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# Effects of the new Histone Deacetylase Inhibitor, PXD101, in Bladder Cancer

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Histone deacetylase inhibitors (HDACIs) mediate gene expression and chromatin assembly, and induce growth arrest and apoptosis of tumor cells, thus representing a new strategy for human cancer therapy. Changes in apoptosis signaling pathways and the effect on cell growth and cell-cycle arrest of a new HDACI, PXD101, on T-24 bladder cancer cells form the basis of this study.

T-24 cells were incubated with PXD101 at varying concentrations and times, and viable cell count and proliferation curves were constructed. Cell cycle analysis was conducted with Fluorescent Activated Cell Sorting and changes in apoptosis signaling proteins that were previously found to be regulated by survivin-siRNA in T-24 cells were assessed by Western blot.

Treatment of T-24 bladder cancer cells with the HDAC inhibitor PXD101 causes a profound decrease in cell growth and viability, a specific G2/M phase arrest and an increase in apoptotic cells populations. PXD101 treatment also causes changes in upstream mitochondrial apoptosis mediators, including TNFR1 and caspases 2 and 8, and downstream apoptosis mediators, such as caspase 3 and survivin. PXD101 treatment of tumor cells is associated with a profound decrease in survivin and caspases' levels, and with an increase in TNFR1 protein levels, both changes indicative of induction of apoptosis.

Therefore, the new HDACI PXD101, alone or in combination with inhibitors of other tumor relevant factors and chemotherapies with complementary mechanisms of action, shows promise for its use as a suitable new agent for bladder cancer treatment.

# ACKNOWLEDGEMENTS

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# Introduction

A multicellular organism functions as a society, with individual cells organizing into collaborative assemblies and reproducing by cell division through an elaborate set of signals that serve as controls. Each cell rests, divides, differentiates or dies as needed for the prosperity of the organism. Molecular disturbances that upset this harmony mean trouble for such a society and in animals with countless cells and disturbances, mutations are a constant challenge. A single mutation that goes unchecked may give rise to a growing mutant clone of cells that will prosper at the expense of neighboring cells and in the end, destroy the entire society.

Such is the recipe for cancer, and to this day, cancer accounts for nearly one-quarter of deaths in the United States, exceeded only by heart disease. In 2003, there were 556,902 cancer deaths in the US, **Figure 1**. The risk of an American male developing cancer over his lifetime is one in two and approximately one in three women in the United States will develop cancer over her lifetime.

| | Cause of Death | No. of deaths | % of all deaths |
|---|---|---|---|
| 1. | Heart Diseases | 685,089 | 28.0 |
| 2. | Cancer | 556,902 | 22.7 |
| 3. | Cerebrovascular diseases | 157,689 | 6.4 |
| 4. | Chronic lower respiratory diseases | 126,382 | 5.2 |
| 5. | Accidents | 109,277 | 4.5 |
| 6. | Diabetes mellitus | 74,219 | 3.0 |
| 7. | Influenza and pneumonia | 65,163 | 2.7 |
| 8. | Alzheimer disease | 63,457 | 2.6 |
| 9. | Nephritis | 42,453 | 1.7 |
| 10. | Septicemia | 34,069 | 1.4 |

**Figure 1: United States Mortality Data, Leading Causes of Death for 2003 [1].**

# Bladder Cancer

It is estimated that almost 1.4 million new cases of cancer were diagnosed in 2006. Of these, 61,420 cases were due to urinary bladder cancer, making it the fourth most common and ninth most common site of new cancer diagnoses in men and women, respectively. Bladder cancer is the second most common genitourinary tract cancer and between 1985 and 2000, the number of bladder cancers diagnosed annually in the United States increased by 33% [2, 3]. The incidence in men is nearly three times higher than in women [3], and 1.5 times higher in whites than in African Americans. Incidence rates in Hispanic Americans of each sex are roughly half those in whites [4].

| Men 720,280 | | Women 679,510 | |
|---|---|---|---|
| Prostate | 33% | 31% | Breast |
| Lung & bronchus | 13% | 12% | Lung & bronchus |
| Colon & rectum | 10% | 11% | Colon & rectum |
| Urinary bladder | 6% | 6% | Uterine corpus |
| Melanoma of skin | 5% | 4% | Non-Hodgkin lymphoma |
| Non-Hodgkin lymphoma | 4% | 4% | Melanoma of skin |
| Kidney | 3% | 3% | Thyroid |
| Oral cavity | 3% | 3% | Ovary |
| Leukemia | 3% | 2% | Urinary bladder |
| Pancreas | 2% | 2% | Pancreas |
| All Other Sites | 18% | 22% | All Other Sites |
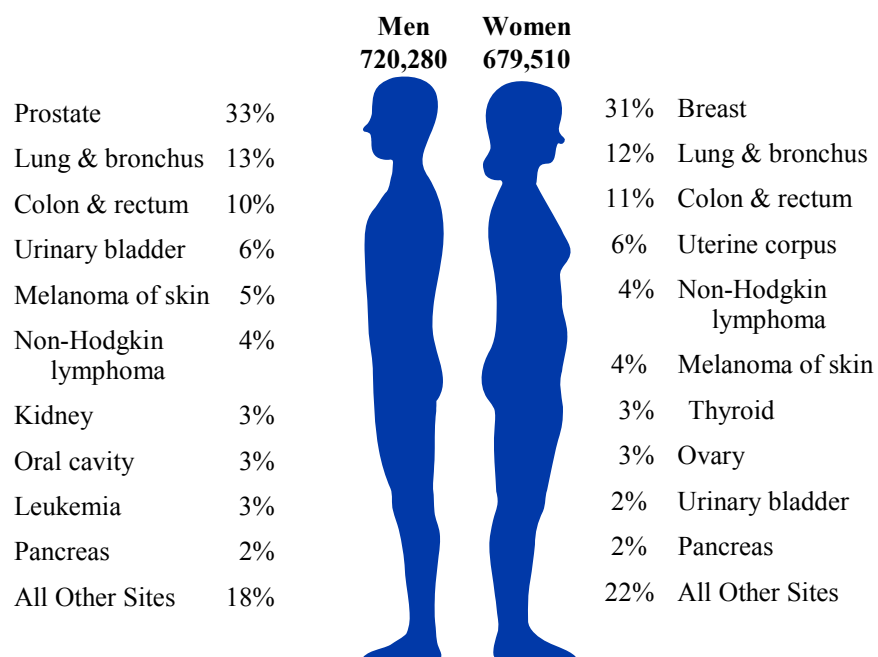
**Figure 2: 2006 Estimated US Cancer Incidence By Site and Sex. Data exclude basal and squamous cell skin cancers [5].**

It was estimated that in 2000 there were 12,200 bladder cancer deaths, 8100 men and 4100 women, making bladder cancer the seventh most common cause of cancer deaths in American men [3]. Bladder cancer accounts for 2.9% of all cancer deaths in

men and 1.5% in women. Even though mortality rates have decreased since the 1970s among African Americans and stabilized since the late 1980s among whites [6], an estimated 13,060 deaths occurred in 2006, as shown in **Figure 3**. The 5-year relative survival rate for bladder cancer is 83% for whites and 64% for African Americans. The survival rate has improved somewhat since the 1970s, mainly due to earlier detection.

### Estimated New Cancer Cases and Deaths by Sex for All Sites, US, 2006*

| | Estimated New Cases | | | Estimated Deaths | | |
|---|---|---|---|---|---|---|
| | Both Sexes | Male | Female | Both Sexes | Male | Female |
| All sites | 1,399,790 | 720,280 | 679,510 | 564,830 | 291,270 | 273,560 |
| Oral cavity & pharynx | 30,990 | 20,180 | 10,810 | 7,430 | 5,050 | 2,380 |
| Tongue | 9,040 | 5,870 | 3,170 | 1,780 | 1,150 | 630 |
| Mouth | 10,230 | 5,440 | 4,790 | 1,870 | 1,100 | 770 |
| Pharynx | 8,950 | 6,820 | 2,130 | 2,110 | 1,540 | 570 |
| Other oral cavity | 2,770 | 2,050 | 720 | 1,670 | 1,260 | 410 |
| Digestive system | 263,060 | 137,630 | 125,430 | 136,180 | 75,210 | 60,970 |
| Esophagus | 14,550 | 11,260 | 3,290 | 13,770 | 10,730 | 3,040 |
| Stomach | 22,280 | 13,400 | 8,880 | 11,430 | 6,690 | 4,740 |
| Small intestine | 6,170 | 3,160 | 3,010 | 1,070 | 560 | 510 |
| Colon† | 106,680 | 49,220 | 57,460 | 55,170 | 27,870 | 27,300 |
| Rectum | 41,930 | 23,580 | 18,350 | | | |
| Anus, anal canal, & anorectum | 4,660 | 1,910 | 2,750 | 660 | 220 | 440 |
| Liver & intrahepatic bile duct | 18,510 | 12,600 | 5,910 | 16,200 | 10,840 | 5,360 |
| Gallbladder & other biliary | 8,570 | 3,720 | 4,850 | 3,260 | 1,280 | 1,980 |
| Pancreas | 33,730 | 17,150 | 16,580 | 32,300 | 16,090 | 16,210 |
| Other digestive organs | 5,980 | 1,630 | 4,350 | 2,320 | 930 | 1,390 |
| Respiratory system | 186,370 | 101,900 | 84,470 | 167,050 | 93,820 | 73,230 |
| Larynx | 9,510 | 7,700 | 1,810 | 3,740 | 2,950 | 790 |
| Lung & bronchus | 174,470 | 92,700 | 81,770 | 162,460 | 90,330 | 72,130 |
| Other respiratory organs | 2,390 | 1,500 | 890 | 850 | 540 | 310 |
| Bones & joints | 2,760 | 1,500 | 1,260 | 1,260 | 730 | 530 |
| Soft tissue (including heart) | 9,530 | 5,720 | 3,810 | 3,500 | 1,830 | 1,670 |
| Skin (excluding basal & squamous) | 68,780 | 38,360 | 30,420 | 10,710 | 6,990 | 3,720 |
| Melanoma – skin | 62,190 | 34,260 | 27,930 | 7,910 | 5,020 | 2,890 |
| Other nonepithelial skin | 6,590 | 4,100 | 2,490 | 2,800 | 1,970 | 830 |
| Breast | 214,640 | 1,720 | 212,920 | 41,430 | 460 | 40,970 |
| Genital system | 321,490 | 244,240 | 77,250 | 56,060 | 28,000 | 28,060 |
| Uterine cervix | 9,710 | | 9,710 | 3,700 | | 3,700 |
| Uterine corpus | 41,200 | | 41,200 | 7,350 | | 7,350 |
| Ovary | 20,180 | | 20,180 | 15,310 | | 15,310 |
| Vulva | 3,740 | | 3,740 | 880 | | 880 |
| Vagina & other genital, female | 2,420 | | 2,420 | 820 | | 820 |
| Prostate | 234,460 | 234,460 | | 27,350 | 27,350 | |
| Testis | 8,250 | 8,250 | | 370 | 370 | |
| Penis & other genital, male | 1,530 | 1,530 | | 280 | 280 | |
| Urinary system | 102,740 | 70,940 | 31,800 | 26,670 | 17,530 | 9,140 |
| Urinary bladder | 61,420 | 44,690 | 16,730 | 13,060 | 8,990 | 4,070 |
| Kidney & renal pelvis | 38,890 | 24,650 | 14,240 | 12,840 | 8,130 | 4,710 |
| Ureter & other urinary organs | 2,430 | 1,600 | 830 | 770 | 410 | 360 |
| Eye & orbit | 2,360 | 1,230 | 1,130 | 230 | 110 | 120 |
| Brain & other nervous system | 18,820 | 10,730 | 8,090 | 12,820 | 7,260 | 5,560 |
| Endocrine system | 32,260 | 8,690 | 23,570 | 2,290 | 1,020 | 1,270 |
| Thyroid | 30,180 | 7,590 | 22,590 | 1,500 | 630 | 870 |
| Other endocrine | 2,080 | 1,100 | 980 | 790 | 390 | 400 |
| Lymphoma | 66,670 | 34,870 | 31,800 | 20,330 | 10,770 | 9,560 |
| Hodgkin lymphoma | 7,800 | 4,190 | 3,610 | 1,490 | 770 | 720 |
| Non-Hodgkin lymphoma | 58,870 | 30,680 | 28,190 | 18,840 | 10,000 | 8,840 |
| Multiple myeloma | 16,570 | 9,250 | 7,320 | 11,310 | 5,680 | 5,630 |
| Leukemia | 35,070 | 20,000 | 15,070 | 22,280 | 12,470 | 9,810 |
| Acute lymphocytic leukemia | 3,930 | 2,150 | 1,780 | 1,490 | 900 | 590 |
| Chronic lymphocytic leukemia | 10,020 | 6,280 | 3,740 | 4,660 | 2,590 | 2,070 |
| Acute myeloid leukemia | 11,930 | 6,350 | 5,580 | 9,040 | 5,090 | 3,950 |
| Chronic myeloid leukemia | 4,500 | 2,550 | 1,950 | 600 | 300 | 300 |
| Other leukemia‡ | 4,690 | 2,670 | 2,020 | 6,490 | 3,590 | 2,900 |
| Other & unspecified primary sites‡ | 27,680 | 13,320 | 14,360 | 45,280 | 24,340 | 20,940 |

