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Existence of Inhibitor of Apoptosis in Tumor Exosomes

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Existence of Inhibitor of Apoptosis in Tumor Exosomes

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

BIR	Baculovirus IAP repeat
CDDP	Cisplatin
CEA	Carcinoembryonic antigen
cIAP1/2	Cellular inhibitor of apoptosis 1/2
CldA	Cladribine
CM	Conditioned media
DEX	Dendritic cell-derived exosomes
DNMT	DNA methyltransferase
EGFR	Epithelial growth factor receptor
5FU	5-fluorouracil
5FdU	5-fluorodeoxyurodine
Gem	Gemcitabine
hCNT	Human concentrative nucleoside transporters
hENT1	Human equilibrative nucleoside transporter 1
HU	Hydroxyurea
IAP	Inhibitor of apoptosis
MALT	Mucosa-associated lymphoid tissue
MMP	Matrix metalloproteinases
PCR	Polymerase chain reaction
RING	Really interesting new gene
RNAi	RNA interference
RR	Ribonucleotide reductase
RRI	Ribonucleotide reductase inhibitor

shRNA	Small hairpin RNA
TEX	Tumor exosomes
TF	Transcription factor
TS	Thymidine synthase
TSI	Thymidine synthase inhibitor
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

ABSTRACT OF THE DISSERTATION

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Pancreatic cancer is a deadly and aggressive disease. The only option for metastatic pancreatic cancer is chemotherapy where only the antimetabolites gemcitabine (Gem) and 5-fluorouracil (5FU) are used clinically. However, resistance to these antimetabolites remains a problem highlighting the need to discover and develop new antimetabolites that will improve a patient's overall survival. Cancer is a disease that has acquired numerous molecular, biochemical and cellular changes. Resistance to apoptosis is one of the characteristics of cancer, of which the inhibitor of apoptosis (IAP) family of proteins plays an important role. It has also been shown that cancer cells secrete vesicles called tumor exosomes (TEX) which are loaded with bioactive molecules which strongly influences the tumor microenvironment. Both protein and mRNA of the IAP Survivin, cIAP1, cIAP2 and XIAP are released into the extracellular space by not only the pancreatic cancer cell line PANC-1, but also from other cancer and non-cancer cell lines. IAP release may play an important role in pancreatic cancer's lack of response to antimetabolite agents and eventual progression to chemoresistance. These findings can be used to design and develop novel compounds that can be used in combination with Gem or 5FU which are designed to target exosomes, in particular IAP packaging, which may make a vital impact in the treatment for metastatic pancreatic cancer.

CHAPTER ONE

INTRODUCTION

INHIBITOR OF APOPTOSIS (IAPS) IN TUMOR EXOSOMES

Pancreatic Cancer

Pancreatic cancer is an aggressive disease that silently attacks. Today, it is the 4th leading cause of cancer deaths in the United States [1-3]. There are several risk factors that lead to the development of pancreatic cancer which include lifestyle factors, such as smoking and a diet high in fat and nitrite, diseases such as chronic pancreatitis and diabetes, and genetic factors [4] . Symptoms, such as jaundice and back or abdominal pain, are usually presented once the disease has progressed to the advanced stages [2]. Since there are no screening tests and patients are asymptomatic in the early stages of the disease, pancreatic cancer is usually diagnosed in the advanced stages, affecting approximately 85% of the patients. At the time of the diagnosis, the survival time given to the patients is 4-8 months, where less than 1% survive more than 5 years [5-7]. Patients that have metastatic pancreatic adenocarcinoma are treated with the only first-line FDA approved treatment GEMZAR or gemcitabine (Gem) [8]. Gem is an antimetabolite agent that is designed to inhibit replication of DNA and normal cellular metabolic processes [9,10], which will be discussed further in the next chapter. At present, numerous efforts are being made to improve treatment strategies for metastatic pancreatic cancer, such as the search for new antimetabolite drugs, as well as using combinations of therapeutic agents.

Inhibitor of Apoptosis

Cancer is a disease that has acquired a number of molecular, biochemical and cellular changes, which is common in most, even all types of cancer. These changes affect normal cellular physiology, which are essential for malignant growth. The changes include independence from growth signals, loss of sensitivity to antigrowth signals, resistance to apoptosis, unlimited ability to replicate, angiogenesis maintenance, and invasion of tissue and metastasis [11]. Out of these acquired capabilities of cancer cells, we are most interested in the resistance to apoptosis. The inhibitor of apoptosis (IAP) family of proteins is of special interest, which includes cIAP1, cIAP2, XIAP and Survivin.

IAPs are characterized by an ~70 amino acid baculovirus IAP repeat (BIR) domain and a RING domain in the C-terminus of each family member [12,13]. IAPs are known to be endogenous caspase inhibitors [14]. Activated caspase-3, -7 and -9 are inhibited by cIAP1, cIAP2 and XIAP by directly binding to the caspases using their BIR domains [15-18]. Survivin is the smallest IAP family member and is the only IAP that has only one BIR domain and no RING domain, making Survivin structurally unique among the rest of the family [19]. Another unique feature of Survivin is its multifunctional role in various cellular activities, which includes the regulation of mitosis, protection from cell death, and adaptation to stressful environments [20,21]. Survivin is found to be localized in the cytoplasm, mitochondria and nucleus, with its subcellular location determining its function [22,23]. It has been shown that Survivin's role in the regulation of mitosis is carried out by a nuclear Survivin pool [24].

Alternatively, mitochondrial Survivin is able to suppress cell death in tumor cell lines and plays a part in tumorigenesis in immunocompromised mice [25].

Survivin in Cancer & Treatment

Survivin expression is normally seen during the embryonic and fetal developmental stages, but is either low in expression or absent in tissues that are terminally differentiated. Survivin has also been shown to be present in highly proliferative adult cells, such as thymocytes, $CD34^+$ bone-marrow-derived stem cells, T cells, vascular endothelial cells and gastrointestinal tract mucosa. Expression levels of Survivin in these cells are significantly lower compared to tumor cells, where there is a striking overexpression of this IAP in virtually every cancer type. High levels of Survivin expression in cancer cells have been associated with dismal prognosis, disease progression, metastatic dissemination, therapy resistance and overall dismal disease outcome [21,26,27]. In pancreatic cancer cells, radioresistance was enhanced by Survivin, which functioned as an inducible radioresistance factor [28]. Another study has shown that both Survivin mRNA and protein levels were higher in Cisplatin-treated gastric cancer cells compared to untreated cells [29]. Both these studies give indication that Survivin plays an essential role in chemotherapy and radiotherapy resistance, increasing the ability of cancer cells to evade apoptosis, thus providing cytoprotection to malignant cells [30].

To date, Survivin is one of the most tumor specific transcriptome [21], and in addition to its presence in both solid tumor and hemapoeitic malignancy, this IAP makes an exciting target for anti-cancer treatment. There have been many efforts in recent years

to develop novel anti-cancer therapeutics targeting Survivin to both inhibit tumor growth as well as increase tumor cells' sensitivity to conventional chemotherapeutic agents [27,31]. Thus far, there are numerous strategies to target Survivin from mRNA to protein levels. Small molecule inhibitor YM155 acts by inhibiting transcription of Survivin mRNA, while anti-sense oligonucleotides, hammerhead ribozymes and siRNA are designed to degrade Survivin mRNA and/or inhibit protein translation. Strategies to inhibit Survivin at the protein level include small molecule antagonist Sheperdin, which prevents Hsp90/Survivin interaction, as well as expression of two Survivin dominant negative mutants Cys84Ala and T34A into tumor cells introduced by plasmid or viral vectors [27,31].

In recent years, many studies have been done to determine whether downregulation of Survivin could reverse chemotheray and radiotherapy resistance in cancer cells. Several groups have shown that inhibition of Survivin expression by shRNA, RNAi, as well as emodin, a natural compound, resensitizes a variety of cancer cells, including squamous cell carcinoma of the tongue [30], osteosarcoma [32], breast cancer [33], and pancreatic cancer [34,35] to cisplatin, adriamycin, and gemcitabine. All the Survivin based therapies mentioned previously have shown to be successful in decreasing Survivin expression levels, inhibiting further growth of malignant cells and increasing sensitivity to chemo,- and radiotherapies.

Existence of Secreted Membrane Vesicles in Cancers

Tumors are known to shed membrane vesicles [36]. In particular, human and mouse tumor cells have been shown to secrete tumor cell-derived

exosomes (TEX), constitutively into the extracellular space [37]. The morphology, density and certain membrane markers expressed, such as LAMP1, MHC class I, HSP70 and HSP80, on the released TEX are similar to the dendritic cell-derived exosomes (DEX) [38]. Despite similarities to DEX, there are differences in the molecular profiles and biological roles of TEXs, both of which give an indication of the cell of origin [39]. The specific protein content found on and within exosomes not only reflects their origin, but in addition, establishes their functional role [40]. TEX secreted from neoplastic cells express diverse tumor antigens, which signifies the type of tumor cells from where TEXs were released [41]. *In vitro,* it has been shown that TEX released from breast carcinoma cells contain HER2, while carcinoembryonic antigen (CEA) was found in the exosomes secreted from colon carcinoma cells, and proteins MelanA/Mart-1 and gp100 that are expressed in melanoma cells are found on the released TEX [38,42]. This phenomenon is also evident *in vivo,* where plasma from cancer patients contain membrane vesicles that are characterized by the expression of tumor antigens which reflect the tumor of origin [43,44].

When immunocompetent and nude mice were pre-treated with murine mammary TEX, an accelerated growth of the tumor was observed [45]. This observation led to various studies to try to elucidate the role of secreted membrane vesicles in cancer. TEX can be described as “multi-purpose carriers” which have important roles in the communication, protection, as well as the exchange of genetic information with neighboring cells [46]. The production and secretion of TEX is important for the tumor. They serve a protective function, have a supportive role in the survival and growth of the tumor cells, are involved in the promotion of host tissue invasion and subsequent

metastasis, and facilitate evasion from the immune response [47,48]. Acting in a paracrine fashion, the diverse function of TEX is speculated to be due to the various bioactive molecules found within and on the vesicles having a strong influence on the surrounding environment [41,43,44,49].

The promotion of angiogenesis is due in part to the upregulation of vascular endothelial growth factor (VEGF) [50] and release of matrix metalloproteinases (MMPs) in neighboring, even distant endothelial cells, which are brought by TEX containing tetraspanin family members [51], epithelial growth factor receptor (EGFR) [52], platelet-derived tissue factor (TF) [53] or developmental endothelial locus-1 protein [43]. TEX has also been implicated in the further growth of tumor by the exchange of genetic material. mRNA was detected within exosomes released from glioblastoma cells. Neighboring microvascular endothelial cells that take up the exosomes and translate the mRNA become liable for further tumor growth leading to the stimulation of angiogenesis [50]. In addition, tissue invasion and stromal remodeling can be facilitated by proteases and MMP transport and release via exosomes [54,55]. Recent studies have shown that TEX provide a protective role to the cancer cells, which can be manifested in different ways. Survivin, a member of the inhibitor of apoptosis (IAP) protein family, was found to be released from tumor cells via exosomes [56]. The protective role of TEX can be attained by the accumulation and packaging of chemotherapeutic drugs or its metabolites into the vesicles, thus decreasing cellular levels of the drug, a factor leading to drug resistance [57,58]. This phenomenon has been observed in various cancer cells. Cisplatin enhanced the shedding of the vesicle from melanoma cells [5], while doxorubicin was found in the exosomes released from ovarian carcinoma cells [58]. Despite the beneficial

roles of TEX for the tumor cells and the tumor microenvironment, TEX can be a useful tool for detecting the malignant condition. Serum levels of exosomes taken from cancer patients are significantly increased. These vesicles taken from serum [59], as well as from malignant tumor fluids, such as ascites fluids [60], pleural effusions [38] and urine [61] positively correlate with the tumor progression.

Constitutive and Inducible Vesicle Secretion in Cancer and Cancer Therapy

In the tumor microenvironment, various changes are taking place, which could have an effect on the release of vesicles, such as exosomes. Environmental changes, such as stress induced by chemo- and radio-therapy, can modulate TEX release and the biome they contain. This phenomenon may induce the tissues to adapt to changes taking place in the microenvironment [62]. Tumor cells that have undergone radiation or chemotherapy treatment have been shown to increase the release of TEX [63,64]. Interestingly, when treated with chemotherapeutic agents, there is a significantly enhanced membrane vesicle secretion in chemoresistant cells compared to chemosensitive cells. This activity may be a factor leading to drug resistance [57,58]. TSAP6 is an important cellular component as it regulates the secretion of protein via the non-classical pathway or the ER/Golgi-independent protein secretion pathway needed for the enhanced release of exosomes [63,65,66]. Normally, the secretion of exosomes in various cell types happens at a low rate. However, when p53 is activated, endosomal compartment activities are activated. Simultaneously, there is an increased expression of TSAP6, inducing the release of exosomes at a higher rate [67]. It is suggested that following p53 activation, exosomal release may act as a ‘detoxifier’ to expel unwanted chemotherapeutic agents [5,57,58,66].

Communication to the microenvironment is the other proposed role of TSAP6 and exosomal release after p53 activation, which may act as a warning signal to the neighboring cells, the immune system, and the extracellular matrix, that there are abnormal intracellular events happening [66,67].

TEX can be used as an important biomarker for the disease, which will give information not only on the disease progression, but on the tumor type. As previously mentioned, TEX express specific tumor antigens which reflect the protein content of the tumor, giving an indication of the tumor type. The content of these vesicles can also be useful as markers for the aggressiveness of the disease.

Exosomal Survivin

Survivin is found to be localized in various subcellular locations. Depending on its function, this IAP is shown to be in the cytoplasm, mitochondria and nucleus [23,68]. Recently, our lab has discovered that Survivin exists in the extracellular space [69], which is released by 40-100nm membrane vesicles called exosomes [56]. Various cell types, such as B- and T- lymphocytes, dendritic cells, neurons, intestinal epithelia cells as well as tumor cells release exosomes [70-72]. In particular, it has been shown that both human and mouse tumor cells release tumor cell-derived exosomes (TEX) constitutively [37]. Additionally, specific protein content found both on and within TEX gives an indication on not only their functional and biological roles, but also on their cell of origin, making TEX excellent biomarkers [39,40,73]. Our lab has shown that the extracellular pool of Survivin had the ability to cause neighboring cancer cells to become resistant to therapy, rapidly proliferate and acquire an increased potential to become invasive [69],

providing a protective role to the neighboring tumor cells [74]. The ability of extracellular Survivin to cause these effects in the surrounding cancer cells comes to no surprise as to why an overexpression of this IAP is seen in virtually every human cancer type [21]. TEX as biomarkers can be also used as tools to detect malignant conditions. Serum taken from cancer patients had an increased level of TEX [54,59],which had a positive correlation with the progression of the tumor [74]. In addition to serum, TEX was shown to be isolated from malignant tumor fluids, such as urine [61], ascites fluids [75] and pleural effusions [38]. We have recently shown that exosomal Survivin may be a useful tool for early detection and diagnosis or even monitoring prostate cancer progression. Newly diagnosed and advanced Prostate cancer patients with high or low-grade cancer had significantly higher levels of exosomal Survivin compared to control subjects or patients with pre-inflammatory BPH [76].

References

1. Bockman DE, Buchler M, Beger HG. Interaction of pancreatic ductal carcinoma with nerves leads to nerve damage. *Gastroenterology.* Jul 1994;107(1):219-230.

2. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet.* Mar 27 2004;363(9414):1049-1057.

3. Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N. Engl. J. Med.* . 1992;326:455-465.

4. Lowenfels AB, Maisonneuve P. Risk factors for pancreatic cancer. *Journal of cellular biochemistry.* Jul 1 2005;95(4):649-656.

5. Chen KG, Valencia JC, Lai B, et al. Melanosomal sequestration of cytotoxic drugs contributes to the intractability of malignant melanomas. *Proc Natl Acad Sci USA.* 2006;103(26):9903-9907.

6. Galloway NR, Aspe JR, Sellers C, Wall NR. Enhanced antitumor effect of combined gemcitabine and proton radiation in the treatment of pancreatic cancer. *Pancreas.* Oct 2009;38(7):782-790.

7. Palmer DH, Stocken DD, Hewitt H, et al. A randomized phase 2 trial of neoadjuvant chemotherapy in resectable pancreatic cancer: gemcitabine alone versus gemcitabine combined with cisplatin. *Annals of surgical oncology.* Jul 2007;14(7):2088-2096.

8. Toschi L, Finocchiaro G, Bartolini S, Gioia V, Cappuzzo F. Role of gemcitabine in cancer therapy. *Future Oncol.* Feb 2005;1(1):7-17.

9. Shewach DS, Lawrence TS. Antimetabolite radiosensitizers. *J Clin Oncol.* Sep 10 2007;25(26):4043-4050.

10. Kaye SB. New antimetabolites in cancer chemotherapy and their clinical impact. *Br J Cancer.* 1998;78 Suppl 3:1-7.

11. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100:57-70.

