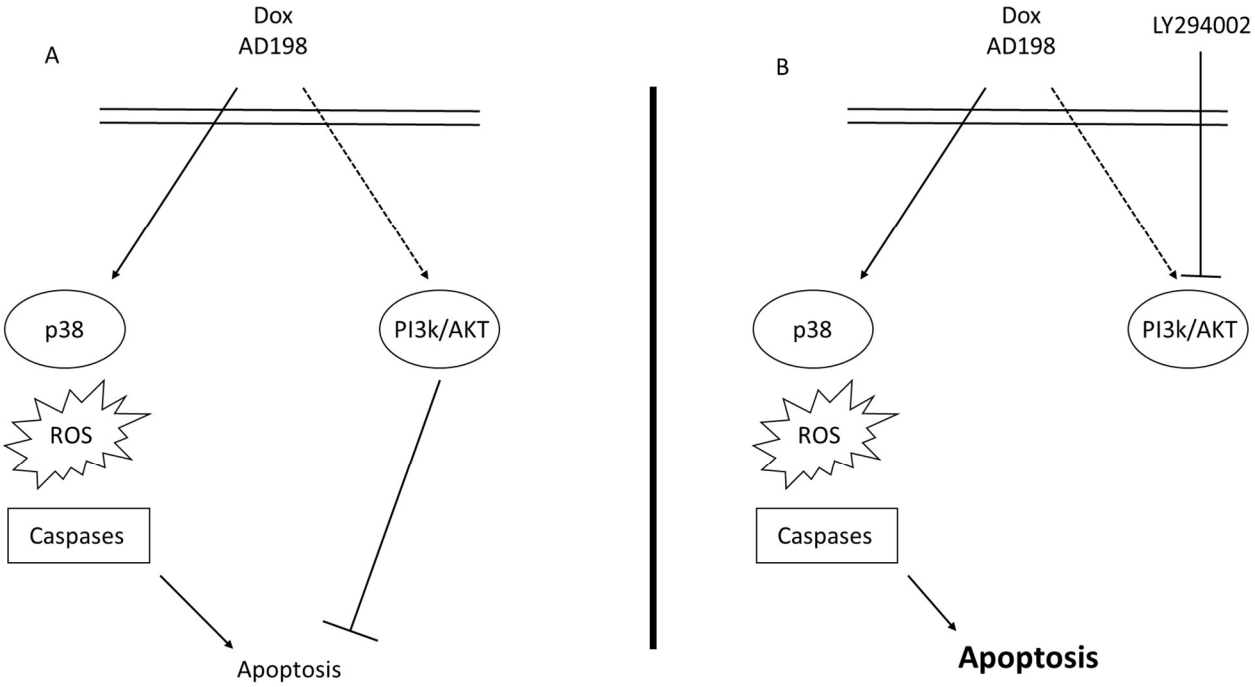


Figure 5.1: Representation of increasing sensitivity to Dox and AD198 by targeting the PI3K/AKT pathway

(a) Both Dox and AD198 decreased cell proliferation through activation of p38, production of reactive oxygen species and activation of caspases. The activation of PI3K/AKT induced by Dox and AD198 inhibited apoptosis through these mechanisms. **(b)** Upon inhibition of the PI3K/AKT pathway by LY294002, the apoptotic effects of Dox and AD198 are further increased through increased activation of p38 and caspases.

VITA

Dmitriy Smolensky was born in St. Petersburg, Russia on December 29th 1981. In 1990 he immigrated to America with his family. After graduating high school in 1999, he joined the United States Army National Guard where he honorably served for 6yrs including 2yrs of active duty. He pursued and received his bachelor's degree in Molecular Biology Biochemistry and Bioinformatics (MB3) from Towson University, Maryland in 2007. After receiving his B.S. degree, he worked under the mentorship of Dr. Hugh O'Neill at Oak Ridge National Laboratory as a research assistant. In 2009 he joined the UT-ORNL Graduate School of Genome Science and Technology where he successfully defended his dissertation for Doctor of Philosophy degree under the mentorship of Dr. Maria Cekanova (2014-2016) and graduated in spring 2016. Dmitriy plans on pursuing his post-doctoral training.