*Rounded to the nearest 10; estimated new cases exclude basal and squamous cell skin cancers and in situ carcinomas except urinary bladder. About 61,980 carcinoma in situ of the breast and 49,710 melanoma in situ will be newly diagnosed in 2006. †Estimated deaths for colon and rectum cancers are combined. ‡More deaths than cases suggests lack of specificity in recording underlying causes of death on death certificates.

**Source:** Estimates of new cases are based on incidence rates from 1979 to 2002, National Cancer Institute's Surveillance, Epidemiology, and End Results program, nine oldest registries. Estimates of deaths are based on data from US Mortality Public Use Data Tapes, 1969 to 2003, National Center for Health Statistics, Centers for Disease Control and Prevention, 2006.

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**Figure 3: Types of cancers in United States, with incidence and resulting death rate.**

# Findings

The most common presenting symptom of bladder cancer is painless hematuria, which occurs in more than 85% of patients [7]. However, hematuria is often intermittent, and the lack of hematuria does not rule out the presence of bladder cancer [8]. If an adult has unexplained gross or microscopic hematuria, cystoscopic examination is warranted. The symptom complex of bladder irritability and urinary frequency, urgency, and dysuria is the second most common presentation and is usually associated with diffuse carcinoma in situ or invasive bladder cancer. These symptoms almost never occur without at least microscopic hematuria.

Other signs and symptoms of bladder cancer include flank pain from ureteral obstruction, lower extremity edema and pelvic mass. Very rarely, patients present with symptoms of advanced disease, such as weight loss and abdominal or bone pain. Hepatomegaly and supraclavicular lymphadenopathy are signs of metastatic disease. Pyuria may be present, either due to a concomitant infection or inflammation of the urothelium around the tumor. Azotemia due to ureteral obstruction or anemia from chronic blood loss also may be found.

Cytologic specimens from bladder washings may allow tumor detection and are useful in screening patients or assessing response to treatment. Urine cytology is more sensitive in patients with high-grade tumors or carcinoma in situ, but even in patients with high-grade tumors, cytology may be falsely negative in as many as 20%of cases.

# Pathology

Nearly all bladder cancers are of epithelial origin, with the vast majority being transitional cell carcinomas. The urothelium of the normal bladder is three to seven layers thick, with a basal cell layer and one or more layers of intermediate cells in between the basal layer and the superficial basal layer. The most superficial layer is composed of large, flat, umbrella cells and the entire urothelium rests on the lamina propria basement membrane.

Epithelial hyperplasia describes an increase in the number of cell layers without nuclear or architectural abnormalities, whereas urothelial metaplasia refers to a non-transitional epithelial appearance of the bladder lining, with associated squamous or adenomatous metaplasia. Atypical hyperplasia is similar to epithelial hyperplasia, except that there are also nuclear abnormalities and a partial derangement of the umbrella cell layer [9]. The term dysplasia denotes epithelial changes that are intermediate between normal urothelium and carcinoma in situ. Dysplastic cells have large, round, notched, basally situated nuclei that do not exhibit the normal epithelial polarity. Dysplastic epithelium does not have an increased number of cell layers or mitotic figures [7], but a large portion of patients with moderate dysplasia tend to develop high-grade urothelial cancer [10].

Carcinoma in situ may appear cystoscopically as a velvety patch of erythematous mucosa, although it may be endoscopically invisible. It consists of a poorly differentiated transitional cell carcinoma confined to the urothelium, **<u>Figure 4</u>**. Carcinoma in situ may be asymptomatic or may produce severe symptoms of urinary frequency, urgency and dysuria. Urine cytopathology is positive in 80% to 90% of patients with carcinoma in situ

[11]. Carcinoma in situ is present in 25% or more of patients with high-grade superficial tumors [9],  and between 40 and 83% progress to muscle-invasive cancer[12]. It also is present in 20 to 75% of high-grade muscle-invasive cancers [13]. About 20% of patients treated with cystectomy for diffuse carcinoma in situ are found to have microscopic muscle-invading cancer [14].
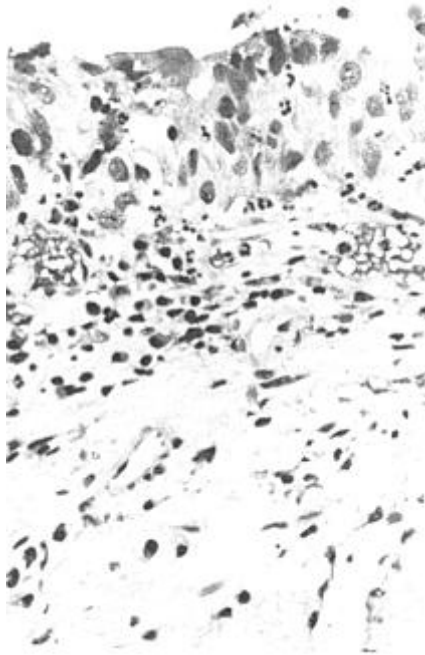

**Figure 4: Carcinoma in situ [15].**

# Transitional Cell Carcinoma

The most common bladder cancers are transitional cell (TCC), squamous cell and adenocarcinomas. More than 90% of bladder cancers are TCC, which exhibit an increased number of epithelial cell layers with papillary foldings of the mucosa, loss of cell polarity, abnormal cell maturation with increased nuclear-cytoplasmic ratio, prominent nucleoli, clumping of chromatin and increased number of mitoses. TCC manifests in a variety of patterns of tumor growth, including papillary, sessile, infiltrating, nodular, mixed, and flat intraepithelial growth (carcinoma in situ).

Approximately 70% of bladder tumors are papillary, 10% are nodular and 20% are mixed.

No uniformly accepted grading system for TCC exists, but most systems are based on the degree of anaplasia of tumor cells [16, 17]. A strong correlation exists between tumor grade and stage [18], with most well-differentiated and moderately differentiated tumors being superficial and most poorly differentiated tumors being muscle invasive. There is a significant correlation between tumor grade and prognosis, however, the correlation between tumor stage and prognosis is even stronger.

## Nontransitional Cell Carcinomas

Squamous cell carcinoma accounts for only 5% of bladder cancers in Europe and the United States [19, 20], but as much as 75% in Egypt, where *Schistosoma haematobium* cystitis is causally related to the development of squamous cell carcinoma of the bladder [21]. Adenocarcinomas account for less than 2% of primary bladder cancers [20], and are prevalent in intestinal urinary conduits, augmentations, neobladders and bladder exstrophy [22]. About 5% of all cases are mixed, and other epithelial cancers include carcinoid, carcinosarcomas and melanomas. Rare nonepithelial cancers of the bladder include pheochromocytomas, choriocarcinomas and mesenchymal tumors.

# Causes

Chemical carcinogens have been implicated in the pathogenesis of bladder cancer, but there are undoubtedly a large portion of cases with no obvious exposure to such agents. Occupational exposure accounts for roughly 20% of bladder cancer cases in the United States [23], and the latency period is around 30-50 years, with more intensive exposures leading to shorter latency periods [24]. Most bladder carcinogens are aromatic

compounds. Nevertheless, the list is non-exhaustive, from aniline dyes [25], to combustion gases and soot from coal, chlorinated aliphatic hydrocarbons [26], dietary nitrites and nitrates [27] and aldehydes used in the rubber and textile industries [28].

Although the mechanisms of carcinogenesis in the presence of inflammation are not understood [29] there appears to be a clear association of inflammation and squamous cell carcinoma of the bladder. Chronic cystitis in the form of severe, long-term infections leads to an increased risk [30]. There also is an association with the presence of long-term indwelling catheters, since 2-10% of paraplegics with long-term indwelling catheters develop malignancy. Cigarette smoking also has been reported to be significantly associated with an increased risk of transitional and squamous cell bladder carcinomas [31], leading to a fourfold higher incidence of bladder cancer compared to people who have never smoked [32, 33], with the risk correlating with the number of pack years.

Use of cyclophosphamide leads to a ninefold increased risk of developing bladder cancer [32], with a relatively short latent period between 6 and 13 years. These are high grade and muscle infiltrating tumors at the time of diagnosis, with an equal incidence in both sexes [34]. The urinary metabolite acrolein is believed to be the responsible agent [35], and mesna, 2-mercaptoethanesulfonic acid, reduces the risk of bladder cancer in patients who develop hemorrhagic cystitis [36].

Specific genetic associations are still unknown, but are likely to involve activation of oncogenes or loss of function of tumor suppressor genes. Some of the most common genetic abnormalities identified in bladder cancer include loss of chromosomes 11p or 17p, which map to the proto-oncogene c-ras and the tumor suppressor gene p53, respectively[37].