12. Srinivasula SM, Ashwell JD. IAPs: what's in a name? *Mol Cell.* 2008;30:123-135.

13. Altieri DC. Survivin and IAP proteins in cell-death mechanisms. *Biochem J.* 2010;430:199-205.

14. Salvesen GS, CS D. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol.* 2002;3(6):401-410.

15. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. *Genes Dev.* 1999;13:239-252.

16. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol.* 1999;9:323-328.

17. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature.* 1997;388:300-304.

18. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* 1997;16:6914-6925.

19. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, JP. N. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat Struc Biol.* 2000;7:602-608.

20. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer.* 2008;8:61-70.

21. Altieri DC. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer.* 2003;3:46-54.

22. Li F. Survivin study: what is the next wave? *J Cell Physiol.* 2003;197:8-29.

23. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol.* 1999;1:461-466.

24. Fortugno P, Wall NR, Giodino A, et al. Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtuble function. *Journal of Cell Science.* 2002;115:575-585.

25. Dohi T, Beltrami E, Wall NR, Plescia J, DC A. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J Clin Invest.* 2004;114:1117-1127.

26. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulators of mitosis and apoptosis and novel targets for cancer therapeutics. *Clin Cancer Res.* 2008;14:5000-5005.

27. Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy: fulfilled promises and open questions. *Carcinogenesis.* Jun 2007;28(6):1133-1139.

28. Asanuma K, Moriai R, Yajima T, et al. Survivin as a radioresistance factor in pancreatic cancer. *Japanese journal of cancer research : Gann.* Nov 2000;91(11):1204-1209.

29. Ikeguchi M, Kaibara N. survivin messenger RNA expression is a good prognostic biomarker for oesophageal carcinoma. *Br J Cancer.* Oct 7 2002;87(8):883-887.

30. Jian-Hui Xu, An-xu Wang, Hong-Zhang Huang, Jian-Guang Wang, Chao-Bin Pan, Bin Zhang. Survivin shRNA Induces Caspase-3-Dependent Apoptosis and Enhances Cisplatin Sensitivity in Squamous Cell Carcinoma of the Tongue. *Oncology Research.* 2010;18:377-385.

31. Lladser A, Sanhueza C, Kiessling R, Quest AF. Is survivin the potential Achilles' heel of cancer? *Advances in cancer research.* 2011;111:1-37.

32. Jing-Wei Wang, Yi Liu, Hai-mei Tian, Wei Zhang. Effect of Survivin-siRNA on Drug Sensitivity of Osterosarcoma Cell Line MG-63. *Chin J Cancer Res.* 2010;22(1):68-72.

33. Yongxin Yang, Yu Gao, Lingli Chen, Yongzhuo Huang, Yaping Li. Downregulation of Survivin Expression and Enhanced Chemosensitvity of MCF-7 Cells to Adriamycin by PDMAE/Survivin shRNA Complex Nanoparticles. *International Journal of Pharmaceutics.* 2011;405:188-195.

34. Qingqu Guo, Ying Chen, Bo Zhang, Muxing Kang, Quiping Xie, Yulian Wu. Potentiation of the Effect of Gemcitabine by Emodin in Pancreatic Cancer is Associated with Survivin Inhibition. *Biochemical Pharmacology.* 2009;77:1674-1683.

35. Wen-Song Liu, Hai-Jiao Yan, Ren-Yi Qin, et al. siRNA Directed against Survivin Enhances Pancreatic Cancer Cell Gemcitabine Chemosensitivity. *Dig Dis Sci.* 2008;54:89-96.

36. Taylor DD, Gercel-Taylor C. Tumour-derived exosomes and their role in cancer-associated T-cell signalling defects. *Br J Cancer.* Jan 31 2005;92(2):305-311.

37. Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med.* 2001;7(3):297-303.

38. Andre F, Schartz NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet.* Jul 27 2002;360(9329):295-305.

39. Wieckowski E, TL W. Human tumor-derived vs dendritic cell-derived exosomes have distinct biologic roles and molecular profiles. *Immunologic Research.* 2006;36(1-3):247-254.

40. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med.* 1998;4(5):594-600.

41. Iero M, Valenti R, huber V, et al. Tumour-released exosomes and their implications in cancer immunity. *Cell Death Differ.* 2008;15(1):80-88.

42. Andreola G, Rivoltini L, Castelli C, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *The Journal of experimental medicine.* May 20 2002;195(10):1303-1316.

43. Hegmans JP, Bard MP, Hemmes A, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am J Pathol.* 2004;164(5):1807-1815.

44. Mears R, Craven RA, Hanrahan S, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics.* 2004;4(12):4019-4031.

45. Liu C, Yu S, Zinn K, et al. Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol.* 2006;176(3):1375-1385.

46. Nieuwland R, Sturk A. Why do cells release vesicles? *Thromb Res.* 2010;125:49-51.

47. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res.* 2007;67(7):2912-2915.

48. Anderson HC, Mulhall D, Garimella R. Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. *Lan Invest.* 2010;90(11):1549-1557.

49. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. *J Biochem.* 2006;140(1):13-21.

50. Skog J, Wurdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2008;10(12):1470-1476.

51. Gesierich S, Berezovskiy I, Ryschich E, Zoller M. Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. *Cancer Res.* Jul 15 2006;66(14):7083-7094.

52. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proceedings of the National Academy of Sciences of the United States of America.* Mar 10 2009;106(10):3794-3799.

53. Osterud B. The role of platelets in decrypting monocyte tissue factor. *Disease-a-month : DM.* Jan 2003;49(1):7-13.

54. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount of proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res.* 1998;18(5A):3433-3437.

55. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer Res.* Oct 1 2004;64(19):7045-7049.

56. Khan S, Jutzy JMS, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. *Apoptosis.* 2011;16(1):1-12.

57. Safaei R, Larson BJ, Cheng TC, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. *Mol Cancer Ther.* 2005;4(10):1595-1604.

58. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. *Cancer Research.* 2003;63(15):4331-4337.

59. Ginestra A, Miceli D, Dolo V, Romano FM, Vittorelli ML. Membrane vesicles in ovarian cancer fluids: a new potential marker. *Anticancer Res.* 1999;19(4C):3439-3445.

60. Adams M, Navabi H, Croston D, et al. The Rationale for Combined Chemo/Immunotherapy using Toll-like receptor 3 (TLR3) Agonist and Tumour-derived Exosomes in Advanced Ovarian Cancer. *Vaccine.* 2005;23:2374-2378.

61. Nilsson J, Skog J, Nordstrand A, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br J Cancer.* May 19 2009;100(10):1603-1607.

62. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009;9(8):581-593.

63. Yu X, Harris SL, Levine AJ. The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res.* May 1 2006;66(9):4795-4801.

64. Lehmann BD, Paine MS, Brooks AM, et al. Senescence-associated exosome release from human prostate cancer cells. *Cancer Res.* Oct 1 2008;68(19):7864-7871.

65. Nickel W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *European journal of biochemistry / FEBS.* May 2003;270(10):2109-2119.

66. Lespagnol A, Duflaut D, Beekman C, et al. Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice. *Cell Death Differ.* Nov 2008;15(11):1723-1733.

67. Yu X, Riley T, Levine AJ. The regulation of the endosomal compartment by p53 the tumor suppressor gene. *The FEBS journal.* Apr 2009;276(8):2201-2212.

68. Fengzhi Li, Xiang Ling. Survivin Study: An Update of "What is the Next Wave?". *Journal of Cellular Physiology.* 2006;208(3):476-486.

69. Khan S, Aspe JR, Asumen MG, et al. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential *Br J Cancer.* 2009;100:1073-1086.

70. Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci.* 2000;113(9):3365-3374.

71. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunol Lett.* 2006;107(2):102-108.

72. Simpson RJ, Lim JWE, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomic.* 2009;6(3):267-283.

73. Andre F, Schartz NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. *2002.* 2002;360(9329):295-305.

74. Khan S, Jutzy JMS, Aspe JR, et al. The application of membrane vesicles for cancer therapy2011.

75. Adams M, Navabi H, Croston D, et al. The rationale for combined chemo/immunotherapy using a Toll-like receptor 3 (TLR3) agonist and tumour-derived exosomes in advanced ovarian cancer. *Vaccine.* Mar 18 2005;23(17-18):2374-2378.

76. Khan S, Jutzy JMS, VAlenzuela MMA, et al. Plasma-derived Exosomal Survivin, a Plausible Biomarker for Early Detection of Prostate Cancer. *PLoS One.* 2012;7(10):e46737.

CHAPTER TWO

ANTIMETABOLITE TREATMENT FOR PANCREATIC CANCER

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Abstract

Pancreatic cancer is a deadly and aggressive disease. Less than 1% of diagnosed patients survive 5 years with an average survival time of only 4 - 8 months. The only option for metastatic pancreatic cancer is chemotherapy where only the antimetabolites gemcitabine and 5-fluorouracil are used clinically. Unfortunately, efforts to improve chemotherapy regimens by combining, 5-fluorouracil or gemcitabine with other drugs, such as cisplatin or oxaliplatin, have not increased cell killing or improved patient survival. The novel antimetabolite zebularine shows promise, inducing apoptosis and arresting cellular growth in various pancreatic cancer cell lines. However, resistance to these antimetabolites remains a problem highlighting the need to discover and develop new antimetabolites that will improve a patient's overall survival.

Introduction

In the United States, pancreatic cancer is the 4th leading cause of cancer death aggressively and silently attacking the patient [1-3]. Pancreatic cancer is only identified in more advanced stages when the patient is symptomatic, as there are no screening tests for this disease [4]. At the time of diagnosis, approximately 85% of the patients have advanced pancreatic cancer resulting is a short median survival time of 4-8 months where less than 1% survive more than 5 years [5, 6]. Currently, the best treatment is surgical resection where approximately 20% of patients increase their life span by approximately 2 years [7]. For metastatic pancreatic adenocarcinoma, chemotherapy using gemcitabine (GEMZAR) is currently the only first-line FDA approved treatment [8]. Antimetabolite drugs are designed to stop DNA replication and normal cellular metabolic processes by different mechanisms and have been investigated for almost 70 years [9, 10]. Currently, efforts to improve the treatment for metastatic pancreatic cancer explore using combinations of therapeutic agents as well as searching for new antimetabolite drugs. This review will discuss the different antimetabolite agents (Table 1) used to treat pancreatic cancer, both clinically approved and experimental, their mechanisms of action, and therapy resistance.

5-Fluorouracil

The pyrimidine 5-fluorouracil (5FU) has been under investigation for the treatment of human cancers since 1954 when it was observed that uracil is utilized more efficiently by tumor cells than normal cells [11]. The knowledge that fluorine substitutions of hydrogen in metabolites often resulted in a toxic compound inspired the

design of 5FU (Figure 1) and testing as a tumor-inhibiting compound [11-13]. Since its discovery, 5FU has been used as a treatment for many solid tumors such as colon, breast, head and neck cancers, and advanced pancreatic cancer. For 20 years, 5FU was regarded as the only effective drug against advanced pancreatic cancer. However, despite numerous efforts to improve therapy outcomes, the best response rate was approximately 20% [12, 14, 15].

Mechanism of Action

Like uracil, 5FU is salvaged to form 5-fluorouridine and then phosphorylated by nucleoside and nucleotide kinases as well as reduced by ribonucleotide reductase forming three different active metabolites (Figure 2). After incorporation of 5-fluorouridine triphosphate (FUTP) into cellular RNA, RNA processing and post-transcriptional modification can be inhibited [15, 16]. During DNA synthesis, 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) inhibits thymidylate synthase resulting in an imbalanced pool of deoxynucleotide triphosphates, particularly decreased deoxythymidine triphosphate (dTTP) and increased deoxyuridine triphosphate (dUTP). Absent dTTP, stalled DNA polymerases can incorporate 5-fluorodeoxyuridine triphosphate (FdUTP) or dUTP which are subsequently recognized as damaged DNA setting up a futile cycle of misincorporation and repair [15, 16]. When DNA damage exceeds a cells ability to repair misincorporated FdUTP or dUTP, single strand and double strand breaks accumulate favoring cell death. Given these cellular actions of 5FU, its toxicity is generally considered a function of transport into the cell and metabolism to active metabolites,

Table 1. Anti-metabolite Drugs (experimental & clinical)

Demethylase	Ribonucleotide Reductase Inhibitor	Polymerase Inhibitor	Deaminase Inhibitor	Thymidine Synthase Inhibitor	DNA Crosslinker
Zeb	HU	Gem	Zeb	5FU	CDPD
DAC	Gem	AraC		5’FdU	
5'FdC	CldA				

Zeb (zebularine), HU (hydroxyurea), Gem (gemcitabine), 5FU (5flourouracil), CDPD (platinum), DAC (5-Aza-2’-deoxycytidine), AraC (1-β-D-arabinofuranosylcytosine), 5’FdU (5’-Flouro-deoxyUridine), 5’FdC (5flouro-deoxyCytidine), CldA (2-chloro-2’-deoxyadenosine)

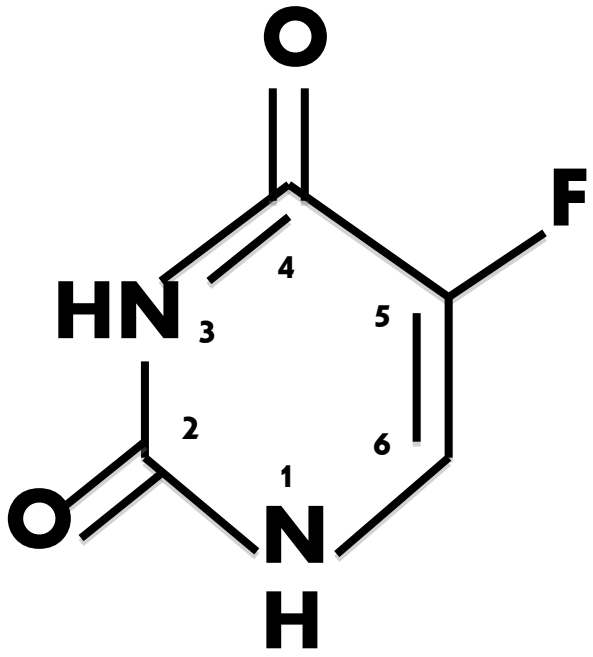

Figure 1 - Structure of 5FU with the fluor group in carbon 5-position. 5FU is a pyrimidine analog drug whose mechanism of action is through irreversible inhibition of thymidylate synthase (TS). Clinically is have been used in the treatment of anal, breast, colorectal, esophageal, stomach, pancreatic and skin cancers.

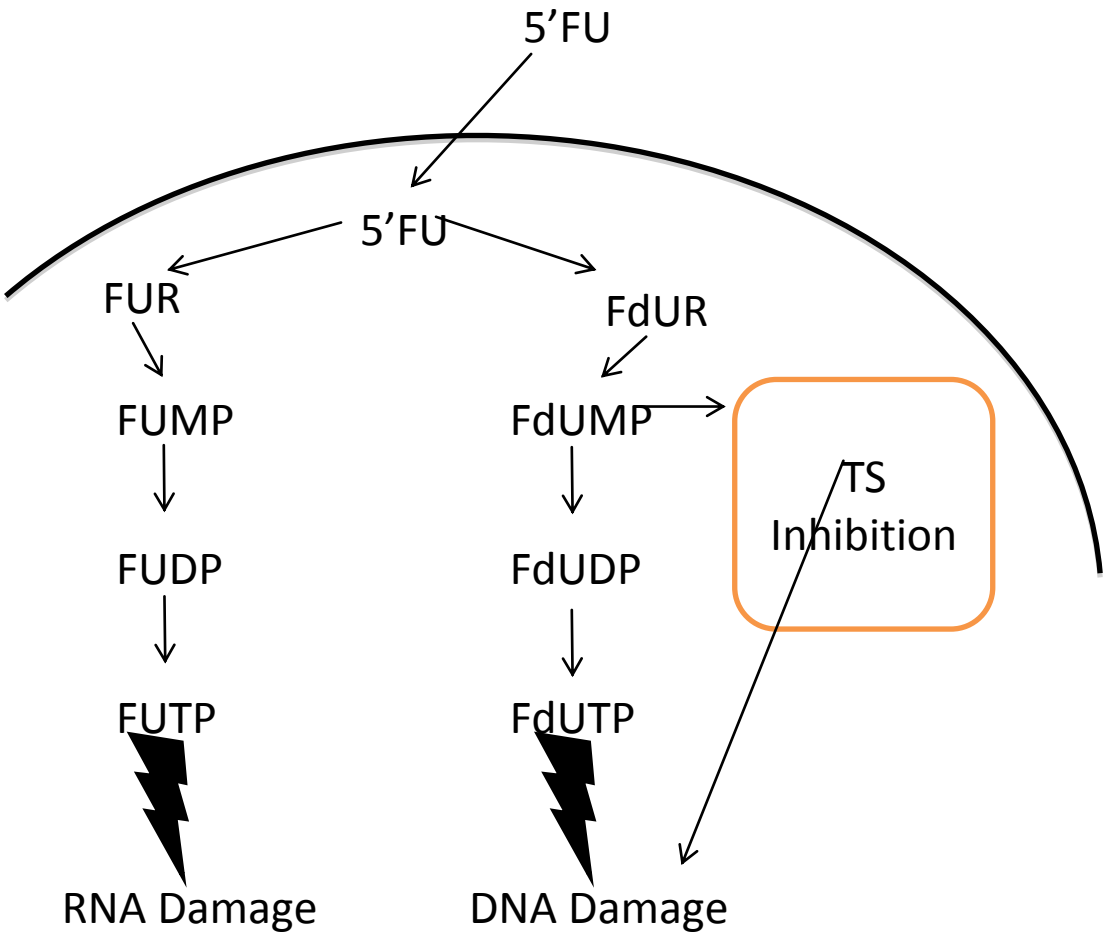

Figure 2 - Mechanism of 5FU leading to RNA and DNA damage. Thymidylate synthase inhibition is the main mechanism of action of 5FU through its active metabolite FdUMP. Synthesis of the pyrimidine thymidine, which is required for DNA synthesis, is the result of blocking thymidylate synthase. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) to for thymidine monophosphate (dTMP). The use of 5FU in cancer causes there to be a reduction leading to a scarcity of dTMP so that rapidly dividing cancer cells die from a lack of thymine.

particularly FdUMP, while resistance occurs when 5FU metabolism is decreased or DNA repair is efficient.