# Natural History

Malignant transitional cells invading the lamina propria and the muscularis propria can gain access to blood vessels and lymphatics. Roughly 5% of patients with well-differentiated or moderately differentiated superficial papillary cancer and approximately 20% with high-grade superficial disease (including carcinoma in situ) ultimately manifest vascular or lymphatic spread, indicating that many patients with superficial malignancies have their initial lesion pathologically understaged and already harbor muscle-invading disease [38].

In the United States, about 60% of all newly diagnosed bladder cancers are well-differentiated or moderately differentiated, superficial papillary TCC [39]. The majority of these patients develop tumor recurrences after endoscopic resection, with up to 25% recurring as higher-grade tumors [13]. Approximately 10% of patients with superficial papillary tumors subsequently develop invasive or metastatic cancer [11, 12], even after a prolonged tumor-free remission of over 5 years [10]. 40% of newly diagnosed bladder cancers are high-grade lesions, more than half of which are muscle invading or more extensive at the time of diagnosis [39]. High-grade superficial tumors tend to recur and develop invasive and metastatic disease far more frequently than low-grade tumors [38].

Most patients with occult metastases develop overt clinical evidence of distant metastases within 1 year [40]. The common sites of metastases are liver, 38%, lung, 36%, bone, 27%, adrenal glands, 21%, and intestine, 13% [15]. High grade disease also spreads by implantation in abdominal wounds or denuded urothelium [41]. Despite advances in treatment of systemic urothelial cancer, few patients with distant metastases survive 5 years [42].

# Staging and Treatment

Because tumor stage is important in determining therapy, accurate staging of bladder cancer is desirable. The main staging system of bladder cancer, also termed Tumor-Node-Metastasis system (TNM), has been revised and developed by the International Union Against Cancer and the American Joint Committee on Cancer [43].

| PRIMARY TUMOR (T) | | | Treatment Option |
| --- | --- | --- | --- |
| Ta | Noninvasive papillary carcinoma | Single, low grade, non-recurrent | Complete Transurethral resection (TUR) |
| | | Multiple, high grade, or recurrent | Complete TUR followed by intravesical BCG or chemotherapy |
| Tis | Carcinoma in situ: flat tumor | | Complete TUR followed by intravesical BCG |
| T1 | Tumor invades lamina propria | | Complete TUR followed by intravesical BCG or chemotherapy |
| T2 | Tumor invades muscle | | Radical cystectomy or Neoadjuvant chemotherapy followed by radical cystectomy or Radical cystectomy followed by adjuvant chemotherapy or Neoadjuvant chemotherapy followed by irradiation |
| T2a | Tumor invades superficial muscle | | |
| T2b | Tumor invades deep muscle (outer half) | | |
| T3 | Tumor invades perivesical fat | | |
| T3a | Microscopically | | |
| T3b | Macroscopically (extravesical mass) | | |
| T4 | Tumor invades prostate, uterus, vagina, pelvic wall, or abdominal wall | | |
| T4a | Tumor invades prostate, uterus, vagina | | |
| T4b | Tumor invades pelvic or abdominal wall | | |
| NODAL INVOLVEMENT (N) | | | |
| N0 | No regional lymph node metastasis | | |
| N1 | Metastasis in a single lymph node, 2 cm or less in greatest dimension | | Systemic chemotherapy followed by selective surgery or irradiation |
| N2 | Metastasis in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension | | |
| N3 | Metastasis in a lymph node more than 5 cm in greatest dimension | | |
| DISTANT METASTASIS (M) | | | |
| M0 | No distant metastasis | | |
| M1 | Distant metastasis | | Systemic chemotherapy followed by selective surgery or irradiation |

**Table 1: TNM Staging System for Bladder Cancer**

Bladder cancer remains a therapeutic challenge, where cystectomy, systemic chemotherapy, radiation, intravesical treatment or a combination of these comprise the spectrum of interventions. Treatments begin from transurethral resection and intravesical therapy of superficial bladder tumors, as indicated in **Table 1**, and include selective surgical cystectomy procedures, before or after chemotherapy or irradiation for muscle invasive disease.

Intravesical instillation with Bacillus Calmette-Guerin (BCG) has proven to be an effective treatment for superficial bladder cancer and for CIS for the last twenty five years, even though the exact mechanism of action remains unknown [44]. Mitomycin C, an antibiotic that inhibits DNA synthesis, is equivalent to BCG for superficial disease in terms of survival rates [45]. Radical cystectomy is the treatment of choice for muscle-invasive bladder cancer, however, only half of these patients will be cured by cystectomy alone [46]. Systemic chemotherapy involves MVAC, methotrexate, vinblastine, adriamycin and cisplatin, which has been the standard combination regimen for nearly two decades. For locally advanced and metastatic bladder cancer, long-term survival is rare. Average survival for patients receiving MVAC is less than 14 months, and approximately 4% remain disease-free at 5 years [47]. Newer agents, such as gemcitabine, have a single-agent response rate of approximately 25% [48].

Even though the mortality rates for bladder cancer have improved over the last thirty years, it is estimated that 13,060 patients in 2006 succumbed of the disease. Current forms of therapy for invasive tumors are limited and there has been little improvement over the last two decades. These daunting statistics beckon for new and better treatment modalities [49].

# Histone Deacetylase Inhibitors as a New Cancer Treatment

Evidence for epigenetic events associated with tumorigenesis centers around DNA methylation and histone acetylation [50]. Post-translational modifications of histones, such as acetylation, phosphorylation and methylation, play an important role in chromatin structure and function and in the regulation of gene expression [51]. Histone acetylation is regulated by two families of enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs), which catalyze, respectively, the addition or the hydrolysis of acetyl groups to lysine residues of nucleosomal histones [52]. The opposing actions of HATs and HDACs allow gene expression to be exquisitely regulated.

Histone hypo-acetylation is involved in the tumorigenesis of many malignancies, and inhibition of HDACs poses as a new strategy in human cancer therapy. To date, many HDAC inhibitors (HDACIs) have been developed and have been shown to be potent inducers of tumor growth arrest, differentiation and apoptosis of tumor cells *in vitro* and in cancer patients in phase I and II clinical trials [53]. Although the precise mechanisms underlying these cellular responses to HDACIs have yet to be characterized, HDACI efficacy may be due at least in part to suppression of cancer cell migration, invasion, metastasis, blood supply and angiogenesis [53].

There are several structurally diverse classes of HDACIs. These include short-chain fatty acids, cyclic and non-cyclic hydroxamates, cyclic peptides or tetrapeptides, benzamides, ketones and hybrids of hydroxamic acid and cyclic tetrapeptide. One of the first known HDACI was sodium N-butyrate [54], a potent growth inhibitor and differentiating agent for many tumor cell lines. During initial clinical trials, limited efficacy was observed with some toxicity and Phase II clinical trials are still on-going.

Phenylacetate and phenylbutyrate gave the same results, as well as the anti-epileptic agent, valproic acid, which has been reported to delay the growth of primary breast cancers and to act against metastases found in the lung [55]. However, all these compounds are weak inhibitors, even in high concentrations and non-selective, with pleiotropic effects [56].

## Histone Deacetylase Inhibitors and Bladder Cancer

T-24 cells are poorly differentiated bladder urothelial cells, derived from an invasive high-grade bladder tumor with metastatic potential [57]. When treated with several HDACIs, such as suberoylanilide hydroxamic acid or Trichostatin A, T-24 cells show changes in a core set of genes that are involved in cell cycle progression, DNA synthesis and apoptosis [58]. Specific changes include an up-regulation of tumor suppressor p21, an accumulation of cells in the G2/M phase of the cell cycle and a decrease in survivin levels [59]. p21 is aberrantly expressed in bladder cancer [60] and when activated, modulates survivin expression [61, 62].

Survivin is a member of the family of inhibitors of apoptosis proteins (IAP), and is expressed in nearly all human carcinomas, including TCC, but not in terminally differentiated adult tissues [63]. It is highly expressed in bladder cancer and bladder cancer cell lines, but not in normal urothelium or in cultured urothelial cells [64, 65], and its expression in bladder tumors correlates with abbreviated survival [64]. Furthermore, the presence of survivin protein and mRNA in urine [66] correlates with the presence of TCC and that when measured in subjects following intravesical BCG or mitomycin C treatment, survivin may serve as a marker for recurrent disease [67].

As a pilot study in the Weiss lab, T-24 cells were treated with valproic acid, a relatively weak HDACI with inhibitor activity in the millimolar range, to assess effects on the IAP survivin (Justin Cohen, unpublished results). Incubation of T-24 cells with 10 mM valproic acid for 24 and 48 hrs caused a 65 and 57% decrease in cell proliferation, respectively, and reduced survivin protein levels by approximately 50% relative to actin protein levels at 0.1 mM (**Figure 5**).
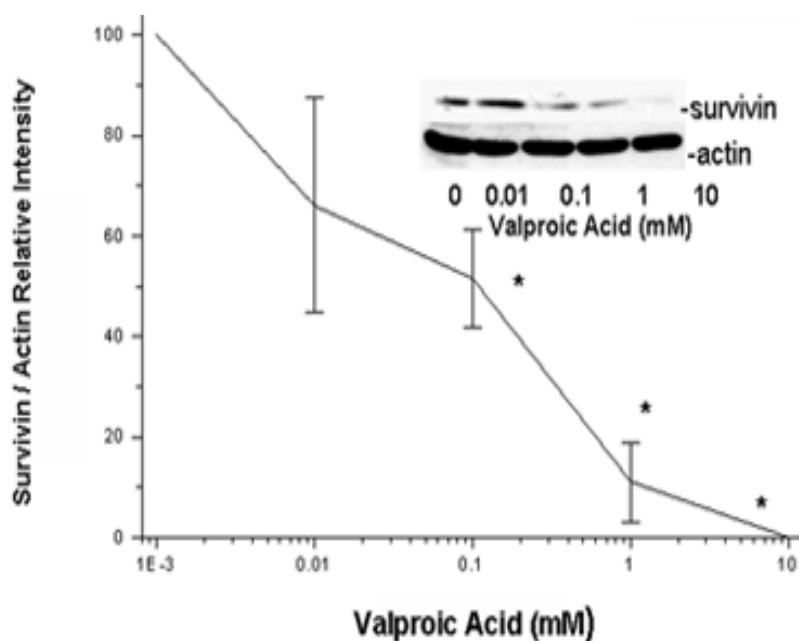


**Figure 5: Effect of Valproic acid on survivin levels in T-24 cells at 24 hours.**

Given the importance of this IAP, the Weiss lab and others have investigated the possibility of a new cancer treatment strategy for TCC by reducing the levels of survivin using RNA interference [68]. This is a process by which a 20-25 nucleotide small interference RNA (siRNA) can lead to specific gene silencing by forming a RNA-induced silencing complex with double-stranded RNA. Because survivin is important in bladder cancer tumorigenesis, this new technique has implications for gene therapy of TCC. This led to siRNA-mediated down-regulation of survivin in two bladder cancer cell

lines, which was associated with reduced viability, a specific G2/M arrest, the induction of apoptosis, the occurrence of multi-nucleated cells and an increase in cytosolic cytochrome c, a marker of mitochondrial damage. The Weiss group also investigated the effects of survivin-siRNA on T-24 bladder cancer cells using microarray analysis of a set of 114 apoptosis related genes. Significant decreases were noted in 14 genes, as shown in **Table 2**.