Resistance

One mechanism of 5FU resistance may result from high levels of thymidylate synthase expression in pancreatic cancer patients. Head and neck[17] and gastric[18] cancer patients with low tumoral thymidylate synthase expression exhibited increased sensitivity to 5FU treatment, while a lack of response was seen in advanced colorectal patients[19] with high thymidylate synthase expression. Interestingly, the opposite was observed where node-positive breast[20] and Dukes' B and C rectal[21] cancer patients with high expression levels of thymidylate synthase responded well to 5FU therapy. It is not currently known why this phenomenon was seen, but 5FU therapy-outcome may be associated with the tumor type that is being treated or with the biome of stress-associated molecules expressed and/or induced. One retrospective study of pancreatic cancer patients found that 5FU resulted in longer survival for patients with low thymidylate synthase expression [22]. Further translational studies are needed to better understand the role of thymidylate synthase expression and therapy outcome [10, 16]. These and other studies on the mechanism of resistance continue and may prove instrumental in understanding resistance leading to better therapeutic design and combinations.

An additional mechanism of resistance is decreased expression 5FU transport into pancreatic cancer cells. In human pancreatic cancer cell lines, the sensitivity to 5FU directly correlated with the expression level of the human equilibrative nucleoside transporter 1 (hENT1) [23]. However, increased median survival time in pancreatic

cancer patients treated with 5FU was not significantly different [24]. Additional studies are needed to understand the differences in resistance to 5FU in cell lines as opposed to pancreatic cancer patients.

Gemcitabine

Gemcitabine (2', 2'-difluoro-2'deoxycytidine, dFdC) was originally considered as an antiviral drug [25], but was later shown to demonstrate anti-cancer activity in both *in vivo* and *in vitro* models of solid and hematological cancers [14, 25, 26]. Today, gemcitabine is the only FDA approved single chemotherapy agent against metastatic pancreatic cancer, showing a better 1-year survival rate, median survival, and clinical benefit when compared to 5FU [8].

Mechanism of Action

Gemcitabine is a 2'-deoxycytidine analogue with fluorine substituted for hydrogen at the 2' position of the furanose ring (Figure 3). Gemcitabine is a broad-spectrum agent, which has different mechanisms of action, depending upon its phosphorylation state (Figure 4) [8, 25]. Uptake of Gem into the cell uses both human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs) [27, 28]. Inside the cell, gemcitabine is phosphorylated by deoxycytidine kinase into gemcitabine monophosphate (dFdCMP), which is further converted into its active di- and triphosphate (dFdCDP and dFdCTP) states by nucleotide kinases [29]. Ribonucleotide reductase is inhibited by dFdCDP leading to a reduction in dCTP levels. Reduced dCTP lessens the negative feedback regulation of deoxycytidine

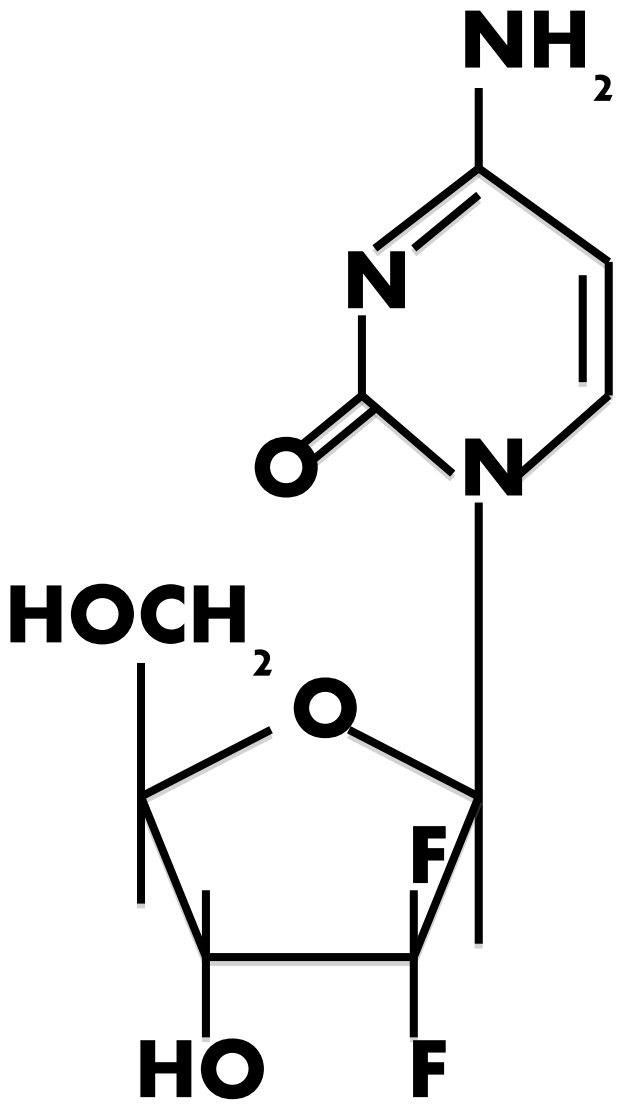

Figure 3 - Gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2' Carbon of deoxycytidine are replaced by fluorine atoms. Like other analogues of pyrimidines, the triphosphate analogue of gemcitabine replaces the important cytidine building block of nucleic acids during DNA replication arresting tumor growth and resulting in apoptosis. Gemcitabine has been used to treat various carcinomas including lung, pancreatic, bladder and breast cancers. It is being investigated for the possible use against esophageal cancers and lymphomas.

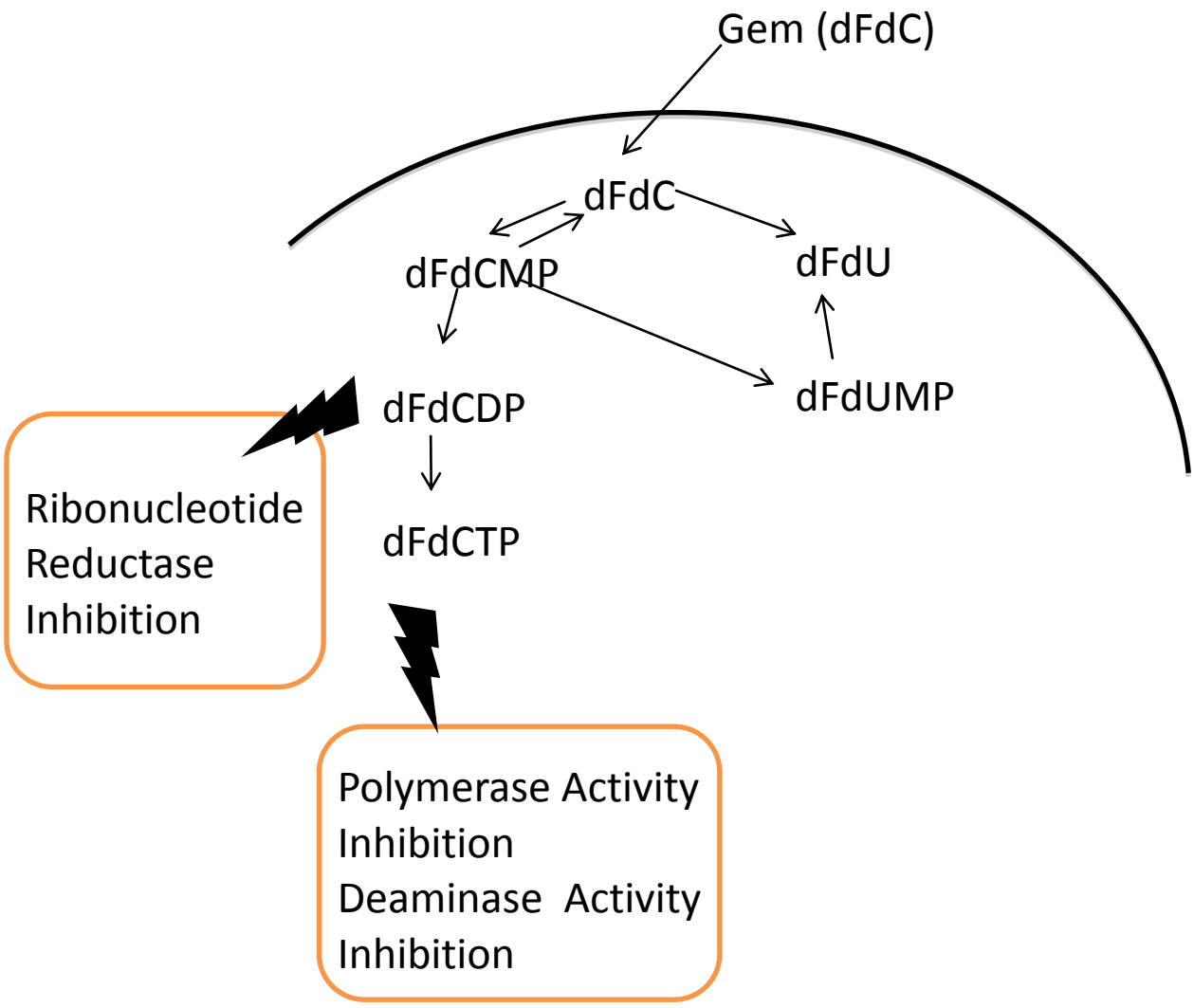

Figure 4 - The broad spectrum mechanism of action of Gem, depending on its phosphorylation state, can inhibit Riobonucleotide Reductase, Polymerase and Deaminase activities. Once these enzymes are irreversibly inhibited, the cell cannot produce the deoxyribonucleotides required for DNA replication and repair and the cell dies via apoptosis.

kinase and favors the efficient phosphorylation of gemcitabine [30]. The cytotoxic activity of gemcitabine leading to apoptosis is mainly the result of its triphosphate form. DNA polymerase activity is inhibited when dFdCTP is incorporated into the DNA strand leading to a termination of the DNA chain synthesis and single strand breakage[31-33]. Consequently, a depletion of dCTP levels, due to inhibition of ribonucleotide reductase activity, results in the competition of dFdCTP with dCTP leading to an increased incorporation of dFdCTP into the DNA strand [30]. In addition, high intracellular levels of dFdCTP also strongly inhibited dCMP deaminase activity, by directly inhibiting the deaminase as well as indirectly because of the decreased dCTP:dTTP ratio [34].

Resistance

It has been shown *in vitro* that low levels of hENT1, leading to limited gemcitabine intracellular uptake, is a mechanism of chemoresistance[23, 35, 36]. In pancreatic cancer patients, the levels of hENT1 were recently observed to correlation with overall median survival time, where patients with higher levels of hENT1 have better survival rates[24]. Further mechanisms of resistance to gemcitabine observed in cell lines from multiple cancer types resulted from decreases in deoxycytidine kinase activity and increased ribonucleotide reductase activity [37]. Implications for pancreatic cancer patients regarding activity and expression of these enzymes, however, are still unknown [38].

Platinum

Platinum agents are used today in combination therapy regimes with gemcitabine

as second line chemotherapy for metastatic pancreatic cancer. Cisplatin (dis-diamminedichloroplatinum, CDDP, $PtCl_2(NH_3)_2$) is shown in Figure 5 and is an inorganic platinum complex composed of a doubly charged platinum ion, and four ligands - two chloride ions and two amines. Cisplatin is a potent chemotherapy drug discovered in the 1960's. It is widely used today against a variety of tumors including head and neck, non-small cell lung, stomach and bladder cancers, non-Hodgkin's lymphoma and sarcomas [39, 40]. Oxaliplatin (trans-*l*-1,2-diaminocyclohexane oxalatoplatinum) (Figure 6) is a new platinum agent that is more potent *in vitro* and has a better toxicity profile compared to cisplatin, as it only needs a small number of DNA adducts to attain the same cytotoxicity profile as cisplatin. In preclinical studies, oxaliplatin shows efficacy in a number of cancer cell lines, which also includes cell lines that are cisplatin resistant [41, 42]. This provides hope that with minor modification of these platinum compounds, not only will efficacy increase, but resistance will decrease as well.

Mechanism of Action

Once taken up into the cells, the chloride ions are lost and replaced with water molecules transforming cisplatin into a reactive species. Loosely bound, the water molecules easily fall off, exposing the platinum ion which readily forms bonds with DNA bases, forming DNA-DNA cross-links and DNA-protein cross-links. These cross-links between bases are usually formed at sites where adenosine and guanine are adjacent on the same DNA strand. It has been speculated that the *cis*-geometry of cisplatin is important to its anti-tumor activity, as the *trans*-isomer of cisplatin, transplatin, is inactive [43]. Unlike 5FU, cisplatin chemotherapy arrests cells at the G1, S or G2-M

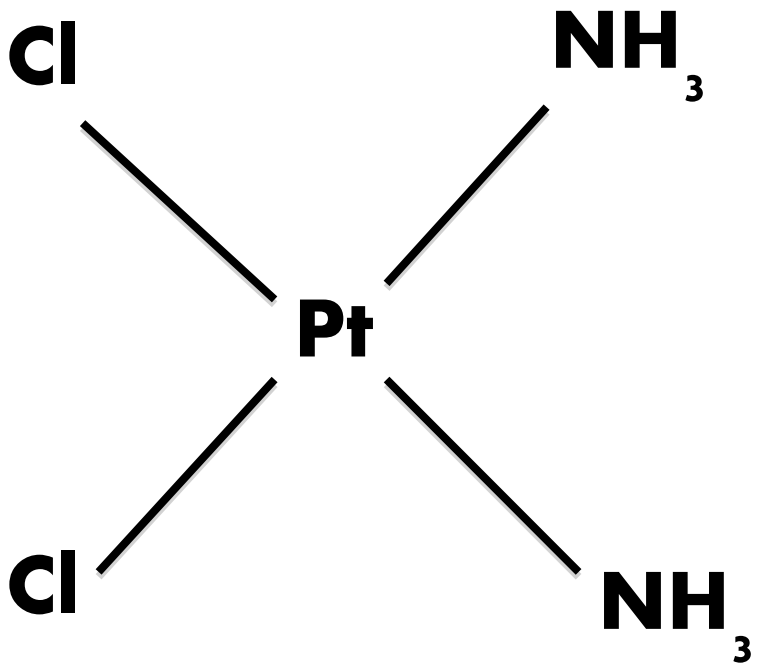

Figure 5 – Cisplatin has two chloride ions and two amine groups attached to the center platinum ion. Cisplatin has been used to treat various cancers which include sarcomas carcinomas of the lung and ovary, lymphomas and germ cell tumors and is especially effective in treatment of testicular cancer.

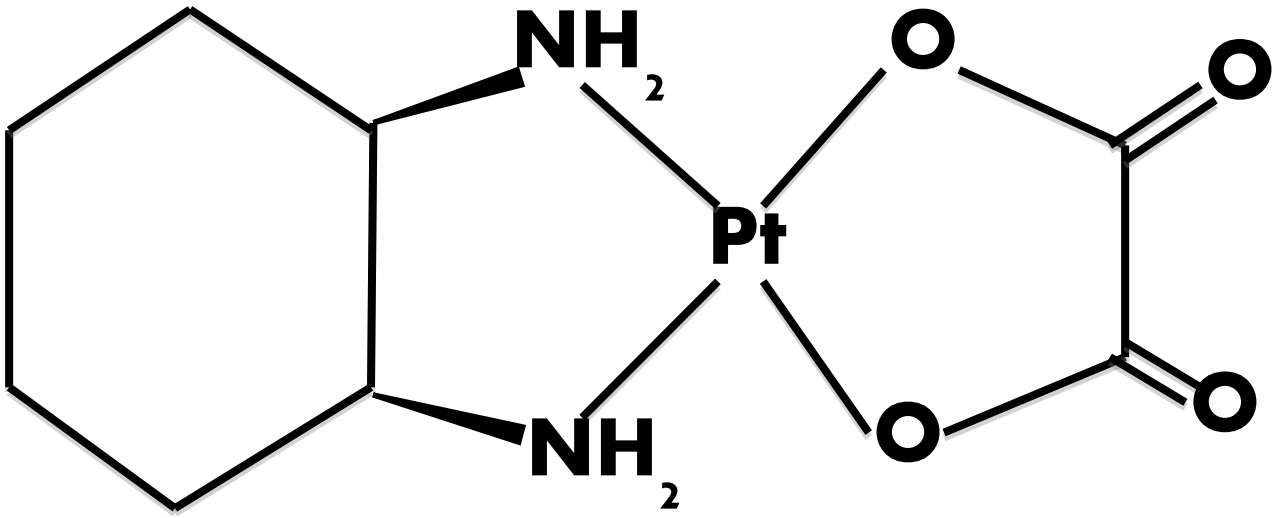

Figure 6 – Similar to Cisplatin, Oxaliplatin contains a doubly charged platinum ion in the center. It, however, contains diamnocyclohexane and carboxylate compounds. These platinum complexes bind to and crosslink DNA in vivo which triggers apoptosis.

phase of the cell cycle, making this drug efficient in killing cells that are in all stages of the cell cycle [39, 44-46].

In oxaliplatin, the two amines and two chloride ions of cisplatin are replaced with diaminocyclohexane and carboxylate compounds, respectively (Figure 6). Similar to cisplatin, once inside the cell, the carboxylate compound is displaced, transforming oxaliplatin into a reactive compound that forms DNA intra-strand cross-links, DNA interstrand cross-links, and DNA-protein cross-links [45]. DNA lesions induced by intrastrand cross-links are formed when the drug binds to two adjacent guanine bases, and to a lesser extent, to adjacent adenosine and guanine bases. Binding of the mismatch repair protein complex to the DNA becomes more difficult due to the conformation of adducts, which may result in poor repair of the lesion. Oxaliplatin has been reported to inhibit TS activity, much like 5FU [44, 45].

Resistance

There are several mechanisms whereby tumor cells become resistant to both cisplatin and Oxaliplatin. The toxicity of cisplatin and oxaliplatin is reduced in cells with an efficient repair of damaged DNA where enzymes involved in nucleotide excision repair remove the platinum-DNA adducts [39]. The relationship between enhanced platinum resistance, a decrease in drug sensitivity, and increased DNA repair protein levels has been described [39, 47, 48]. Another mechanism is through a decrease in intracellular platinum concentration resulting from a reduction in drug uptake and an increase of platinum expulsion out of the cell or detoxification by glutathione and metallothionein and an increased level of glutathione and metallothionein has been

shown in some cases to correlate with cisplatin resistance [49]. This resistance is not due to only one mechanism, but on a variety of mechanisms targeting various systems [39, 44, 45]. The mechanisms of resistance for cisplatin and oxaliplatin differ from the mechanisms of resistance for gemcitabine resulting in a benefit from combining these agents in a therapeutic regimen.

Combination Therapy with Platinum Agents

Cisplatin and oxaliplatin are not used as single agents against pancreatic cancer, but rather, in combination with either gemcitabine or 5FU when treatment with gemcitabine alone has failed. There have been multiple studies on the effects of cisplatin used in combination with gemcitabine. One phase III study showed that compared to patients treated with gemcitabine alone, the overall median survival and progression-free survival of patients on the Gemcitabine-cisplatin combination therapy improved, but did not reach statistical significance [50]. Furthermore in another study, comparable results in patients treated with Gemcitabine alone or in combination with cisplatin were observed [46]. However, they also noted that the combination therapy was more toxic than gemcitabine alone. Nevertheless, studies do show favor for a Gem-cisplatin combination, where disease progression and the median 1-year event-free survival is encouraging [42]. Oxaliplatin has been used in combination with both Gemcitabine and 5FU. One study has shown that patients with inoperable pancreatic cancer tolerated the combination of Gemcitabine with oxaliplatin well and was recorded to be highly effective[51] while a phase II trial showed moderate activity [41]. When in combination with 5FU, clinical benefits were recorded and toxicity levels were acceptable [52]. These platinum agents,

when combined with Gemcitabine or 5FU, may be a promising treatment regime for pancreatic cancer patients.

Zebularine

Epigenetic changes accompany pancreatic tumorigenesis as well as the acquisition of resistance to chemotherapy [53, 54]. Therapeutic agents that alter the epigenetic state of pancreatic cancer cells are under investigation as cytotoxic agents as well as agents to reverse acquired resistance to first-line agents. Lacking an amino group on the C-4 position of the pyrimidine ring, zebularine ((1-β-D-Ribofuranosyl)-2(1*H*)-pyrimidinone), a cytidine analogue (Figure 7), was originally developed as a cytidine deaminase inhibitor. It is also a novel DNA methytransferase (DNMT) inhibitor and unlike other DNMT inhibitors, zebularine is more stable in aqueous solution and is less toxic *in vitro* and *in vivo* [55-57]. Continuous exposure of numerous cancer cell lines to zebularine slowed tumor cell growth as compared to normal human fibroblast cell lines indicating its promise as a chemotherapy agent for cancer treatment [58].

Mechanism of Action

Once inside cells, zebularine is phosphorylated by uridine-cytidine kinase. Nucleotide kinases phosphorylate zebularine monophosphate to form zebularine triphosphate, which is then incorporated into DNA. The 2(1*H*)-pyrimidinone ring is important as its incorporation into the DNA strand leads to DNMT1 depletion and DNA methylation inhibition. When zebularine replaces cytosine in a CpG dinucleotide and a DNA methyltransferase attempts to methylate zebularine, an irreversible covalent

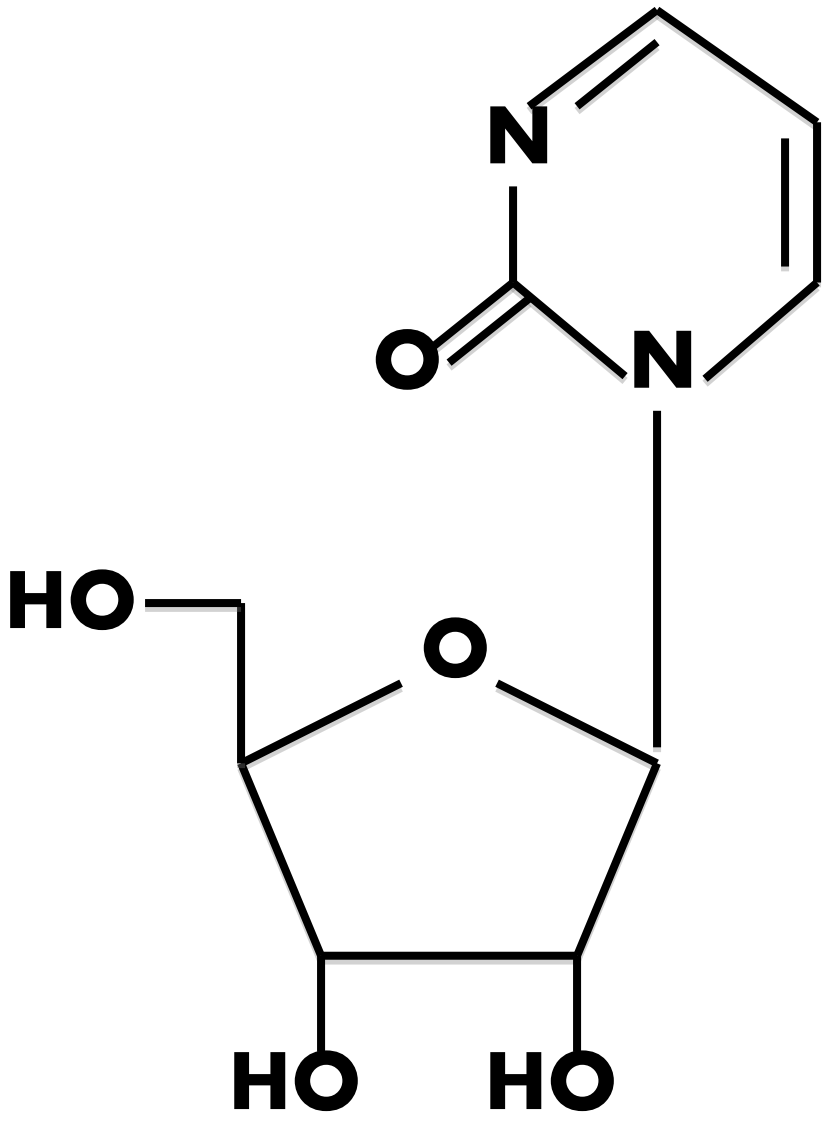

Figure 7 – Zebularine's structure includes a 2(1*H*)-pyrimidinone ring. It is a nucleoside analog of cytidine and works by inhibiting cytidine deaminase by binding to the active site as a covalent hydrates. It has also been shown to inhibit DNA methylation and tumor growth in vivo and in vitro. Though entirely experimental at this time, it has been suggested that it could be used as a chemoprevention agent or even in epigenetic therapy.

complex is formed thus inhibiting DNA methylation [58]. In a transgenic mouse model of breast cancer, zebularine slows tumor growth and induces cell death by both necrosis and apoptosis [55]. Other studies show that zebularine decreases levels of DNMT1, DNMT3a, and DNMT3b in breast cancer cell lines [59] as well as DNMT1 and partially DNMT3b in bladder cancer cells [58]. A reduction in DNMT1 and DNMT3b was also shown in the mammary tumors in transgenic mice [55]. The growth inhibition property of zebularine may be due to drug incorporation into the DNA. However, the amount of zebularine in DNA was low in normal cells and growth was minimally affected, while the opposite was seen in cancer cells[58]. Understanding incorporation aspects of this agent may prove useful in developing more effective analogues.

Zebularine and Pancreatic Cancer

Studies have shown that zebularine effectively slows cellular growth in CFPAC-1, a pancreatic cancer cell line, by inducing the *p21* and/or *p16* genes [58]. The p21 protein in response to DNA damage, directly stops DNA replication and arrests cellular growth. They have also shown a decrease in DNMT1 through the incorporation of the 2(1*H*)-pyrimidinone ring, as stated above [58]. In addition, studies also showed that zebularine, as a single agent, induced apoptosis and growth arrest by inhibition of DNMT1 in three pancreatic cancer cell lines: YAP C, DAN G and Panc-89 [60]. Though there are minimal studies showing the potential use of zebularine in pancreatic cancer, initial reports show promise for the use of zebularine in treating pancreatic cancer. More studies, however, are needed to fully test the full potential of zebularine *in vivo.*

Conclusion

The only effective treatment option available for patients with advanced metastatic pancreatic adenocarcinoma remains the antimetabolite gemcitabine. Despite efforts to improve therapy regimens by using 5FU or Gem in combination with alkylating agents, the prognosis for treating metastatic pancreatic cancer remains bleak. Therefore, it is imperative to continue studying and developing novel antimetabolite agents, such as zebularine, to improve treatment options and improve overall survival rates.

Conflict of Interest

The authors declare that they have no competing interests.

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References

1. Bockman DE, B.M., Berger HG., *Interaction of pancreatic ductal carcinoma with nerves leads to nerve damage.* Gastroenterology, 1994. 107: p. 219-230.

2. Li D, X.K., Wolff R, Abbruzzesse JL., *Pancreatic cancer.* Lancet, 2004. 363(9414): p. 1049-1057.

3. Warshaw AL, F.-d.C.C., *Pancreatic Carcinoma.* N Engl J Med, 1992. 326: p. 455-465.

4. Moss RA, L.C., *Current and emerging therapies for the treatment of pancreatic cancer.* OncoTargets and Therapy, 2010. 3: p. 111-127.

5. Galloway NR, A.J., Sellers C, Wall N., *Enhanced antitumor effect of combined gemcitabine and proton radiation in the treatment of pancreatic cancer.* Pancreas, 2009. 38(7): p. 782-790.

6. Palmer DH, S.D., Hewitt H, Markham CE, Hassan AB, Johnson PJ, Buckels JA, Bramhall SR., *A randomized phase 2 trial of neoadjuvant chemotherapy in resectable pancreatic cancer: gemcitabine alone versus gemcitabine combined with cisplatin.* Ann Surg Oncol, 2007. 14(7): p. 2088-96.

7. Kleef J, M.C., Friess H, Büchler MW., *Pancreatic cancer: from bench to 5-year survival.* Pancreas, 2006. 33(2): p. 111-118.

8. Toschi L, F.G., Bartolini S, Gioia V, Cappuzzo F., *Role of gemcitabine in cancer therapy.* Future Oncol, 2005. 1(1): p. 7-17.

9. Shewach DS, L.T., *Antimetabolite radiosensitizers.* Journal of Clinical Oncology, 2007. 25(26): p. 4043-4050.

10. Kaye, S., *New antimetabolites in cancer chemotherapy and their clinical impact.* Br J Cancer, 1998. 78 Suppl 3: p. 1-7.

11. Rutman, R.J., A. Cantarow, and K.E. Paschkis, *Studies in 2-acetylaminofluorene carcinogenesis. I. The intracellular distribution of nucleic acids and protein in rat liver.* Cancer Res, 1954. 14(2): p. 111-4.

12. Thomas, D., Zalcberg, JR., *5-fluorouracil: a pharmacological paradigm in the use of cytotoxics.* Clin Exp Pharmacol Physiol, 1998. 25(11): p. 887-95.

13. Heidelberger, C., et al., *Fluorinated pyrimidines, a new class of tumour-inhibitory compounds.* Nature, 1957. 179(4561): p. 663-6.

14. Burris H A, r., Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD., *Improvements in survival and clinical benefit with*

gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol, 1997. **15**(6): p. 2403-13.

15. Noordhuis P, H.U., Van der Wilt CL, Van Groeningen, Smid K, Meijer S, Pinedo HM, Peters GJ., *5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibiton of human colorectal cancers.* Annals of Oncology, 2004. 15: p. 1025-1032.

16. Longley DB, H.D., Johnston PG., *5-Fluorouracil: Mechanisms of action and clinical strategies.* Nat Rev Cancer, 2003. 3: p. 330-338.

17. Johnston PG, M.R., Recant W, Behan KA, Dolan ME, Ratain MJ, Beckman E, Weichselbaum RR, Allegra CJ, Vokes EE, *Thymidylate synthase expression and response to neoadjuvant chemotherapy in patients with advanced head and neck cancer.* J Natl Cancer Inst, 1997. 89: p. 308-313.

18. Lenz H-J, L.C., Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L., *Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. .* J Clin Oncol, 1996. 14(4): p. 176-182.

19. Leichman L, L.H., Leichman CG, Groshen S, Danenberg S, Danenberg K, Baranda J, Spears CP, Boswell W, Silberman H, Ortega A, Stain S, Beart R, Danenberg P., *Quantitation of intratumoral thymidylate synthase expression predicts for resistnace to protracted infusion of 5-fluorouracil and weekly leucovorin in dissemintated colorectal cancers: preliminary report from an ongoing trial.* Eur J Cancer, 1995. 31A: p. 1306-1310.

20. Pestalozzi BC, P.H., Gelber RD, Goldhirsch A, Gusterson BA,Trihia H, Lindtner J, Cortes-Funes H, Simmoncini E, Byrne MJ, Golouh R, Rudenstam CM, Castiglione-Gertsch M, Allegra CJ, Johnston PG., *Prognostic importance of thymidylate synthase expression in early breast cancer.* J Clin Oncol, 1997. 15(5): p. 1923-31.

21. Johnston PG, et al., *The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer.* J Clin Oncol, 1994. 12(12): p. 2640-7.

22. Hu, Y.C., et al., *Thymidylate synthase expression predicts the response to 5-fluorouracil-based adjuvant therapy in pancreatic cancer.* Clin Cancer Res, 2003. 9(11): p. 4165-71.

23. Tsujie, M., et al., *Human equilibrative nucleoside transporter 1, as a predictor of 5-fluorouracil resistance in human pancreatic cancer.* Anticancer Res, 2007. 27(4B): p. 2241-9.

24. Greenhalf, W., et al., *Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial.* J Natl Cancer Inst, 2014. 106(1): p. djt347.

25. Mini E, et al., *Cellular pharmacology of gemcitabine.* Annals of Oncology, 2006. 17: p. v7-v12.

26. Giovannetti E, et al., *Synergistic cytotoxicity and pharmacogenomics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines.* Clin Cancer Res, 2004. 10: p. 2936-2943.

27. Ueno H, Kiyosawa K, and Kaniwa N, *Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy?* Br J Cancer, 2007. 97(2): p. 145-51.

28. Bachet J-P, Marechal R, and V.L. J-L., *Treatment of pancreatic cancer: What can we really predict today?* Cancers, 2010. 2.

29. Heinemann V, et al., *Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine.* Cancer Res, 1988. 48(14): p. 4024-31.