| Inhibitors of Apoptosis (IAPs) | | Survivin siRNA/ Scramble SiRNA ratio |
|---|---|---|
| BIRC5 | SURVIVIN | 0.02 |
| **TNF receptors & genes related to NF-κB signaling** | | |
| TNFRSF25 | DR3/Apo-1/cd95 (TNF RECEPTOR SUPERFAMILY MEMBER 25) | 0.18 |
| TNFRSF1A | TNFR1  (TNF RECEPTOR SUPERFAMILY MEMBER 1A) | 0.40 |
| LTBR | LYMPHOTOXIN β RECEPTOR(TNF RECEPTOR SUPERFAMILY MEMBER 3) | 0.46 |
| TRADD | TNFRSF1A-ASSOCIATED VIA DEATH DOMAIN | 0.74 |
| Caspases and related genes | | |
| CARD10 | CASPASE RECRUITMENT DOMAIN FAMILY MEMBER 10 | 0.17 |
| CASP4 | CASPASE-4, APOPTOSIS-RELATED CYSTEINE PROTEASE | 0.31 |
| CASP2 | CASPASE-2, APOPTOSIS-RELATED CYSTEINE PROTEASE  ICH-2 | 0.31 |
| **Akt/PKB & related genes** | | |
| Akt /PKB | Akt / PKB (v-Akt MURINE THYMOMA VIRAL ONCOGENE HOMOLOGUE 1) | 0.14 |
| ABL1 | c-Abl (v-Abl ABELSON MURINE LEUKEMIA VIRAL ONCOGENE HOMOLOGUE 1) | 0.20 |
| **Bcl-2 related genes** | | |
| BCL2L11 |    BCL2-LIKE11 (APOPTOSIS FACILITATOR,  BimL) | 0.15 |
| BCL2L13 | BCL2-LIKE 13 (APOPTOSIS FACILITATOR) | 0.19 |
| BNIP1 | NIP-1 (BCL2/ADENOVIRUS E1B 19 kDa INTERACTING PROTEIN 1) | 0.19 |
| BCL2L12 | BCL2-LIKE 12 (APOPTOSIS FACILITATOR) | 0.21 |
| BAK1 | Bak (BCL2 -ANTAGONIST/KILLER 1) | 0.29 |
| **Housekeeping genes** | | |
| GAPD | GAPDH | 0.85 |
| ACTB | BETA- ACTIN | 0.97 |

**Table 2: Changes in apoptotic gene products after treatment of T-24 cells with**

**survivin-siRNA for 72 hours.**

Downregulation of survivin in T-24 cells using survivin-siRNA is associated with a decreased cell growth, a specific G2/M arrest, an increase in cytochrome-c release and altered production of genes related to apoptosis, including four TNF receptors [69], three caspases, and the Bcl-2 related gene, BAK1 [65].

# A new Histone Deacetylase Inhibitor, PXD101

PXD101 is a new hydroxamate HDACI developed by TopoTarget Prolifix in the UK and Curagen in the US. PXD101 was found to inhibit HDAC activity in HeLa extracts with an $IC_{50}$ value of 27 nM. It also inhibited HDAC activity in various cell lysates from ovary, colon, lung or breast with an $IC_{50}$ in the 9–100 nM range. Antitumor activity at a dose of 10 mg/kg daily was observed in xenografts of the human ovarian cell line A2780, with no effect on body weight or apparent toxicity to the mice at ≤40 mg/kg. Antitumor activity was also noted [70] in cell lines relatively resistant to current cytotoxic drugs. PXD101 is well tolerated in rodents and non-rodents and orally bioavailable in dogs. PXD101 entered Phase I clinical trials at the end of 2003 [71]. Synergism with *cis*-platin and 5-Fu has also been reported [72].
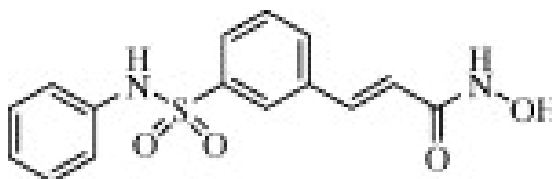


**Figure 6: Histone Deacetylase Inhibitor, PXD101**

No significant improvement in the treatment of bladder cancer has occurred in the past two decades and therefore new modalities of treatment are needed. Survivin is highly expressed in TCC, and its down-regulation causes profound changes in mitosis and apoptosis, which may be related to changes in TNF receptors. HDAC inhibitors inhibit the growth of bladder cancer cells, and also downregulate survivin and other anti-apoptotic proteins in cancer cells. Therefore, we propose to study the effects of PXD101 on survivin levels in T-24 bladder cancer cells, as the chemopreventative role of this new HDACI may involve down-regulation of survivin and other apoptotic signaling proteins.

# Statement of Purpose

Bladder cancer remains a therapeutic challenge as current forms of therapy for invasive tumors are limited. Daunting statistics beckon for new and better treatment modalities. HDACIs mediate gene expression and chromatin assembly, and induce growth arrest and apoptosis of tumor cells, thus representing a new strategy for human cancer therapy. HDACIs inhibit the growth of bladder cancer cells, and also downregulate survivin and other anti-apoptotic proteins in cancer cells. Survivin is highly expressed in TCC and down-regulation of survivin causes profound changes in mitosis and apoptosis. Therefore, changes in apoptosis signaling pathways and the effect on cell growth and cell-cycle arrest of a new HDACI, PXD101, on T-24 bladder cancer cells form the basis of this study.

# Materials and Methods

All experiments and data analysis were conducted by Hristos Z. Kaimakliotis.

**Materials:** Anti-survivin polyclonal antibody was purchased from NovusBiologicals, Inc, Littleton, CO. Anti-caspase-8 monoclonal antibodies was purchased from Cell Signaling Technology, Beverly, MA. Anti-actin goat polyclonal and anti-TNFR1 monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-caspase-3 polyclonal antibody was purchased from R&D Systems, Inc. Anti-caspase-2 polyclonal antibody was purchased from BD Pharmingen Bioscience, San Diego CA. Anti-Bak polyclonal antibody was purchased from Upstate, Charlottesville, VA. Peroxidase-conjugated affiniPure $F(ab')_2$ fragment donkey anti-rabbit IgG and rat anti-mouse antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA.

**Preparation of cells treated with PXD101:** T-24 cells were plated on 6 well plates in McCoy's media with serum and glutamate. Cells were treated after 24 hours, when they were 30-50% confluent. Cells were incubated with PXD101 for up to 72 hours, then trypsinized, treated with trypan blue and counted or prepared for Western blot or FACS cell sorting.

**FACS of propidium iodide (PI) treated cells:** Trypsinized cells were washed with PBS and fixed with 95% ethanol. Two hours prior to cell sorting, ethanol was removed after centrifugation. Since RNA also binds PI, cells were treated with 400 μg RNAse type 1A (Sigma, St. Louis, MO) at 37°C for 30 minutes, followed by PI (50 μg/ml) for 45 minutes (4°C). Cells were sorted and analyzed with ModFit to determine DNA content.

**Western blot analysis:** After removal of media, 20 mmol/L Tris buffer (pH 7.4) containing 1 mmol/L EDTA, 1 mmol/L EGTA, 1% triton X-100, phosphatase and protease inhibitors, was added to culture wells to lyse the cells (10 minutes, 4°C). Then, 2x SDS sample buffer with 5% β-mercaptoethanol was added, cells were scraped and collected. Pairs of samples were normalized for each time point and concentration of PXD101, based on actin quantification determined after Western blot. To normalize the samples, standard loading volumes were used and samples were subjected to Western blot analysis and normalized volumes were determined by adjusting loading volumes with respect to actin immunoreactivity of the protein bands on the resulting blot.

For all Western blots, after separation of proteins by SDS-PAGE, proteins are transferred to nitrocellulose membranes. After incubation with the appropriate primary and secondary antibodies, immunoreactive proteins were detected using chemiluminescence (ECL, Amersham Biosciences, Uppsala, Sweden). Band densities from photographic film of the blot were quantified using digital image analysis (Eastman Kodak Company, Rochester, NY).

**Cell proliferation assay:** Cell proliferation in the presence and absence of the HDACI, PXD101 was measured by the cleavage of the tetrazolium salt WST-1 to formazan by mitrochondrial dehydrogenases (EMD Bioscience, Darmstadt, Germany).

**Statistics:** For each experiment a PXD101 concentration curve was constructed, using vehicle in control samples. Results of at least 3 different experiments performed in duplicate were expressed as a mean % change over control $\pm$ SEM. Significance was determined by ANOVA ($p<0.05$).

# Results

Changes in apoptosis signaling pathways and the effect on cell growth and cell-cycle arrest of the new HDACI, PXD101, on T-24 bladder cancer cells form the basis of this study and results are presented below.
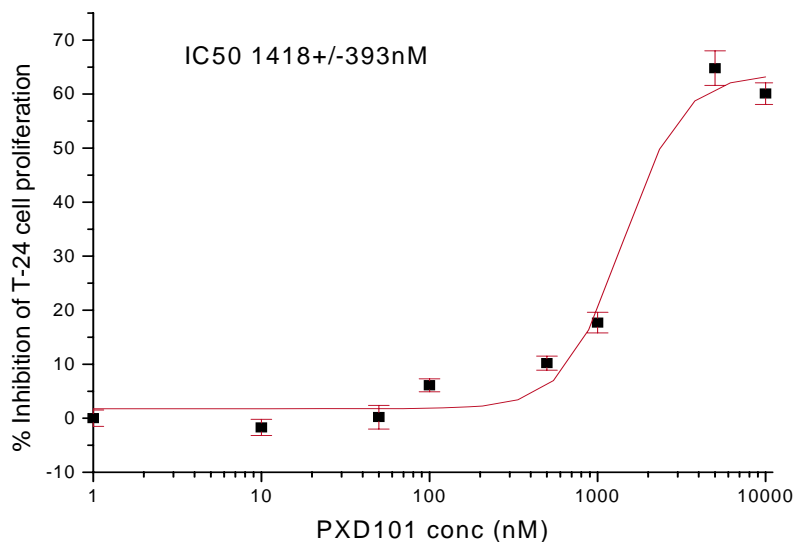
## Cell Proliferation and Viable Cell Counts