30. Heinemann V, et al., *Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine.* Mol Pharmacol, 1990. 38(4): p. 567-72.

31. Gandhi V and Plunkett W, *Modulatory activity of 2',2'-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides.* Cancer Res, 1990. 50(12): p. 3675-80.

32. Huang P, et al., *Action of 2',2'-difluorodeoxycytidine on DNA synthesis.* Cancer Res, 1991. 51(22): p. 6110-7.

33. Ross DD and Cuddy DP, *Molecular effects of 2',2'-difluorodeoxycytidine (Gemcitabine) on DNA replication in intact HL-60 cells.* Biochem Pharmacol, 1994. 48(8): p. 1619-30.

34. Heinemann V, et al., *Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate: a mechanism of self-potentiation.* Cancer Res, 1992. 52(3): p. 533-9.

35. Mackey JR, et al., *Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines.* Cancer Res, 1998. 58(19): p. 4349-4357.

36. Michalski, C.W., et al., *Ex vivo chemosensitivity testing and gene expression profiling predict response towards adjuvant gemcitabine treatment in pancreatic cancer.* Br J Cancer, 2008. 99(5): p. 760-7.

37. Bergman, A.M., H.M. Pinedo, and G.J. Peters, *Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine).* Drug Resist Updat, 2002. 5(1): p. 19-33.

38. Andersson R, et al., *Gemcitabine chemoresistance in pancreatic cancer: molecular mechanisms and potential solutions.* Scand J Gastroenterol, 2009. 44(7): p. 782-6.

39. Florea AM and Busselberg D, *Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects.* Cancers (Basel), 2011. 3(1): p. 1351-71.

40. Costello BA, et al., *Phase I trial of everolimus, gemcitabine and cisplatin in patients with solid tumors.* Invest New Drugs, 2014.

41. Alberts SR, et al., *Gemcitabine and oxaliplatin for metastatic pancreatic adenocarcinoma: a North Central Cancer Treatment Group phase II study.* Ann Oncol, 2003. 14(4): p. 580-5.

42. Almhanna K and Kim R, *Second-line therapy for gemcitabine-refractory pancreatic cancer: is there a standard?* Oncology (Williston Park), 2008. 22(10): p. 1176-83; discussion 1190, 1192, 1196.

43. Boudvillain, M., et al., *Intrastrand cross-links are not formed in the reaction between transplatin and native DNA: relation with the clinical inefficiency of transplatin.* Nucleic Acids Res, 1995. 23(13): p. 2381-8.

44. Goodsell DS, *The molecular perspective: Cisplatin.* Stem Cells, 2006. 24(3): p. 514-5.

45. Alcindor T and Beauger N, *Oxaliplatin: a review in the era of molecularly targeted therapy.* Curr Oncol, 2011. 18(1): p. 18-25.

46. Chao Y, et al., *A randomized controlled trial of gemcitabine plus cisplatin versus gemcitabine alone in the treatment of metastatic pancreatic cancer.* Cancer Chemother Pharmacol, 2013. 72(3): p. 637-42.

47. Stordal, B., N. Pavlakis, and R. Davey, *Oxaliplatin for the treatment of cisplatin-resistant cancer: a systematic review.* Cancer Treat Rev, 2007. 33(4): p. 347-57.

48. Siddik, Z.H., *Cisplatin: mode of cytotoxic action and molecular basis of resistance.* Oncogene, 2003. 22(47): p. 7265-79.

49. Wang, X. and Z. Guo, *Towards the rational design of platinum(II) and gold(III) complexes as antitumour agents.* Dalton Trans, 2008(12): p. 1521-32.

50. Heinemann V, et al., *Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer.* J Clin Oncol, 2006. 24(24): p. 3946-52.

51. Chen Y, et al., *Transarterial infusion with gemcitabine and oxaliplatin for the treatment of unresectable pancreatic cancer.* Anticancer Drugs, 2014.

52. Azmy A, Abdelwahab S, and Yassen M, *Oxaliplatin and Bolus-Modulated 5-Fluorouracil as a Second-Line Treatment for Advanced Pancreatic Cancer: Can Bolus Regimens Replace FOLFOX When Considered for Second Line?* ISRN Oncol, 2013. 2013: p. 358538.

53. Omura, N. and M. Goggins, *Epigenetics and epigenetic alterations in pancreatic cancer.* Int J Clin Exp Pathol, 2009. 2(4): p. 310-26.

54. Dhayat, S., et al., *Epigenetic markers for chemosensitivity and chemoresistance in pancreatic cancer--a review.* Int J Cancer, 2011. 129(5): p. 1031-41.

55. Chen M, et al., *DNA methyltransferase inhibitor, zebularine, delays tumor growth and induces apoptosis in a genetically engineered mouse model of breast cancer.* Mol Cancer Ther, 2012. 11(2): p. 370-82.

56. Yoo CB, Cheng JC, and Jones PA, *Zebularine: a new drug for epigenetic therapy.* Biochem Soc Trans, 2004. 32(Pt 6): p. 910-2.

57. Cheng JC, et al., *Inhibition of DNA methylation and reactivation of silenced genes by zebularine.* J Natl Cancer Inst, 2003. 95(5): p. 399-409.

58. Cheng JC, et al., *Preferential response of cancer cells to zebularine.* Cancer Cell, 2004. 6(2): p. 151-8.

59. Billam M, Sobolewski MD, and Davidson NE, *Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells.* Breast Cancer Res Treat, 2010. 120(3): p. 581-92.

60. Neureiter D, et al., *Apoptosis, proliferation and differentiation patterns are influenced by Zebularine and SAHA in pancreatic cancer models.* Scand J Gastroenterol, 2007. 42(1): p. 103-16.

CHAPTER THREE

CELL DEATH IN RESPONSE TO ANTIMETABOLITES DIRECTED AT RIBONUCLEOTIDE REDUCTASE AND THYMIDYLATE SYNTHASE

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Abstract

New agent development, mechanistic understanding and combinatorial partnerships with known and novel modalities continue to be important in the study of pancreatic cancer and its improved treatment. In this study, known anti-metabolite drugs such as Gemcitabine (ribonucleotide reductase inhibitor (RRI)) and 5-fluorouracil (thymidylate synthase inhibitor (TSI)) were compared with novel members of these two drug families in the treatment of a chemoresistant pancreatic cancer cell line PANC-1. Cellular survival data, along with protein and mRNA expression for Survivin, XIAP, cIAP1 and cIAP2 were compared from both the cell cytoplasm and from exosomes after single modality treatment. While all anti-metabolite drugs killed PANC-1 cells in a time- and dose-dependent manner, neither family significantly altered the cytosolic protein level of the four IAPs investigated. Survivin, XIAP, cIAP1 and cIAP2 were found localized to exosomes where no significant difference in expression was recorded. This inability for significant and long-lasting expression may be a reason why pancreatic cancer lacks responsiveness to these and other cancer killing agents. Continued investigation is required to determine the responsibilities of these IAPs in their role in chemoresistance in pancreatic adenocarcinoma.

KEYWORDS: IAPs, exosomes, pancreatic cancer, antimetabolites, Gemcitabine

Introduction

Pancreatic adenocarcinoma is the 4th leading cause of cancer death [1,2]. Out of all diagnosed patients, only 2-5% survive 5 years, and the average survival time is only 4-6 months [3-6]. There are a number of treatments available for patients, but the option for metastatic pancreatic cancer is limited to chemotherapy, of which only the anti-metabolite drugs gemcitabine (Gem) and 5-fluorouracil (5FU) are clinically used [3,7,8]. Anti-metabolite drugs are designed to stop DNA synthesis and replication. Each anti-metabolite differs from one another in their mechanism of actions, leading to different cytotoxic effects. 5FU is converted into its active form FdUMP which then acts as a thymidine synthase inhibitor. It inhibits the conversion of dUMP to dTMP by binding to thymidine synthase and folate [9,10]. Unlike 5FU, Gem has three mechanisms of action, making this anti-metabolite a broad-spectrum agent. Once Gem enters the cell, it is subjected to phosphorylation by deoxycytidine kinase into gemcitabine monophosphate. Conversion of Gem into its di- and triphosphorylation states by nucleoside kinases converts this anti-metabolite into a ribonucleotide reductase and polymerase inhibitor, respectively [11-13]. There have been numerous efforts to improve chemotherapy treatment regimens by combining these chemotherapies with either 5FU or Gem in combination. Unfortunately, most of these studies have confirmed that combination therapy does not show significant improvements [14-16]. In addition to the failure to improve treatment regimen, patients face the challenge of chemoresistance. Low response rate in patients treated with Gem has been shown to be associated with innate and acquired chemoresistance [17]. Additional studies still need to be conducted to understand resistance to Gem and 5FU in pancreatic cancer patients.

The inhibitor of apoptosis (IAP) family of proteins includes Survivin, XIAP, cIAP1, and cIAP2. IAPs are characterized by a ~70 amino acid baculovirus IAP repeat (BIR) domain and, except for Survivin, a RING domain in the C-terminus of each family member [18,19]. XIAP directly binds to activated caspase-3, -7 and -9 using its BIR domains, inhibiting the caspases' function [20-23]. On the other hand, while cIAP1 and cIAP2 are weak caspase inhibitors [24], these IAPs act as E3 ubiquitin-protein isopeptide ligases on Smac using their RING domains to promote Smac degradation [25]. Survivin, the smallest IAP, is both structurally and functionally unique among the rest of the IAP family, having a multifunctional role in various cellular activities, which includes the regulation of mitosis, protection from cell death, and adaptation to stressful environments [26-28] . This IAP is also found to be localized in the cytoplasm, mitochondria and nucleus, with its subcellular location determining its function [29,30]. Our lab has shown that an extracellular pool of Survivin exists, which causes neighboring cancer cells to become resistant to therapy, to rapidly proliferate, and acquire an increased potential to be invasive [31].

Recently, our lab has discovered that Survivin is released by small (40-100 nm) membrane bound vesicles called exosomes [32]. Tumor cell-derived exosomes (TEX) have been shown to be released constitutively into the extracellular space [33], both in vitro and in vivo [34,35]. TEX have different molecular profiles and biological roles, giving an indication of the cell of origin [36,37]. In addition, specific protein content found on and within TEX and exosomes in general establishes their functional role [38]. The goal of this study is to examine whether anti-metabolite treatments in PANC-1 cells modulate IAP protein and message levels both intracellularly and exosomally. Such IAP

modulation may indicate that these chemotherapeutic agents may contribute to chemoresistance in pancreatic cancer cells.

Materials and Methods

Cell Cultures

The pancreatic carcinoma (PANC-1) cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in DMEM (ATCC) supplemented with 100U penicillin, 100µg/ml streptomycin, 100 µg/ml Normocin (Invitrogen; Grand Island, NY), and 10% fetal bovine serum (FBS: CellGro; Manassas, VA). The cells were grown in a humidified atmosphere of 37°C in 95% O_2 / 5% CO_2 until 60% confluent. Their conditioned medium (CM) for exosome collection was collected after 24 hours treatment with Cladribine (CldA), Gemcitabine (Gem), Hydroxyurea (HU), 5-Fluorodeoxyurodine (5FdU) and 5-Fluorouracil (5FU; Sigma, St. Louis, MO). CldA, Gem, HU, 5FdU were kind gifts of Dr. Jonathan Neidigh at Loma Linda University. All anti-metabolites were dissolved in water and various concentrations were added to cells. For CM collection for exosome isolation, cells were plated 24 hours prior to treatment. Media was changed before anti-metabolite treatment to ensure no apoptotic bodies were present. PANC-1 cells were treated for 24 hours after which CM and cells for Western blots and PCR were harvested.

Apoptosis and Cell Proliferation Analysis

Cells at 60% confluency and 37°C were treated with vehicle (water) or various doses of anti-metabolites for 24, 48 and 72 hours. Cells were harvested and stained with

Annexin V and PI (BioLegend; San Diego, CA) per the manufacturer's directions. Apoptosis and cell proliferation studies were performed and analyzed using a MACS Quant flow cytometer and FlowJo software (Tree Star; Ashland, OR.).

Exosome Isolation

Exosomes were isolated as previously described [39] with the following modifications. Briefly, CM was centrifuged three times prior to ultracentrifugation: 400 x g for 10 mins to remove cells, 2,000 x g for 20 mins to remove cell debris and 10,000 x g for 30 mins to remove nucleic acid and soluble proteins. The supernatant was collected and stored in -80°C until needed. Exosomes were isolated from the CM by ultracentrifugation on a 30% sucrose cushion at 100,000 x g for 16h. The exosomes in the sucrose cushion was extracted and washed once in PBS by ultracentrifugation at 100,000 x g for 2h. The exosome pellet was resuspended in 100ul PBS or lysis buffer.

Exosomes were also isolated using ExoQuick TC™ (Systems Biosciences, Mountain View, CA). Briefly, CM was collected from the treated cells and centrifuged at 3,000 x g for 15 mins. 2ml of ExoQuick TC™ was mixed to 10ml of CM and incubated at 4°C overnight. Following incubation, the CM was centrifuged at 1,500 x g for 30 mins to pellet exosomes. The exosome pellet was resuspended in the appropriate buffer and used for RNA extraction studies.

Exosome Quantification

The amount of exosomes released was semi-quantified by assessing the acetylcholinesterase activity, as our lab has described previously [39]. Briefly, 40µl of the

isolated exosomes were suspended in 110µl PBS. The PBS-diluted exosome fraction was equally divided to 3 individual wells on a 96-well flat-bottomed microplate. 1.25mM acetylcholine and 0.1mM 5,5'-dithiobis(2-nitrobenzoic acid) were added to the exosomes to a total volume of 300µl. The change in absorbance at 412 nm was monitored every 5 min for 30 min.

To determine total exosome number, exosomes were diluted 1:100 in PBS from the total isolated exosome sample and analyzed using a NanoSight LM10-HS microscope (Wiltshire, UK). Size distribution, and total number of exosomes per milliliter were calculated by the nanoparticle tracking analysis software (Wiltshire, UK).

Western Blots

For total cell Western blot analysis, cells are harvested and lysed in cell lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris/HCl, 1 mM PMSF) with sonication. The lysates were centrifuged at 10,000 rpm at 4°C for 20 mins to remove cell debris. For exosome Western blot analysis, exosomes were solubilized in lysis buffer. Protein concentration was determined using the BCA protein assay (Pierce Chemical; Rockford, IL). A total of 50ug cellular protein or 40ug exosomal protein was separated using a 7.5-12% SDS polyacrylamide gels and transferred onto nitrocellulose membrane (BioRad; Hercules, CA). Blots were immunostained with antibodies against Survivin ((1:500-2000), NOVUS Biologicals; Littleton, CO), cIAP1, cIAP2 and XIAP ((1:500-1000), Cell Signaling, Danvers, MA). β-actin ((1:1000), Cell Signaling; Danvers, MA) was used as control for cell samples and Lamp-1 ((1:500, BioLegend, San Diego, CA) was used as control for exosome samples. Goat anti-rabbit and anti-mouse antibodies (LI-

COR Biosciences; Lincoln, NE) were used as secondary antibody. The immunoreactive bands were visualized using the Odyssey imaging system (LI-COR Biosciences).

PCR

Harvested cells and isolated exosomes were resuspended in TRI Reagent® (Molecular Research Center; Cincinnati, OH) and stored at -80°C until needed. RNA was extracted per manufacturer's directions. RNA quantification was performed using a NanoDrop 2000c (Thermo Fisher Scientific; Waltham, MA). Reverse transcription of RNA was performed using the First Strand cDNA Synthesis kit (Syd Labs, Inc.; Malden, MA). Genomic DNA was eliminated prior to reverse transcription of RNA into cDNA. A total concentration of 100ng/ul cDNA was utilized to perform PCR reactions using Phusion® Flash High-Fidelity PCR Master Mix (Finnzymes, Thermo Scientific; Pittsburgh, PA). Forward and reverse primers (IDT, San Diego, CA) have been designed to detect Survivin, cIAP1, cIAP2 and XIAP genes (Table 1).

cDNA was amplified for detection of Survivin, cIAP1, cIAP2, XIAP and GAPDH (housekeeping gene) using the LightCycler 1.0 system real-time thermal cycler and the LightCycler FastStart DNA Master PLUS SYBR Green kit (Roche Applied Science). The assessment of samples as positive for expression was based on 1) observing reproducible cycle threshold (C_T) values in two replicates of the target gene where the GAPDH housekeeping gene showed a C_T value of <30 cycles and 2) melting curve analysis showing superimposable product in negative control samples. Gene-specific primer pairs were designed to span introns using Roche's Assay Design Center (www.roche-applied-science.com). Primers were as follows: GAPDH forward, 5'-GAG TCC ACT GGC GTC

TTC AC; GAPDH reverse, 5'-GTT CAC ACC CAT GAC GAA CA; Survivin forward, 5'-ATG GGT GCC CCG ACG TT; Survivin reverse, 5'-TCA ATC CAT GGC AGC CAG; XIAP forward, 5'-GAC AGT ATG CAA GAT GAG TCA; XIAP reverse, 5'-GCA AAG CTT CTC CTC TTG CAG; cIAP1 forward, 5'-AGC TAG TCT GGG ATC CAC CTC; cIAP1 reverse, 5'-GGG GTT AGT CCT CGA TGA AG; cIAP2 forward, 5'-TGG AAG CTA CCT CTC AGC CTA C; cIAP2 reverse, 5'-GGA ACT TCT CAT CAA CCG AGA. Cycling parameters for all products were initial denaturation of 15 minutes at 95°C followed by 50 cycles of 10 seconds at 95°C (denaturing), 5 seconds at 60°C (annealing), and 15 seconds at 72°C (elongation).

Statistical Analysis

Statistical analysis was performed using a two-way analysis of variance with the aid of GraphPad Prism statistical software (La Jolla, CA, USA), with paired *t*-test used for group analysis. Densitometric analysis was conducted using our Licor Odyssey Images (Licor, Lincoln, NE, USA). Density of individual bands was divided by β-actin, GAPDH or LAMP-1 as the internal controls for cytosolic cellular proteins, block PCR mRNA samples, or exosomal proteins, respectively, with each sample then divided by the particular baseline control.

Results

Anti-metabolite Treatments Induce Growth Inhibition and Cell Death in PANC-1 Cells

CldA treatment of PANC-1 cells with various doses (50nM, 100nM, 1μM, 20μM) showed that lower doses did not inhibit cell growth or show cytotoxic effects (Figs. 1A,

Table 1. Forward and reverse primer dimers for IAP analysis

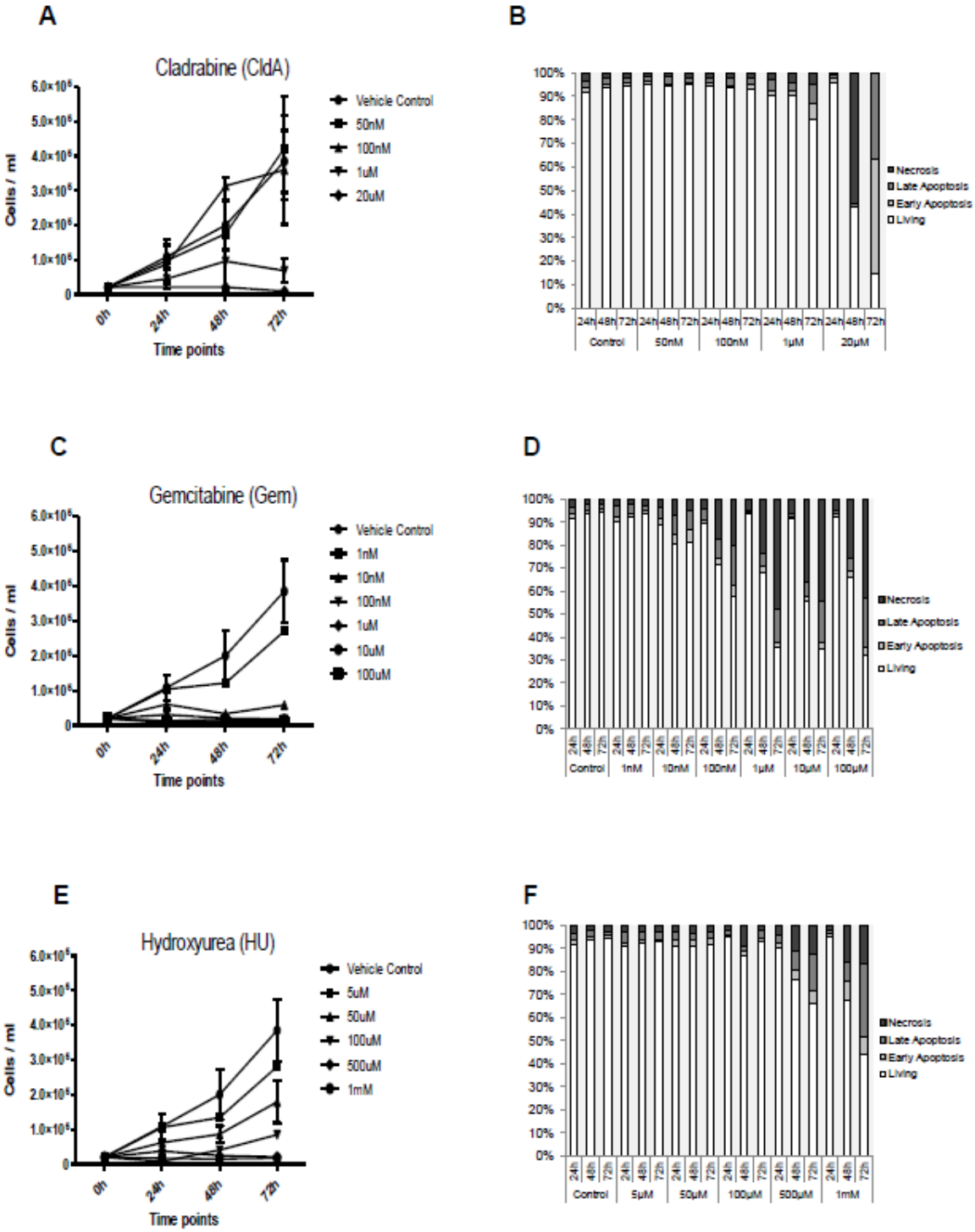
Survivin	Forward	5'-ACCGCATCTCTACATTCAAGA-3'
	Reverse	5'-TCTGTCCAGTTTCAAAAATTC-3'
cIAP1	Forward	5'-CACAAAACTGCCTCCCAAAGA-3'
	Reverse	5'-TTAAGAGAGAAATGTACGAACAGT-3'
cIAP2	Forward	5'-ATGAACATAGTAGAAAACAGCATA-3'
	Reverse	5'-TCATGAAAGAAATGTACGAACTGT-3'
XIAP	Forward	5'-ATGACTTTTAACAGTTTTGAAGGA-'3
	Reverse	5'-TTAAGACATAAAAATTTTTTGCTT-'3
GAPDH	Forward	5'-ACGGATTTGGTCGTATTGGGCG-3'
	Reverse	5'-CTCCTGGAAGATGGTGATGG-3'

1B). At 1μM CldA, there was a decrease in cell proliferation but no significant killing, unlike treatment at 20μM. Treatment with Gem (1nM, 10nM, 100nM, 1μM, 10μM, 100μM) showed a time and dose dependent killing effect, while growth inhibition was evident at all time points and doses except for 1nM (Figs. 1C, 1D). Increasing the concentration of HU (5μM, 50μM, 100μM, 500μM, 1mM) reduced cell proliferation in a time and dose dependent manner. However, the drug's cytotoxic effects were only evident with the two highest doses (Figs. 1E, 1F). Treatment with 5FdU (100pM, 1nM, 10nM, 100nM, 1μM, 10μM) (Figs. 1G, 1H) and 5FU (100nM, 500nM, 1μM, 5μM, 50μM) showed similar cytotoxic and growth inhibition profiles (Figs. 1I, 1J). Of interest, the killing effects of the drugs were time and dose dependent, while cell proliferation was only reduced by the higher doses at all time points (data not shown).

IAPs Expression Levels are not Reduced by Anti-metabolite Treatments and Do Not Play a Role in Inhibiting Cell Death in PANC-1 Cells

To determine whether IAPs play a part in cell death inhibition in PANC-1 cells and play a role in chemoresistance, sub-lethal and lethal doses were chosen to stress the cells for 24, 48 and 72 hours. Ribonucleotide reductase inhibitors did not significantly alter the protein expression of IAPs at 24 and 48 hours (Figs. 2A and 2B). Although not significant, modulation of Survivin expression was shown after 72 hours, in particular with HU treatment at 500μM

Figure 1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Cladrabine (CldA)
Cells / ml
Time points
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24h
48h
72h
Vehicle Control
50nM
100nM
1uM
20uM
B
Necrosis
Late Apoptosis
Early Apoptosis
Living
Control
50nM
100nM
1µM
20µM
C
Gemcitabine (Gem)
Vehicle Control
1nM
10nM
100nM
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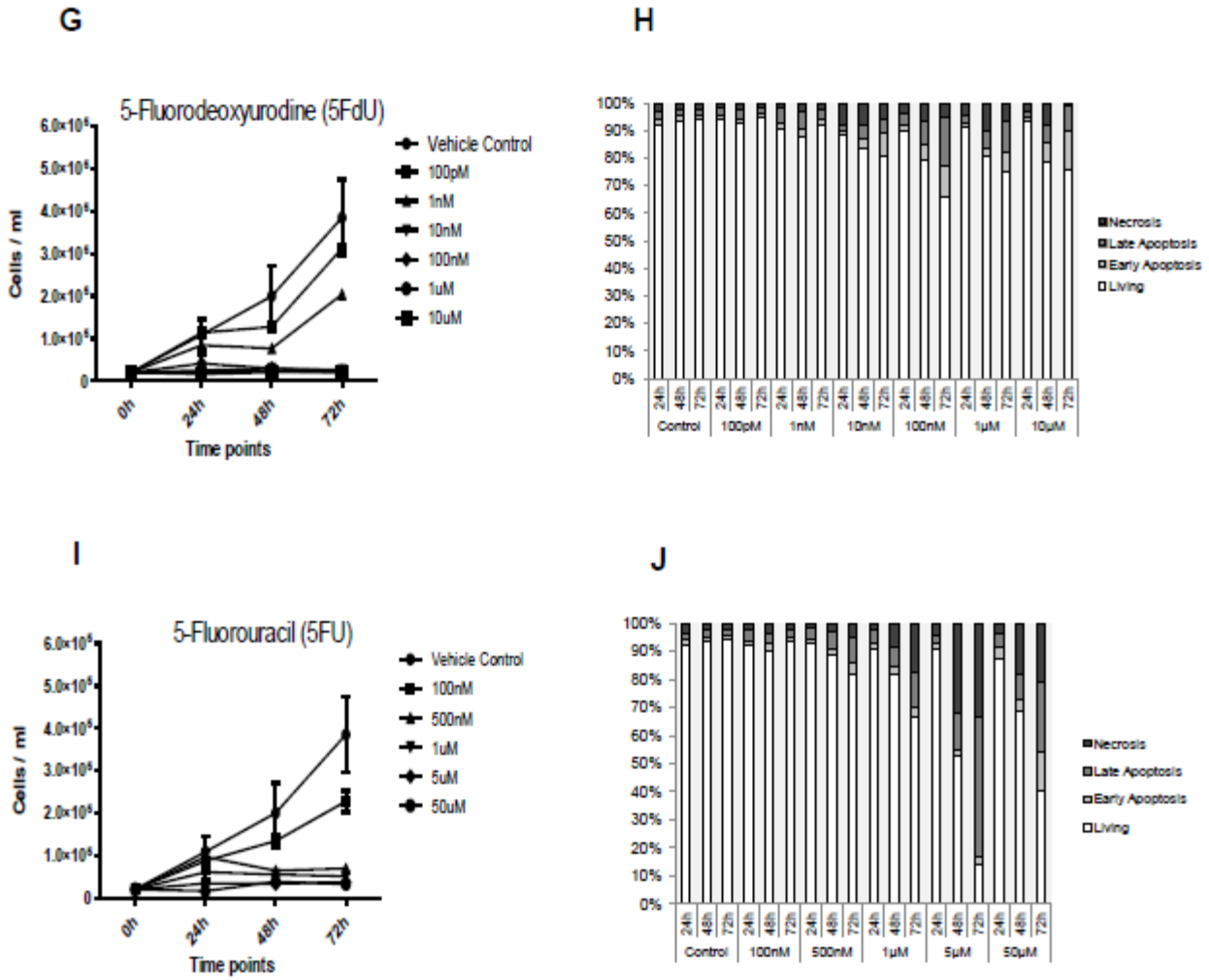
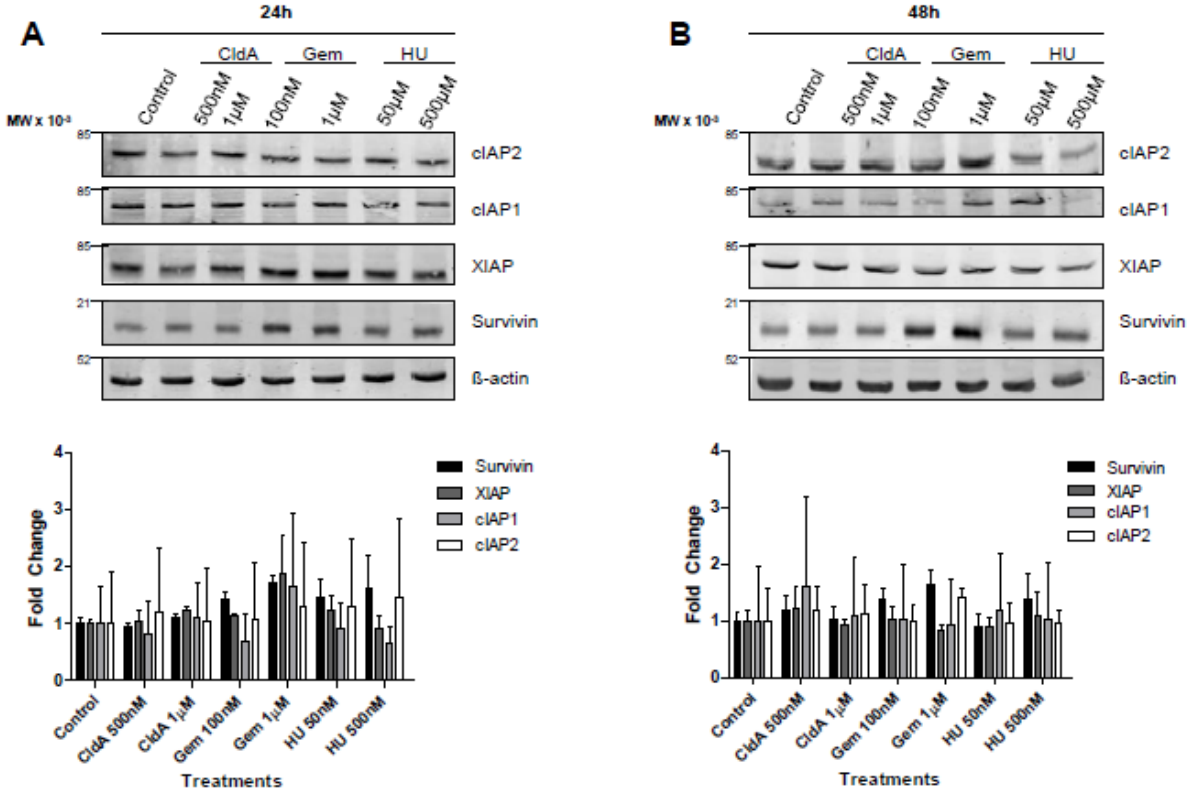


Figure 1 - Reduction of cell proliferation in PANC-1 cells treated with Ribonucleotide Reductase and Thymidine Synthase inhibitors. PANC-1 cells were treated with various concentrations of Ribonucleotide Reductase inhibitors, (A, B) Cladribine, (C, D) Gemcitabine, (E, F) Hydroxyurea, and Thymidine Synthase Inhibitors, (G, H) 5-Fluorodeoxyurodine and (I, J) 5-Fluorouracil for different time periods. It was evident that anti-metabolite treatment causes growth inhibition in PANC-1 cells. To determine the sublethal and lethal doses Annexin/PI assay, along with the cell proliferation assay, was performed on cells treated with (B) Cladribine, (D) Gemcitabine, (F) Hydroxyurea, (H) 5- Fluorodeoxyurodine and (J) 5-Fluorouracil. Cell death in PANC-1 was a time-, and dose-dependent manner.