T-24 cells were incubated with PXD101 at varying concentrations and times, and proliferation and viable cell count curves were constructed. In the presence of 5 μM PXD101 for 48 hours, T-24 cells exhibited a 56.7% ± 4.2 decrease in proliferation (**Figure 7**).
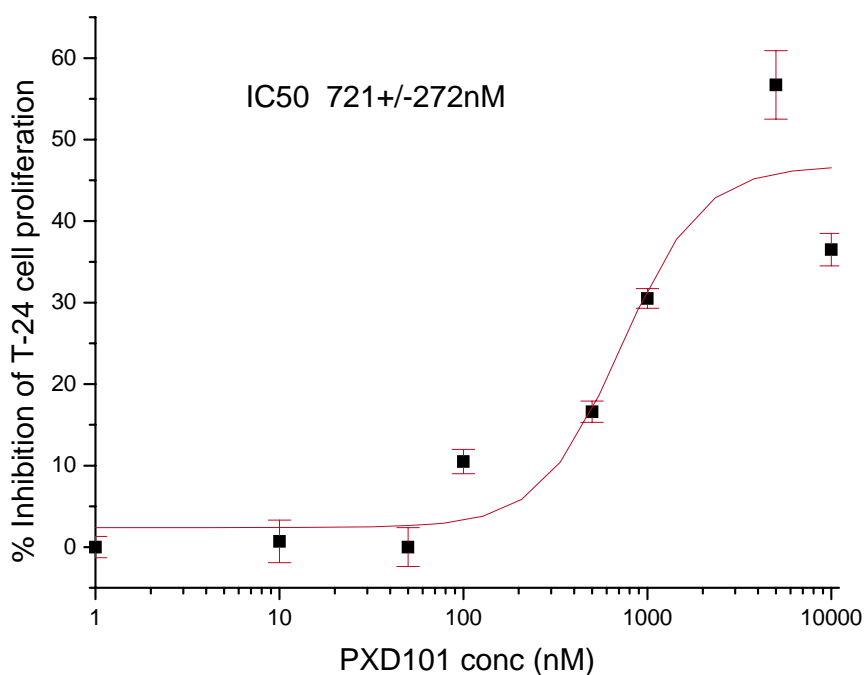


**Figure 7: T-24 cell inhibition at 48 hours in presence of PXD101.**

In the presence of 5 μM PXD101 for 72 hours, T-24 cells exhibited a 64.8% ± 3.2 decrease in proliferation (**Figure 8**).



**Figure 8: T-24 cell inhibition at 72 hours in presence of PXD101.**

Viable cell counts of T-24 cells incubated for 24 and 48 hours with 5 μM PXD101 decreased by 30.6% ± 4.8 and 89.0% ± 3.0, respectively. The $IC_{50}$ of PXD101 for proliferation and viable cell counts was approximately 1 μM (**Figure 9**).
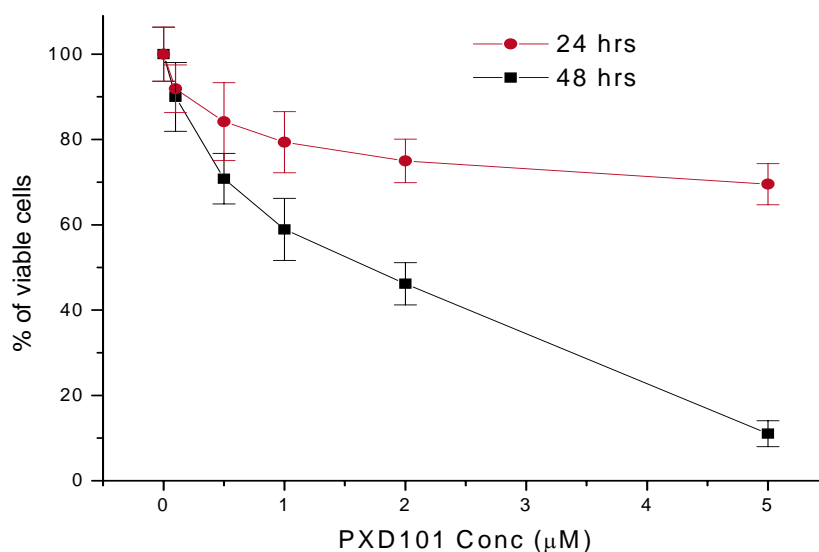


**Figure 9: T-24 viable cell counts in presence of PXD101 for 24 hrs and 48 hrs.**

# FACS Analysis

Cell cycle analysis was conducted with FACS. Treatment of T-24 bladder cancer cells with PXD101 (2 and 5 µM, 48 hrs) increased apoptotic cells (sub-G1 cells) 1.3 and 2.3 fold, respectively (**Figure 10**). Apoptotic cells appeared as a distinct population of decreased size and cell density, as expected.
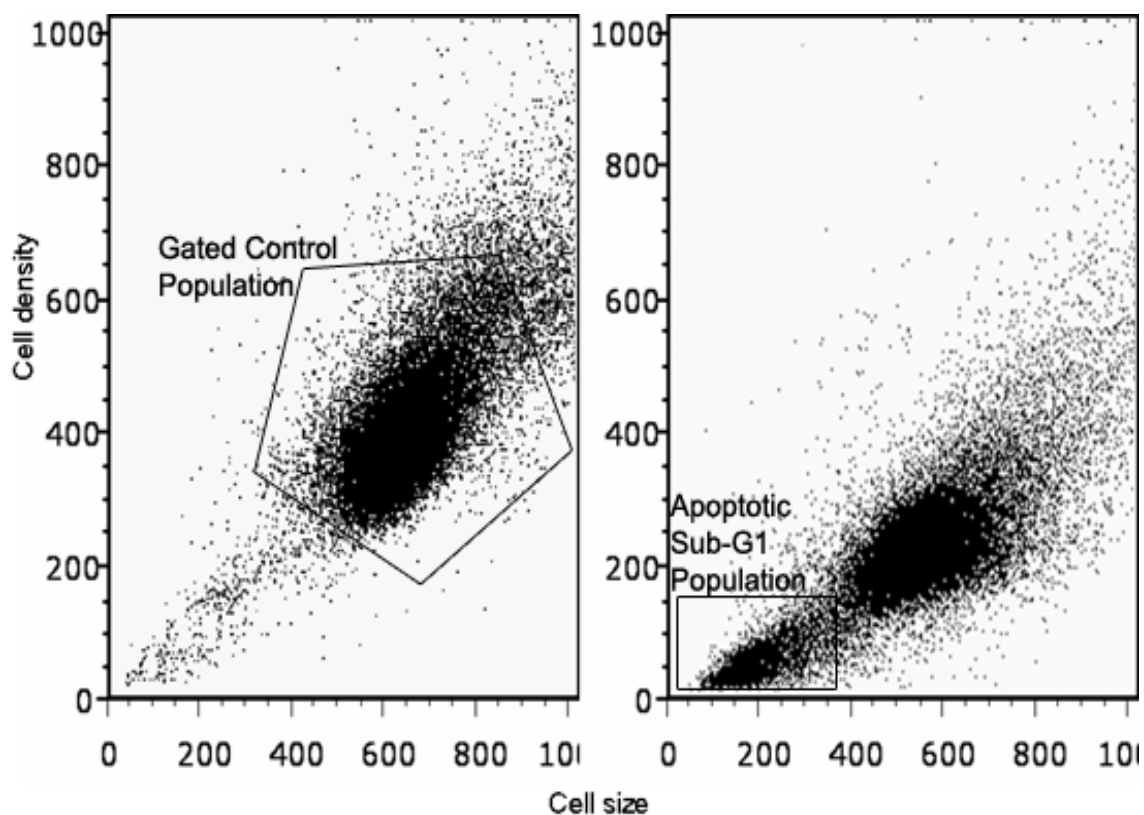


**Figure 10: T-24 bladder cancer cells FACS scatter plots of control (left) vs. treated (right, 5 µM PXD 101 for 48 hours).**

The untreated gated control population was used for cell-cycle population analysis and comparison, and cell distributions. All changes reported in **Table 3** are significant. T-24 bladder cancer cells treated with PXD101 (1, 2 and 5 µM, 48 hrs) decreased G1 phase

cells by 41-54% and increased S and G2/M phase cells 2.5-3.5 fold (**Figure 11**). No

significant changes in cell cycle distribution were noted at 12 or 24 hours.

| | Time (hr) | 0μM PDX101 | 1μM PDX101 | 2μM PDX101 | 5μM PDX101 |
|---|---|---|---|---|---|
| Cell Cycle Changes over Control | | | | | |
| $G_0/G_1$ phase | 48 | | -41.2% $\pm$ 3.8 | -53.7% $\pm$ 3.1 | -53.9% $\pm$ 0.9 |
| S phase | 48 | | +287.4%$\pm$127.4 | +343.0%$\pm$125.7 | +364.8%$\pm$124.3 |
| $G_2/M$ phase | 48 | | +250.5%$\pm$29.8 | +271.3%$\pm$33.3 | +321.5%$\pm$39.1 |
| Cell Cycle Distribution | | | | | |
| $G_0/G_1$ phase | 48 | 84.2.0%$\pm$2.9 | 49.3%$\pm$2.2 | 40.1% $\pm$ 2.0 | 38.9% $\pm$ 1.9 |
| S phase | 48 | 5.5% $\pm$ 1.8 | 14.4%$\pm$0.5 | 17.5% $\pm$ 1.4 | 18.8% $\pm$ 1.8 |
| $G_2/M$ phase | 48 | 7.9% $\pm$ 1.3 | 27.9%$\pm$2.7 | 28.8% $\pm$ 2.6 | 33.6% $\pm$ 3.6 |

**Table 3: FACS analysis of cell cycle distribution and changes in T-24 bladder cancer cells treated with PXD101.**

**Figure 11** shows a representative FACS cell-cycle histogram, comparing treated vs. untreated cells at 48 hours.
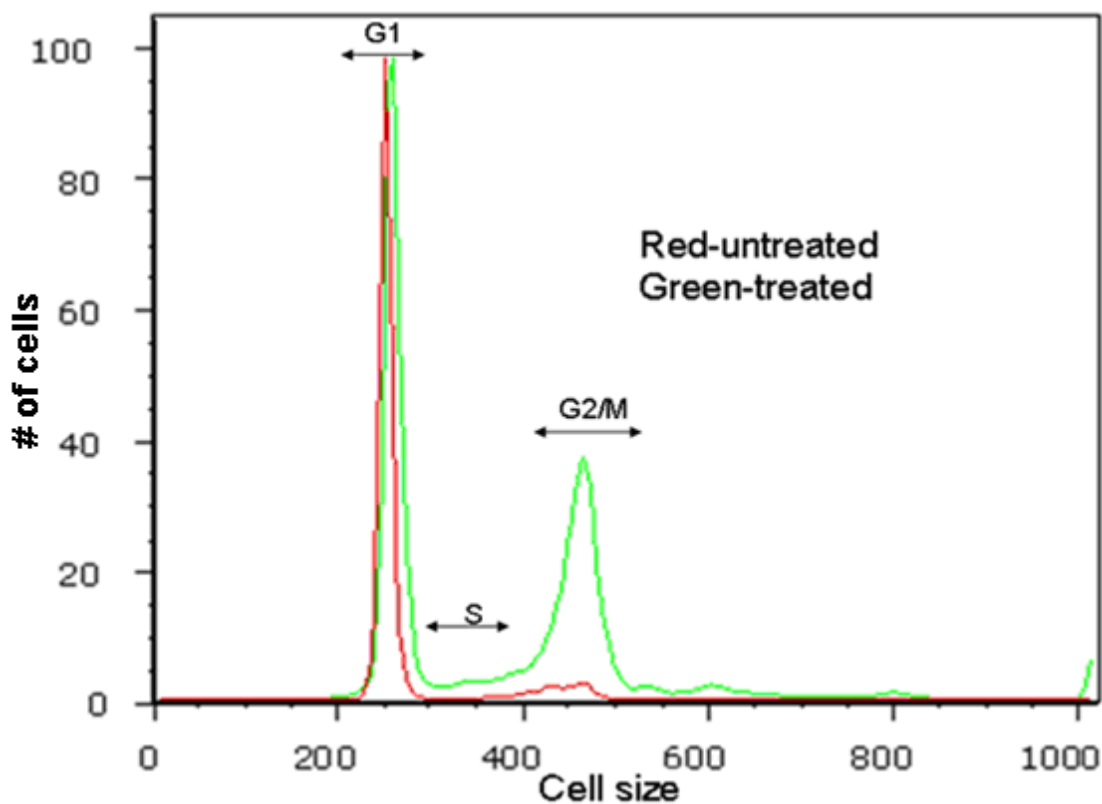


**Figure 11: T-24 bladder cancer cells FACS cell-cycle histogram of untreated (red) vs. treated (green, 5 µM PXD 101 for 48 hours).**

## Western Blot Analysis

Changes in apoptosis signaling proteins that the Weiss laboratory previously found to be regulated by survivin-siRNA in T-24 cells (See **Table 2**) were assessed by Western blot in this study for effects by HDACI PXD101 in T-24 cells. These proteins included survivin, caspases 2, 3 and 8, BAK1 and TNFR1. Actin levels were used to normalize across varying concentrations, as it remains unchanged during induction of apoptosis. Representative blots of each protein assessed are shown in **Figure 12** and all protein level changes are shown in **Figures 14 & 15** and are reported in **Table 4**.
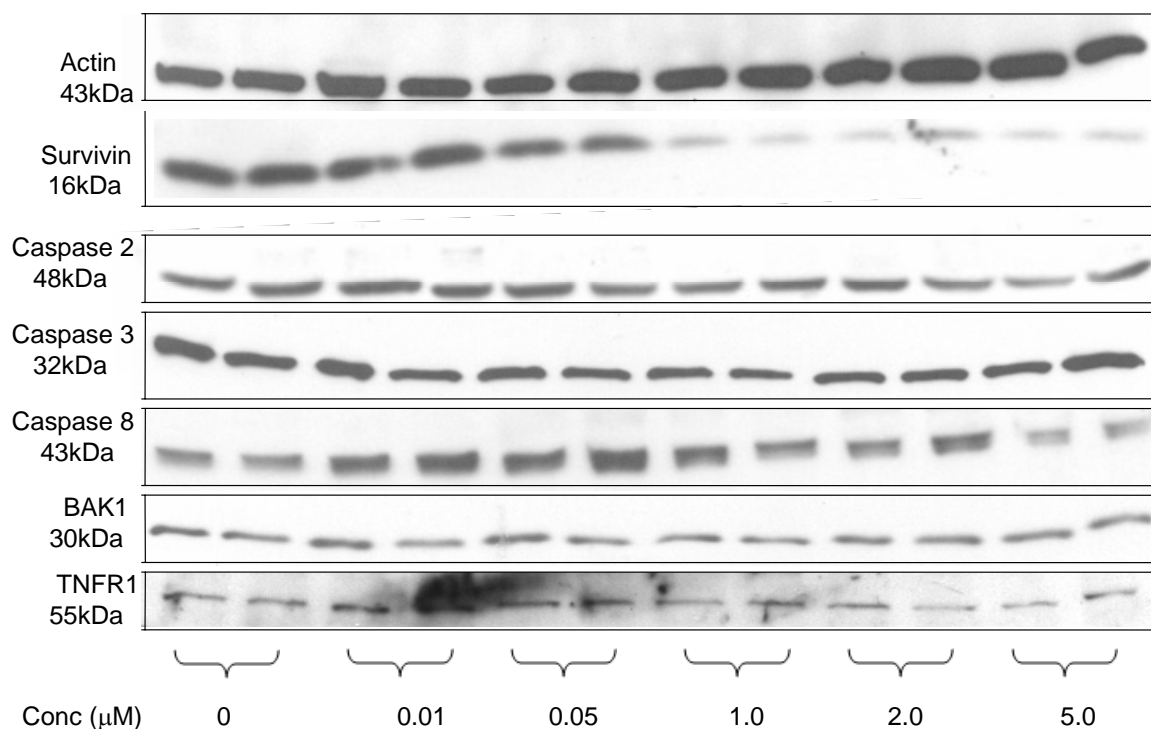


**Figure 12: Representative western blots showing actin, survivin, caspases 2, 3 and 8, BAK1 and TNFR1 levels in T-24 bladder cancer cells treated with PXD101 for 48 hours (experiments were repeated three times for a total n=6).**

The caspases were analyzed using the higher molecular weight pro-caspase band, even though the active protein is the lower molecular cleaved product. One antibody is used for each pro-caspase/caspase pair in question, and although directed at the pro-caspase band, binds both at different rates. The presence of cleaved (activated) caspase was visualized for all three caspases, but only at long film exposures, which deterred its quantitative analysis due to over-exposure and appearance of non-selective binding (**Figure 13**). Decrease in the levels of pro-caspase with the concomitant increase of cleaved activated product indicates up-regulation of caspase pathways and induction of apoptosis.
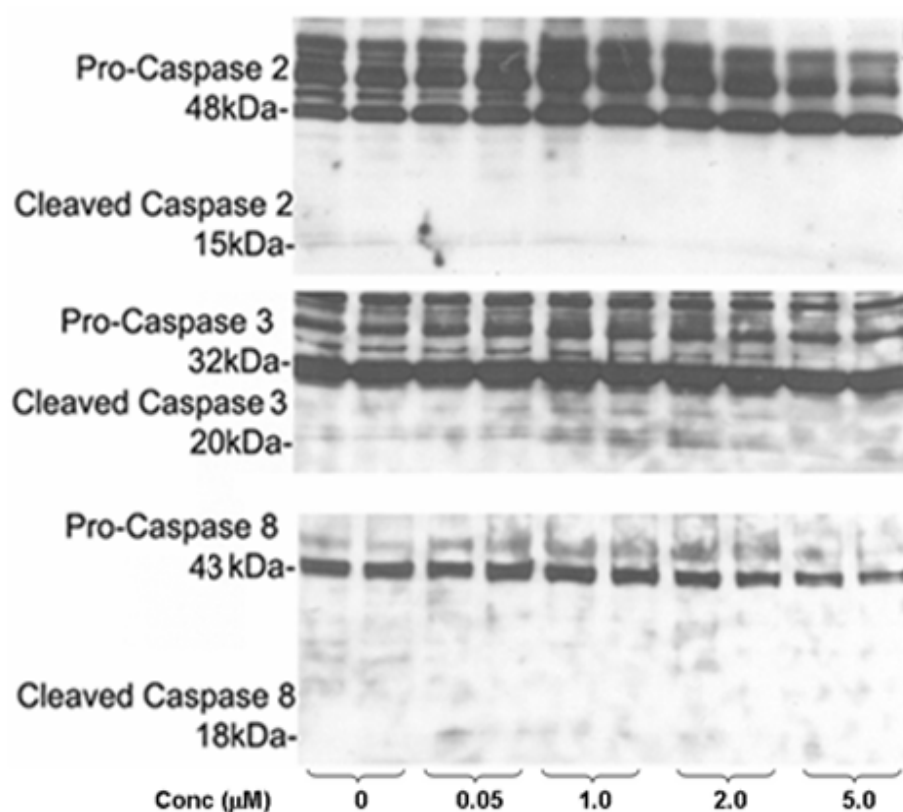


**Figure 13: Representative western blots of pro-caspases 2, 3 and 8 with their respective activated cleaved products in T-24 bladder cancer cells treated with PXD101 for 48 hours (experiments were repeated three times for a total n=6).**

All changes in protein levels are shown in **Figures 14 & 15** and are reported in **Table 4**. Incubation of T-24 cells with PXD101 for 24 hours decreased protein levels of survivin by 30-60% between 0.5-5 μM, and pro-caspase 2 levels by $43.7 \pm 11.9\%$ at 5 μM. The protein levels of TNFR1 and pro-caspase 8 were increased with low concentrations of PDX101 at 24 hours (TNFR1: $112.9 \pm 26.1\%$ and $77.9 \pm 13.9\%$ at 0.1 and 0.5 μM; pro-caspase 8: $140.7 \pm 1.5\%$; 0.5 μM), whereas the protein levels of pro-caspase 3 and of the Bcl2 related protein, BAK1, showed no significant change at 24 hours (**Figure 14**).
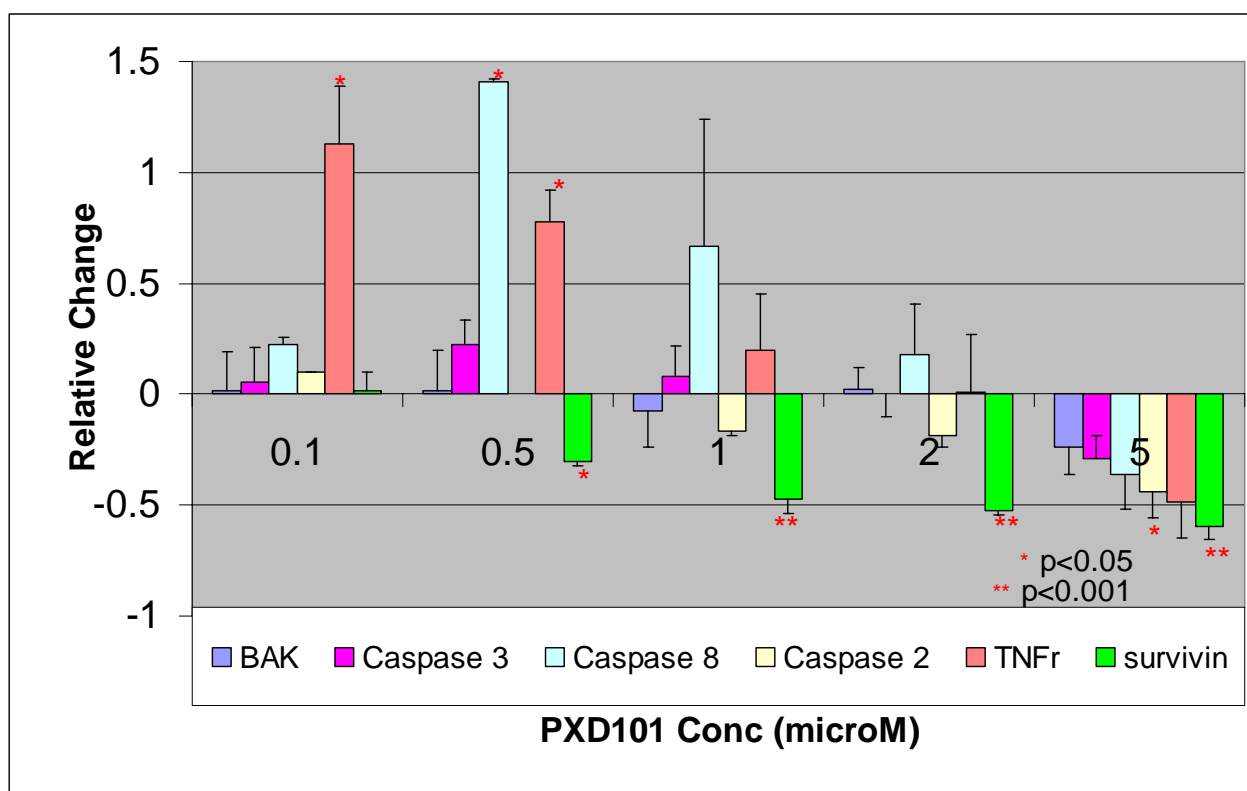


**Figure 14: Changes in levels of apoptosis related proteins, survivin, caspases 2, 3 and 8, BAK1 and TNFR1, in T-24 cells treated with PXD101 at 24 hours (data was generated by quantifying Western blots from three separate experiments, n=6).**

As in the 24 hour experiment, incubation of T-24 cells with PXD101 for 48 hours increased protein levels of TNFR1 by $123.0 \pm 62.9\%$ at the low concentration of 0.1 μM.