Figure 2


A
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CldA
Gem
HU
Control
500nM
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100nM
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50μM
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MW x 10-3
85
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85
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cIAP2
cIAP1
XIAP
Survivin
ß-actin
Fold Change
Survivin
XIAP
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cIAP2
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HU 50nM
HU 500nM
Treatments
B
48h
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Gem
HU
Control
500nM
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100nM
1μM
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500μM
MW x 10-3
cIAP2
cIAP1
XIAP
Survivin
ß-actin
Fold Change
Survivin
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CldA 1μM
Gem 100nM
Gem 1μM
HU 50nM
HU 500nM
Treatments

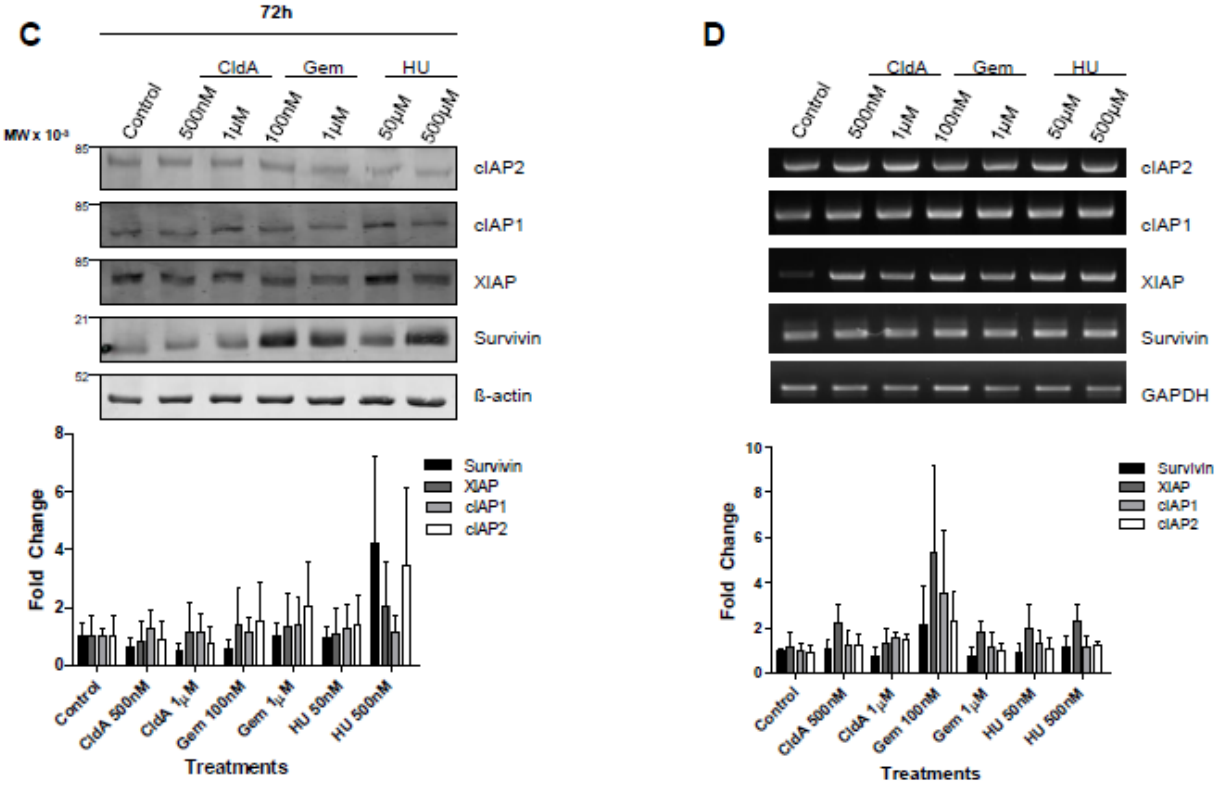


Figure 2 - Modulation of IAPs after treatment of Ribonucleotide Reductase inhibitors. Treatment with sublethal and lethal doses of Ribonucleotide Reductase inhibitors did not decrease the levels of IAP proteins at (A) 24h, (B) 48h and (C) 72h, as well as IAP mRNA (D) in PANC-1 cells. IAP expression was either maintained or increased.

(Fig. 2C). In both mRNA (Fig. 2D and S1A) and protein, IAPs expression levels were either maintained or increased across the doses of ribonucleotide reductase inhibitors.

As shown with the ribonucleotide reductase inhibitors, treatments with thymidine synthase inhibitors did not significantly alter IAPs protein (Figure 3A-C) or mRNA (Figure 3D and S1A) expression levels across all time points and doses. Following the same trend as with the other anti-metabolite family, the IAPs protein and mRNA were either maintained or modestly increased.

Exosome Amount Released Changes with Treatment

To determine whether anti-metabolite drug-treatment stress would affect the amount of exosomes released, an AChE assay was performed. There was no significant difference in the amount of exosomes released in the untreated cells compared to the treated cells (Fig. 4A). Interestingly, the BCA protein assay showed less total protein concentration in the untreated cell sample compared to the treated cells (Fig. 4B). To verify the results, exosomes were examined using a NanoSight LM10-HS which determines the number of exosomes present per ml. Figure 4C shows that compared to the untreated exosome sample, there were more exosomes present in the pooled sample of treated exosomes.

Supplementary material

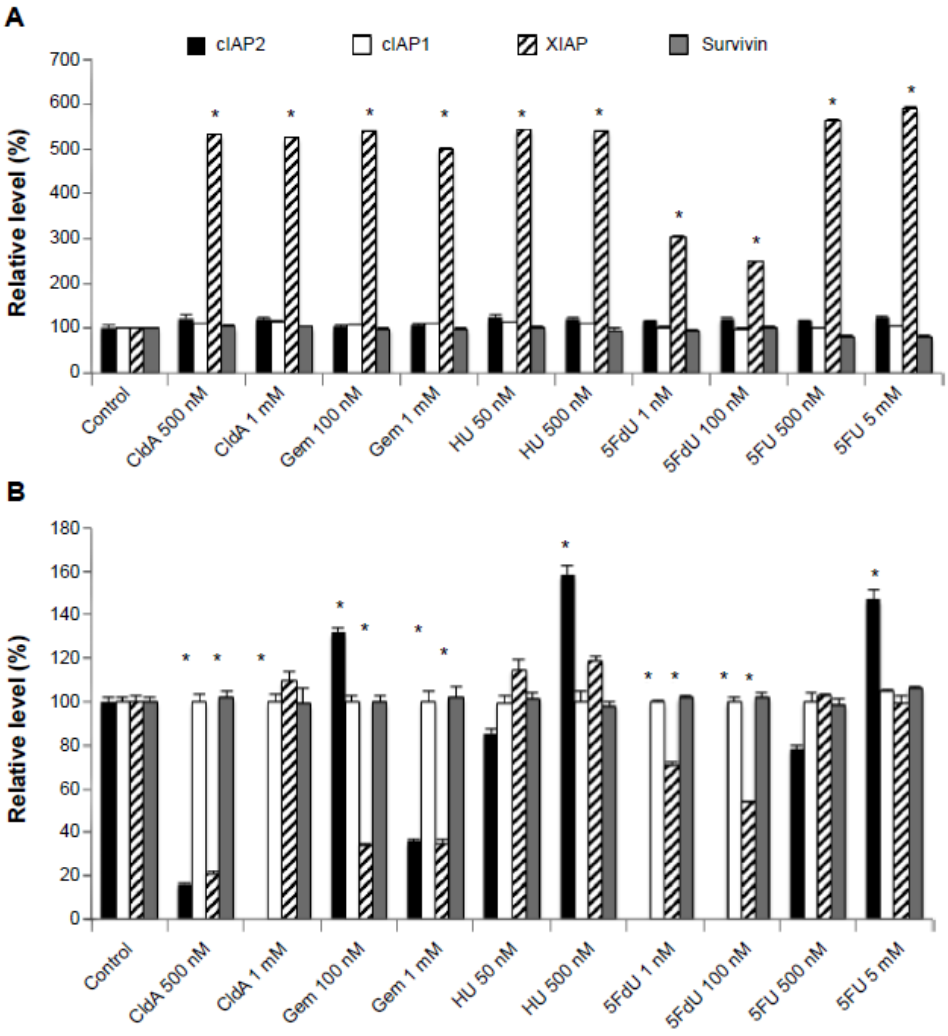
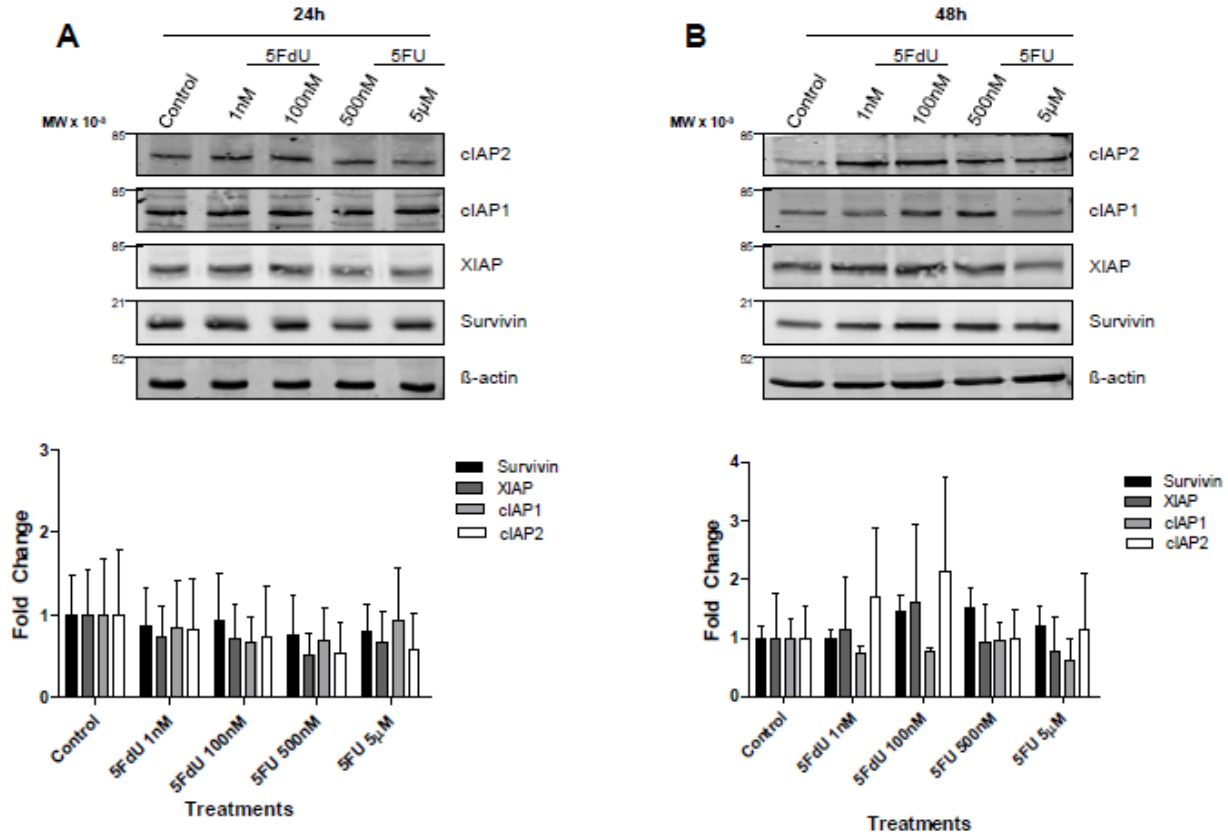


Figure S1 – Ribonucleotide reductase and thymidine synthase inhibitors do not significantly affect cellular (A) or exosomal (B) levels of IAPs.
Notes: PANC-1 cells were treated for 24hours with the indicated amounts of ribonucleotide reductase and thymidine synthase inhibitors. Cell lysates or conditioned medium were extracted for exosomes followed by mRNA. Level of IAP mRNA was determined by real-time polymerase chain reaction. Relative IAP to GAPDH ratios were shown. Data are the mean ± standard deviation of three independent experiments (*P<0.001) as compared with the control.

Figure 3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XIAP
cIAP1
cIAP2
Fold Change
Control
5FdU 1nM
5FdU 100nM
5FU 500nM
5FU 5µM
Treatments
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Control
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cIAP1
XIAP
Survivin
ß-actin
Survivin
XIAP
cIAP1
cIAP2
Fold Change
Control
5FdU 1nM
5FdU 100nM
5FU 500nM
5FU 5µM
Treatments

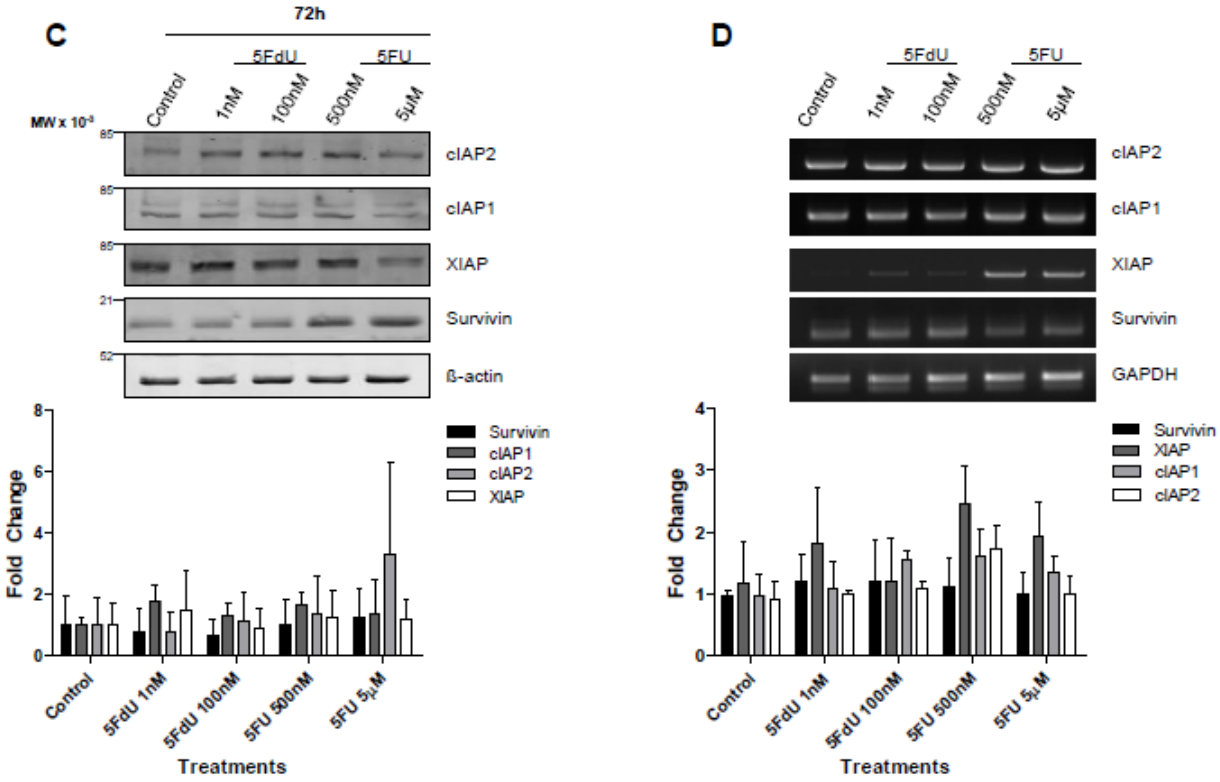


Figure 3 - IAP protein and mRNA levels after treatment with Thymidine Synthase inhibitors. Intracellular IAP protein and mRNA levels (D) were slightly modulated after treatment with sublethal and lethal concentrations of Thymidine Synthase inhibitors at (A) 24h, (B) 48h and (C) 72h.