Incubation with higher concentrations of PXD101 for 48 hours decreased the levels of all the other proteins tested, as shown in (**Figure 15**).
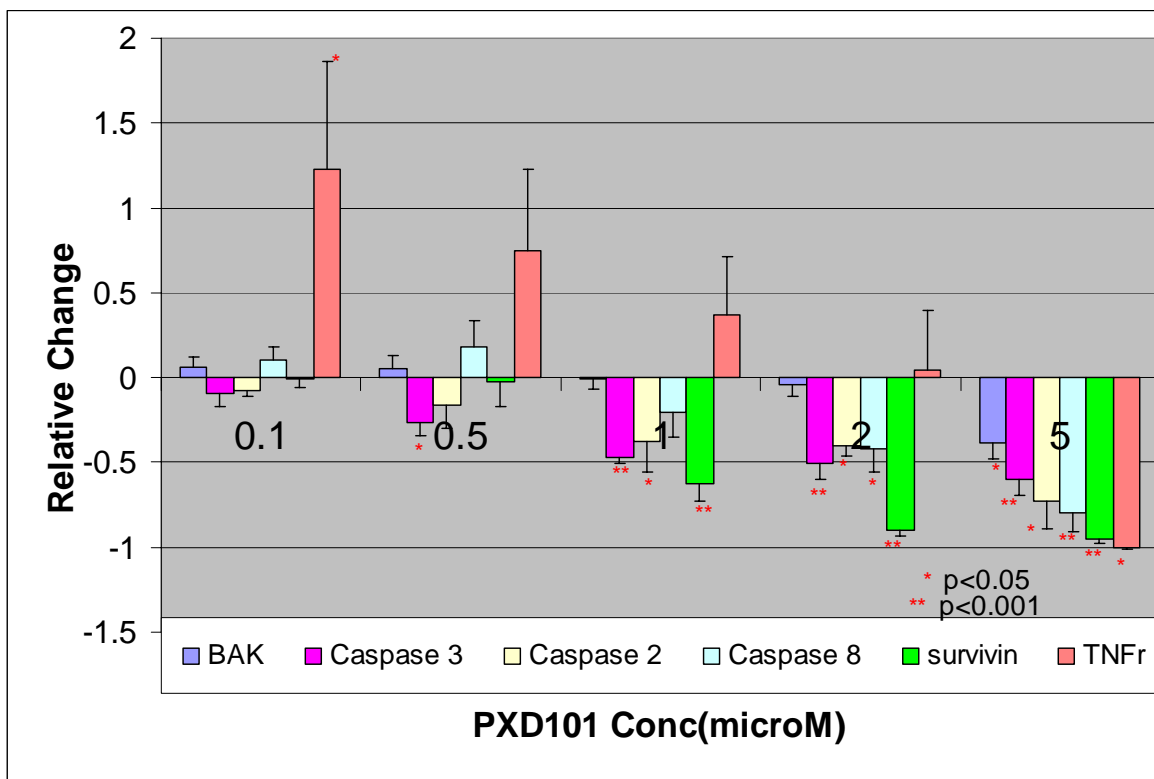


**Figure 15: Changes in levels of apoptosis related proteins survivin, caspases 2, 3 and 8, BAK1 and TNFR1, in T-24 cells treated with PXD101 at 48 hours (data was generated by quantifying Western blots from three separate experiments, n=6).**

| | Time (hr) | 0.1mM PDX101 | 0.5mM PDX101 | 1mM PDX101 | 2mM PDX101 | 5mM PDX101 |
|---|---|---|---|---|---|---|
| BAK1 | 24 | 1.7% + 17.4 | 1.8% + 18.2 | -7.8% + 16.1 | 2.3% + 10.0 | -23.9% + 12.2 |
| Caspase 3 | 24 | 5.4% + 15.8 | 22.3% + 11.1 | 7.8% + 13.8 | 1.0% + 10.3 | -28.8% + 10.2 |
| Caspase 2 | 24 | 10.0% + 0 | 0.5% + 0.1 | -16.9% + 1.5 | -18.3% + 5.4 | -43.7% + 11.9 |
| Caspase 8 | 24 | 22.1% + 3.8 | 140.7%+ 1.5 | 66.8% + 57.4 | 17.8%+ 22.6 | -36.2% + 15.7 |
| Survivin | 24 | 1.4% + .08 | -30.5% + 1.6 | -47.1% + 7.0 | -52.6% + 2.1 | -59.8% + 5.7 |
| TNFR1 | 24 | 112.9%+ 26.1 | 77.9% + 13.9 | -19.5% + 25.9 | 0.8% + 26.2 | -48.8% + 16.1 |
| BAK1 | 48 | 5.7% + 6.7 | 5.6% + 7.3 | -0.9% + 5.7 | -4.3% + 7.0 | -38.8% + 8.8 |
| Caspase 3 | 48 | -9.4% + 7.4 | -26.6% + 7.7 | -46.8% + 3.8 | -50.2% + 9.9 | -60.2% + 8.8 |
| Caspase 2 | 48 | -8.0% + 3.3 | -16.0% + 13.7 | -38.0% + 17.7 | -40.3% + 6.2 | -73.1% + 15.6 |
| Caspase 8 | 48 | 10.0% + 7.9 | 18.5% + 14.7 | -20.1% + 14.9 | -42.0% +13.5 | -79.6% + 11.6 |
| Survivin | 48 | -0.5% + 5.6 | -2.4% + 15.0 | -62.6% + 10.5 | -90.2% + 3.2 | -94.7% + 3.0 |
| TNFR1 | 48 | 123.0%+ 62.9 | 75.0% + 47.8 | 37.0% + 34.3 | -4.0% + 35.8 | -100% + 1.0 |

**Table 4: Relative change in apoptosis proteins survivin, BAK1, TNFR1, caspases 2, 3 and 8, in T-24 cells treated with PXD101 at 24 and 48 hours (red indicates significant changes, n=6).**

# Discussion

Even though the mortality rates for bladder cancer have improved over the last thirty years, it is estimated that over thirteen thousand patients succumbed to the disease in 2006. Current forms of therapy for invasive tumors are limited and there has been little improvement over the last two decades. These daunting statistics beckon for new and better treatment modalities [49].

Histone deacetylase inhibitors (HDACIs) represent a new strategy for human cancer therapy, as they mediate gene expression and chromatin assembly, and induce growth arrest and apoptosis of tumor cells. HDACIs also down-regulate survivin, which is highly expressed in TCC. The Weiss group has previously shown that down-regulation of survivin in T-24 cells using survivin-siRNA is associated with a decreased cell growth, a specific G2/M arrest and altered production of genes related to apoptosis, including TNF receptors [69], caspases, and Bcl-2 related genes [65]. Cytochrome c release into the cytoplasm, the central gate in the activation of apoptosis, was also increased, indicative of mitochrondrial damage [65].

Many of these apoptosis related changes have been reported by the use of several HDACIs in various cancer cell lines. Therefore, changes in apoptosis signaling pathways and the effect on cell growth and cell-cycle arrest of a new HDACI, PXD101, on T-24 bladder cancer cells formed the basis of this study. Western blot analysis of survivin, caspases 2, 3 and 8, Bcl-2-antagonist/killer 1 (BAK1) and TNF receptor superfamily member 1 (TNFR1) was performed. These proteins were chosen for protein analysis because of their high level of gene product expression in T-24 cells and their profound down-regulation when T-24 cells are subjected to survivin-siRNA treatment [65].

In the presence of 5 μM of the new HDACI, PXD101 for 48 and 72 hrs, T-24 bladder cancer cells exhibited 57 and 65% decreases in proliferation, respectively. This decrease in proliferation is greater than changes reported with use of other HDACIs, including valproic acid and phenylbutyrate, at concentrations in this range [73]. Viable cell counts at 24 and 48 hrs (5 μM) were also decreased by 31 and 89% respectively, and the $IC_{50}$ of PXD101 for proliferation and viable cell counts was approximately 1 μM at 48 hours. This is higher than results found in HeLa extracts with an $IC_{50}$ value of 0.03 μM and various cell lysates from ovary, colon, lung and breast with an $IC_{50}$ in the 0.1 μM range [73]. Nonetheless, this relatively low $IC_{50}$ of PXD101 compared to other agents may allow it to be used at lower concentrations, with a lower side effect profile.

Although no changes in cell cycle distribution were noted at 12 or 24 hours, treatment of T-24 cells with PXD101 for 48 hours at 2 and 5 μM increased apoptotic cells (sub-G1 cells) 1.3 and 2.3 fold, respectively. Apoptotic cells appeared as a distinct population of decreased size and cell density, as expected. These dying cells were excluded from analysis of the rest of the cell-cycle phases to provide accurate comparison between live cells of treated and control samples. PXD101 at 1, 2 and 5 μM for 48 hrs, decreased G1 phase cells by 41-54% and increased S and G2/M phase cells between 2.5-3.5 fold. This indicates that mitosis is incomplete, with a specific arrest at the G2/M phase. These results are consistent with other HDACIs' activity and is in accord with the arrest seen when survivin is down-regulated by treatment with survivin-siRNA in T-24 cells [65], hepatocellular carcinoma cells, human sarcoma cells [68] and HeLa cells [74].

As expected, the protein levels of survivin in T-24 cells treated with PXD101 are decreased by 30-60% with 0.5-5 μM PXD101 at 24 hours, and by 62-94% with 1-5 μM

PXD101 at 48 hours. These changes are consistent with survivin-siRNA and other HDACI induced down-regulation of survivin [65], and lead to the apoptotic changes and cell death described earlier, as the IAP is no longer exhibiting its effect in tumor cells.

On the other hand, the TNFR1 protein is dramatically up-regulated at 24 hours of treatment with PXD101 at low concentrations. TNFR1 is increased 113 and 78% with 0.1 and 0.5 μM at 24 hours, and 123.0% at 48 hours with 0.1 μM PXD101. TNF is tumor necrosis factor and increases in TNF receptors and related proteins are considered pro-apoptotic. Up-regulation of TNFR1 and its ligand TNFα, have been shown to occur when apoptosis is induced in leukemic cell with the HDACI, depsipeptide (FK228) [75], further demonstrating the efficacy of PXD101 as an anti-tumor agent.

Activation of the death domain containing receptors, TNF receptor superfamily, is associated with activation of the caspase cascade, cytochrome c release, and apoptosis. To activate TNF receptors, specific adaptor proteins such as FAS-associated death domain (FADD) or receptor-interacting protein [RIP]-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD/CRADD) bind to ligand-bound receptor complexes. Interaction between these adaptor proteins and the prodomain of initiator caspases 2, 8 or 10 triggers sequestration-mediated auto-activation of these caspases. These caspases, in turn, cleave and activate downstream caspases 3, 6, and 7 and trigger apoptosis (**Figure 16**, page 31).

Up-regulation of TNFR1 is reversed at higher concentrations of PXD101 and a dramatic down-regulation of TNFR1 is observed. Several authors have described increases in TNF receptors, ligands and related genes to be associated with induction of apoptosis, but there is growing evidence to suggest that TNFα promotes tumor

development and growth [76]. In a mouse model of metastatic colon adenocarcinoma, TNF$\alpha$ promotes tumor growth [77] and mice deficient in TNF$\alpha$ are resistant to skin carcinogenesis [78]. Furthermore, TNFR1 is the major mediator of TNF$\alpha$-induced tumor formation in skin tumors [79] and during liver carcinogenesis [80]. Thus, while the initial decrease in TNFR1 induced at low concentrations of PXD101 may be considered as part of an activation of apoptotic pathways, the latter increase in TNFR1 at higher PXD101 concentrations may be pro- or anti-apoptotic in T-24 bladder cancer cells.
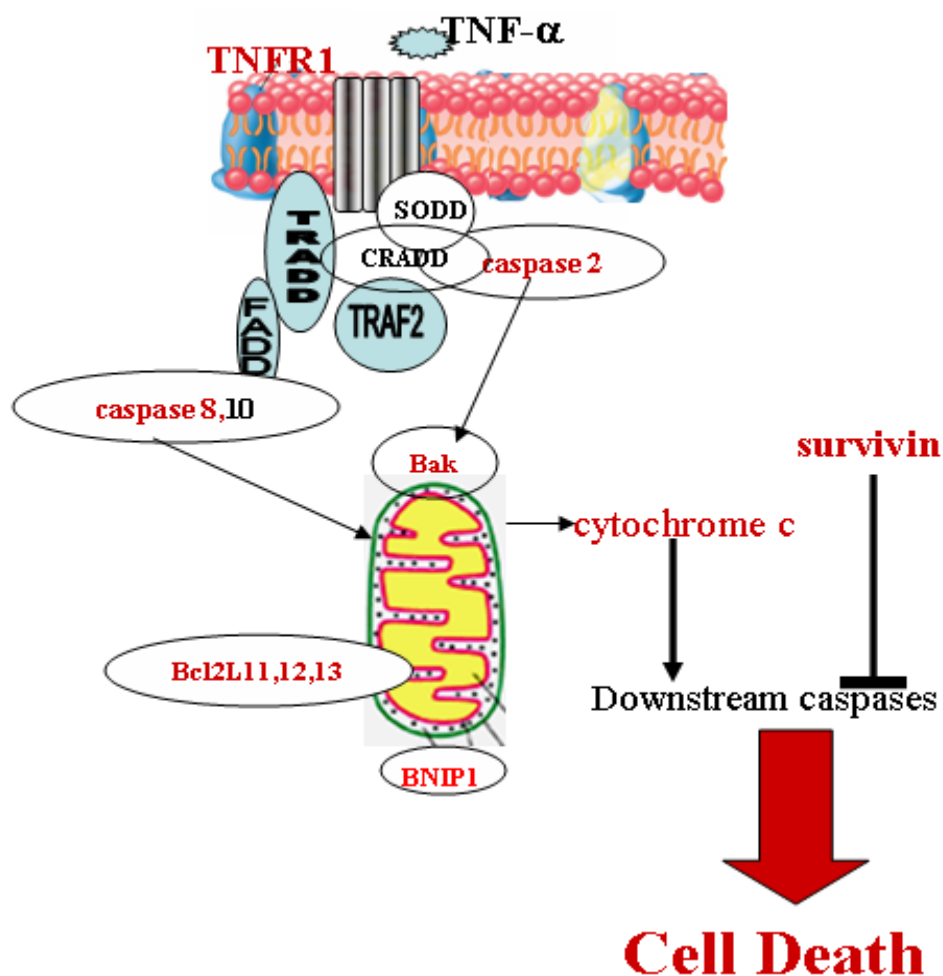


**Figure 16: Signalling pathways in T-24 bladder cancer cells altered by survivin-siRNA. Red lettering indicates that either message or protein levels were modified by survivin-siRNA.**

Similar to TNFR1 protein regulation, pro-caspase 8 levels are up-regulated at low concentrations of PXD101 and down-regulated at high PXD101 concentrations. Caspase 8 is an upstream caspase associated with a number of apoptotic pathways including TNFR1, APO2L/TRAIL and FASL. It is believed that the TNFR1-induced pro-apoptotic signaling pathway requires the formation of two signaling complexes. A rapidly formed plasma membrane bound complex is composed of TNFR1, TRADD, RIP, TRAF2, and c-IAP1 and triggers a NF-κB response, but no apoptosis. A second complex, which lacks TNFR1 but includes FADD and pro-caspases 8 and 10, subsequently forms in the cytoplasm. The complex containing pro-caspase 8 initiates apoptosis, provided that the NF-κB signal from complex I fails to induce the expression of anti-apoptotic proteins such as $FLIP_L$. $FLIP_L$ is an inhibitor of caspase 8 [81]. Thus, up-regulation of both TNFR1 and caspase 8 may be essential for apoptosis.

Along with caspase 8, caspase 2 function as an upstream modulator that can trigger mitochondrial apoptotic pathways and release of cytochrome c [82]. Caspase-2 is required for the translocation of BAX/BAK1 to the mitochondria as well as release of mitochondrial proteins, which leads to apoptosis. Caspase 3 is a downstream effector caspase and is involved in Fas-mediated apoptosis. Decrease of pro-caspase levels with concomitant increase in lower molecular weight activated cleaved product is indicative of caspase pathway activation and induction of apoptosis.

A profound decrease in protein levels of pro-caspases 2, 3 and 8 at higher PXD101 concentrations is noted, with a concomitant appearance of respective cleaved products, further supporting the induction of apoptosis in tumor cells. The cleaved products are only visualized at long film exposures though, deterring quantitative analysis

of cleaved products. The initial up-regulation of pro-caspase 8 at low PXD101 concentration may be due to an initial response by T-24 cells prior to cleavage to the activated product, since caspase 8 is an upstream modulator.

The Bcl-2 family of proteins governs mitochondrial outer membrane permeabilisation and can be either pro-apoptotic or anti-apoptotic, and can induce or inhibit the release of cytochrome c in to the cytosol, which activates caspase-9 and caspase-3, leading to apoptosis. The protein levels of BAK1, a Bcl-2 related protein which promotes the release of cytochrome c, are decreased at high concentrations in T-24 cells after treatment with PXD101. This is similar to our previous results, where expression of many Bcl gene products is altered in T-24 cells after treatment with survivin-siRNA. Survivin-siRNA treatment down-regulates mRNA of apoptosis facilitators, including BCL2-L11, -L12, -L13, BAK1 and BNIP1 (NIP-1, BCL interacting protein 1). BAK1 protein is initially up-regulated by survivin-siRNA, but by 72 hours BAK1 levels decrease. BNIP3, which induces both apoptotic and necrotic death, is also down-regulated in prostate carcinoma cells after treatment with ribosome-mediated inhibition of survivin expression. The complex regulation of these pro-and anti- apoptosis molecules by PXD101 and survivin-siRNA suggests that the relative concentrations of these molecules and the time course of activation or synthesis of these proteins may determine if a cell lives or dies [83].

In summary, T-24 cells treated with PXD101 are characterized by decreased growth and large numbers of cells undergoing apoptosis and arresting in G2/M phase of the cell-cycle. PXD101 treatment is also associated with changes in upstream mitochondrial apoptosis mediators, including TNFR1 and caspases 2 and 8, and

downstream apoptosis mediators, such as caspase 3, BAK1 and survivin. PXD101 treatment of tumor cells is associated with a profound decrease in survivin and pro-caspases' levels, and with an increase in TNFR1 protein levels, both changes indicative of induction of apoptosis. BAK1 levels remain unchanged until high concentrations of the HDACI. This decrease in BAK1 levels, and in fact of all apoptosis related proteins at very high PXD101 concentrations, may be the result of PXD101 treatment or the T-24 cells' response to that treatment, such as generalized cell death with decreased protein synthesis. The down-regulation of survivin may cause T-24 cells to up-regulate TNF receptors and cleave pro-caspases in order to preserve their apoptotic phenotype.

HDACIs have shown profound results in initiating tumor regression and symptomatic improvement in some heavily pre-treated patients at an advanced stage who have experienced multiple relapses. This regression has been achieved with a surprisingly low side-effect profile and a wide therapeutic index [84]. The impressive anti-tumor activity of HDACIs and their lack of toxicity at doses that effectively inhibit tumor growth strongly support these agents as a suitable molecular target for anticancer drug development. Therefore, the new HDACI PXD101, alone or in combination with inhibitors of other tumor relevant factors and with chemotherapies with complementary mechanisms of action, shows promise for its use as a suitable new agent for cancer treatment.

This is the second report indicating up-regulation of TNFR1 with the use of any HDACI and the first to our knowledge that describes an initial increase in protein levels of apoptotic related proteins, only to be followed by a decrease in these levels. Future work on this project will involve investigating the effects of this new HDACI, PXD101,

using real time RT-PCR to assess mRNA levels of the apoptosis-related proteins analyzed above. An ELISA assay of the TNRF1 ligand, TNF$\alpha$, will also be conducted on experiment media, to assess whether there is change in levels of this signaling molecule to further support our findings. A manuscript is currently in preparation to report these results and an abstract has been accepted to be presented at the national meeting of the Society of Genitourinary Surgeons. In addition, our lab has developed transgenic mice that over-express bladder specific survivin [85]. These mice demonstrate increased bladder inflammation and respond to a bladder carcinogen with accelerated tumor formation and shortened survival. The effects of PXD101 in these mice will be tested to determine if they have altered susceptibility to HDACIs and increased survival rates.

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