Figure 4

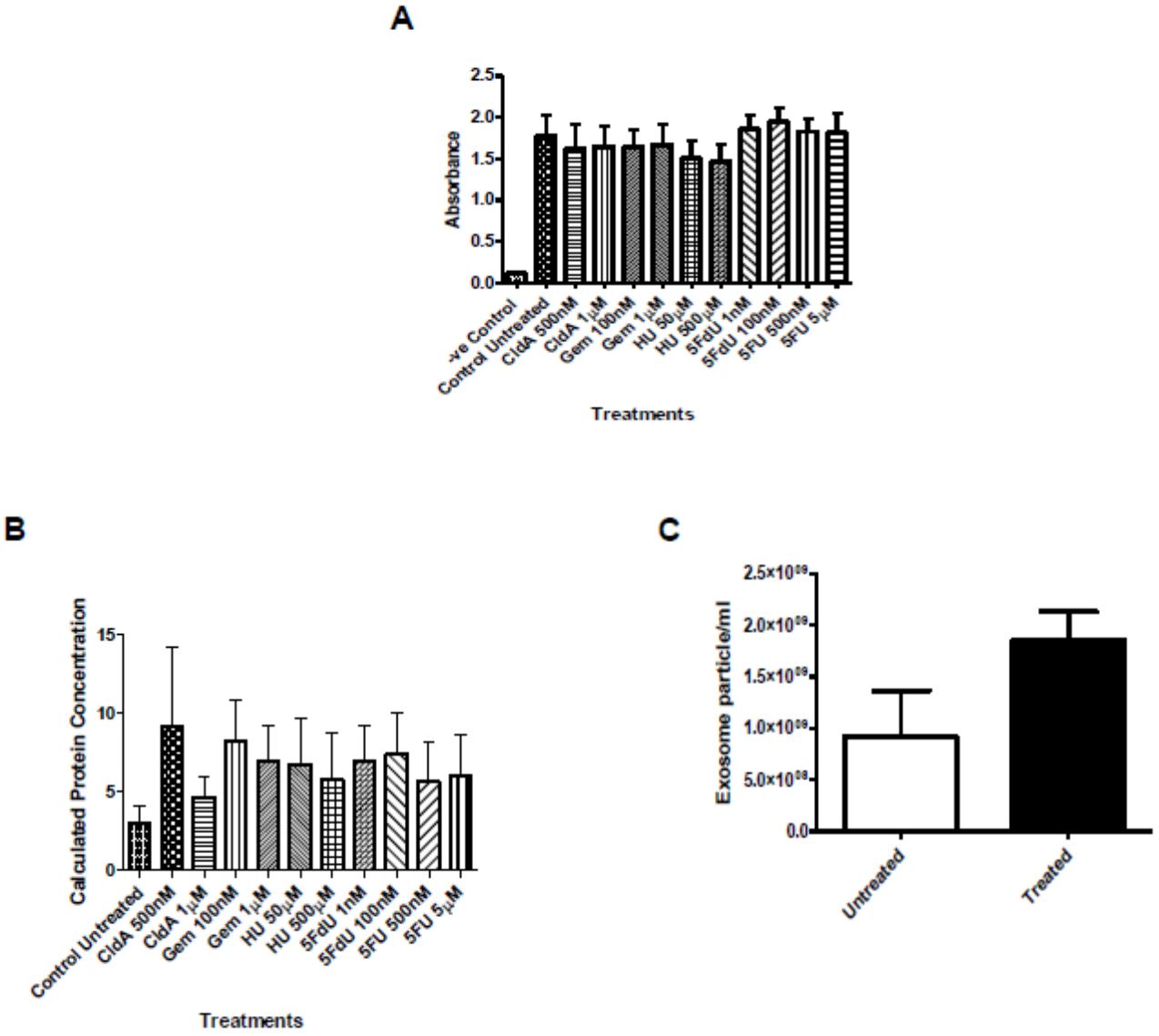
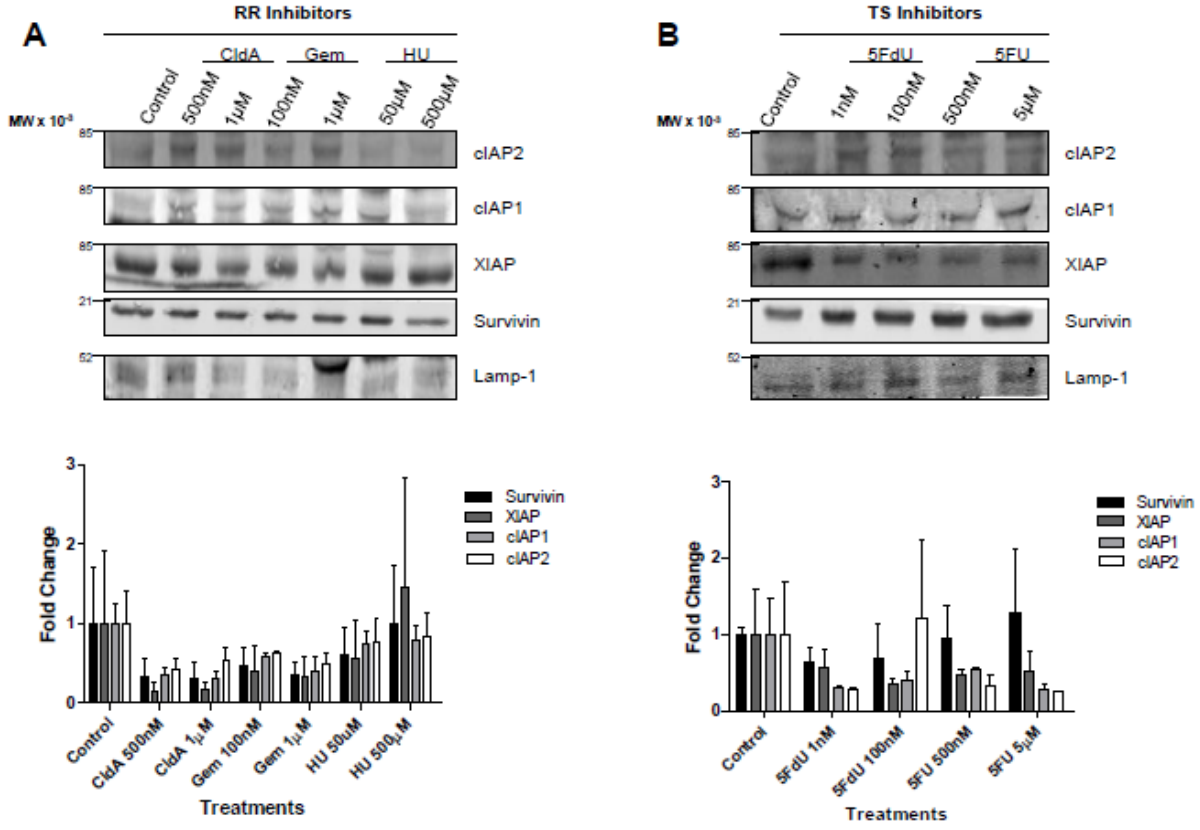


Figure 4 - Exosome release in untreated and treated cells. (A) The acetylcholinesterase activity assay shows that there was no difference in the amount of exosome isolated from conditioned media collected from cells that were treated with vehicle and anti-metabolites. (B) Total exosome protein concentration taken from isolated exosomes from untreated cells was lower compared to the treated exosomes. (C) Total number of exosomes per ml shows that there was more exosomes present in conditioned media taken from treated cells compared to the untreated sample.

Protein and mRNA Inhibitor of Apoptosis Proteins Released Via Exosomes

To further investigate whether IAPs, in addition to Survivin, are released into the extracellular space, exosomes were isolated from conditioned media taken from treated and non-treated cells after 24 hours. Western blotting was performed to determine the presence of IAP proteins in exosomes. As shown in Figures 5A and 5B, not only was Survivin present in exosomes, but so were XIAP, cIAP1 and cIAP2. In addition, treatment with anti-metabolites affected the levels of some released exosomal IAP proteins. Treatment with ribonucleotide reductase inhibitors (Fig. 5A) decreased the levels of exosomal cIAP2, cIAP1 and Survivin at higher concentrations while exosomal IAPs released from cells treated with thymidine synthase inhibitors were only effective in reducing the exosomal levels of XIAP (Fig. 5B). We next wanted to determine whether IAP mRNAs were also present in exosomes. PCR analysis indicated that all IAPs were present in exosomes (Fig. 5C and S1B). Surprisingly, while Survivin and cIAP1 mRNA levels remained unchanged with treatment, the presence of cIAP2 and XIAP mRNA were not consistent across the samples (Fig. 5C and S1B). In addition, XIAP mRNA levels decreased in the majority of the treatments using both families of inhibitors.

Figure 5


A
RR Inhibitors
CldA
Gem
HU
Control
500nM
1μM
100nM
1μM
50μM
500μM
MW x 10-3
85
cIAP2
85
cIAP1
85
XIAP
21
Survivin
52
Lamp-1
Fold Change
Survivin
XIAP
cIAP1
cIAP2
Control
CldA 500nM
CldA 1μM
Gem 100nM
Gem 1μM
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HU 500μM
Treatments
B
TS Inhibitors
5FdU
5FU
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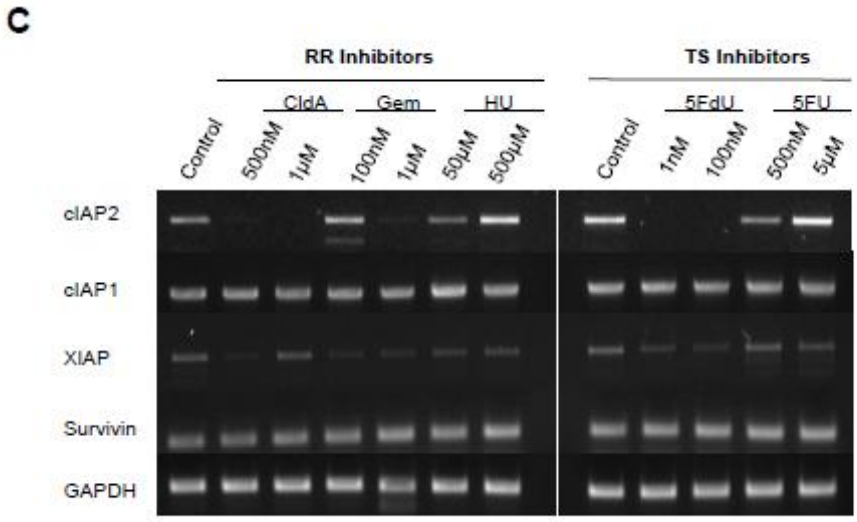

Figure 5 - Presence of IAPs protein and mRNA in exosome. Not only is Survivin protein exported into extracellular space, but XIAP, cIAP1 and cIAP2 are also present in exosomes. (A and B) Exosomes isolated from PANC-1 cells treated with anti-metabolites showed a decrease of IAP protein levels. (C) IAP mRNA is found to be released extracellularly by exosomes. Presence of cIAP2 mRNA, however, was not consistently found in exosomes.

Discussion

In various types of cancer, the function and/or expression of the IAPs is not properly regulated. This can be due to a decrease in levels of endogenous IAP inhibitors, abnormalities in the gene or an increase in either the expression of mRNA or protein [40]. Specifically, the high levels of Survivin expression in cancer cells have been associated with dismal prognosis, disease progression, metastatic dissemination, chemo- and radiotherapy resistance and overall dismal disease outcome [27,41,42]. In many cancer types such as glioblastoma, renal cell carcinoma, liver and pancreatic cancer, the chromosome region of 11q21-23, which include both the cIAP1 and cIAP2 genes, is shown to be amplified in these diseases, making cIAP1/2 protooncogenes. Additionally, in about 50% of surveyed mucosa-associated lymphoid tissue (MALT) lymphoma cases, the BIR domain of cIAP2 is fused to the C-terminus of the paracaspase mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1). This fusion protein in turn constitutively activates NF-κB [40,43]. Overexpression of XIAP correlated with poor clinical outcome, lower survival rates and aggressive tumor growth in diffuse large B lymphoma, colorectal cancer and clear-cell renal cell carcinoma, respectively [40]. In pancreatic adenocarcinoma specifically, it has been shown that Survivin, XIAP, cIAP1 and cIAP2 are constitutively upregulated by NF-κB in cell lines and tissue samples. This abnormal upregulation of IAPs also correlates with chemotherapy resistance [44]. Numerous efforts have been made to target these IAPs to address the problem with resistance to therapy. XIAP silencing by siRNA in pancreatic cancer cell lines has been shown to increase Gem sensitivity [45], as well as an enhancement of cell death when treated with both Gem and proton radiation [42]. Other

studies have targeted Survivin showing that knocking this IAP down with siRNA caused Gem chemosensitivity [46]. Chemotherapy treatment for metastatic pancreatic cancer is limited and modulation of IAP protein and message levels by these anti-metabolites is yet to be determined, both intracellularly and exosomally.

In this study, we first determined that CldA, Gem and HU were all able to decrease cell proliferation, in addition to inducing cell death in a time and dose dependent manner (Fig.1 A-F). We expected IAP protein and mRNA levels to be modulated by the ribonucleotide reductase inhibitors in both sublethal and lethal doses, as cell death was evident in these doses. Surprisingly, we observed that the IAP levels were maintained or even increased (Fig.2). The sublethal dose of Gem was not able to decrease the levels of IAP mRNA, but instead increased expression of all four IAPs. In addition, the lethal dose of Gem consistently increased the levels of IAP protein across all time points.

Like the ribonucleotide reductase inhibitos, cell proliferation was decreased with treatments of 5FdU and 5FU. Cell death was also evident in a time and dose dependent manner (Fig.1 G-J). However, treatments with the sublethal and lethal doses also did not reduce IAP protein and mRNA expression levels (Fig. 3). Since cell death was shown in both sublethal and lethal doses, we expected the IAP protein and mRNA levels to be modulated by these agents. Surprisingly, we observed that the IAP levels were maintained or even increased. This indicates that cell death shown in Fig. 1 may not result from a decrease of IAP levels in these cells, but is through a different mechanism. It is thus not surprising that studies using antimetabolite compounds against leukemias have been recently shown to overcome apoptosis resistance and trigger necroptotic cell death [47]. Additionally, the failure to reduce the levels of IAPs intracellularly by not

only Gem and 5FU, but by all the other agents in both antimetabolite families may play a role in chemoresistance in pancreatic adenocarcinoma patients and why combination therapies do not improve patient survival rates. In studies involving colon cancer cells, cIAP2 reduction has proven to be the only means to increase the efficacy of 5FU [48,49].

Tumor exosomes have been described as "multi-purpose carriers", having a supportive role in the survival and growth of the tumor cells and is involved in promoting host tissue invasion, the subsequent metastasis and facilitating immune response evasion [50-52]. It is speculated that the diverse function of TEX is due to the various bioactive molecules on and within the vesicles, which strongly influences the tumor microenvironment [53-56]. We have also shown that extracellular Survivin has a significant effect on the tumor microenvironment, causing cells to become highly proliferative, invasive and resistant to therapy [31]. In addition to Survivin being exosomal, we found that XIAP, cIAP1 and cIAP2 are also released into the extracellular space via exosomes (Fig. 5).

We looked at the exosomal levels of IAP proteins treated with ribonucleotide reductase inhibitors. We observed that the intracellular IAP expression levels did not reflect the levels of extracellular IAP expression (Fig. 5A). Here, the levels of exosomal IAPs were reduced, with the exception of the treatment of HU (500μM). Looking at the exosomal IAP levels, we saw that 5FU treatments increased Survivin levels in comparison to the other IAPs, as with the cIAP2 with 5FdU treatment at 100nM (Fig. 5B). We expected the exosomal IAP levels would reflect the IAP levels found intracellularly. However, there were modest reductions in the exosomal IAP protein levels in the treated samples. We hypothesize that in an attempt to compensate for

decreasing levels of IAPs in the exosomes, chemotherapy-treated cells released more exosomes into the extracellular space (Fig. 4 and Fig. 5). IAP mRNA were also present in the exosomes (Figures 5C and S1B). The levels of cIAP1 and Survivin appear rather consistent across treatment, but the inconsistent presence of XIAP and cIAP2 mRNA may be due to truncated mRNA that is found within the exosomes. Further work in our lab and others is still evaluating this possibility.

Taken together, the results of this study suggest that protein and mRNA IAPs are found in exosomes and that both cellular and exosomal IAPs should be investigated for their roles in drug resistance in pancreatic cancer. Moreover, though these antimetabolites reduced survival and cell proliferation, levels of the four IAPs studied here only modestly changed and at times increased in both locations depending upon the IAP. Our findings demonstrate for the first time that IAP protein and mRNAs are found in exosomes. More studies, however, are needed to be done in order to fully determine the function of exosomal IAPs in the extracellular space and whether they exhibit similar effects as extracellular Survivin. We also showed that other players are most likely involved in the cell death of PANC-1 cells after anti-metabolite treatments, while the failure to decrease the levels of both protein and mRNA intracellular IAPs may play a role in chemoresistance in pancreatic cancer patients. Although Gem was not able to reduce intracellular IAP protein and mRNA levels, Gem continues to be the first line treatment against metastatic pancreatic cancer. The ability to have different mechanisms of action depending on its phosphorylation state may be why Gem continues to be superior compared to other anti-metabolites [57]. The failure to decrease the levels of both protein and mRNA intracellular IAPs may play a role in chemoresistance in

pancreatic cancer patients. Innate and acquired chemoresistance in patients is a continuing problem in the clinic. Therefore, it is important to continue to find better ways to treat pancreatic adenocarcinoma to try to overcome the problem of resistance and improve overall patient survival rates.

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Conflict of Interest

Dr. Nathan Wall is the interim CEO of Canget BioTekpharma LLC for which he receives travel reimbursements for travel associated with this venture. All other authors declare no conflict of interest.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer Statistics, 2006. *CA Cancer J Clin.* 2006;56:106-130.

2. Niederhuber JE, Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. *Cancer.* 1995;76:1671-1677.

3. Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N. Engl. J. Med.* . 1992;326:455-465.

4. Coleman MP, Gatta G, Verdecchia A, et al. EUROCARE-3 Summary: Cancer survival in Europe at the end of the 20th century. *Ann Oncol.* 2003;14:128-149.

5. Carpelan-Holmstrom M, Nordling S, Pukkala E, et al. Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data Finnish Cancer Registry. *Gut.* 2005;54:385-387.

6. Wagner M, Redaelli C, Lietz M, Seiler CA, Friess H, Buchler MW. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg.* 2004;91:586-594.

7. Ahrendt SA, Pitt HA. Surgical management of pancreatic cancer. *Oncoogy.* 2002;16:725-734.

8. Moss RA, Lee C. Current and emerging therapies for the treatment of pancreatic cancer. *OncoTargets and Therapy.* 2010;3:111-127.

9. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: Mechanisms of action and clinical strategies. *Nat Rev Cancer.* 2003;3:330-338.

10. Thomas DM, JR Z. 5-Fluorouracil: A pharmacological paradigm in the use of cytotoxics. *Clinical and Experimental Pharmacology and Physiology.* 1998;25:887-895.

11. Andersson R, Aho U, Nilsson BI, et al. Gemcitabine chemoresistance in pancreatic cancer: Molecular mechanisms and potential solutions. *Scandinavian Journal of Gastroenterology.* 2009;44:782-786.

12. Mini E, Nobili S, Caciagli B, Landini I, Mazzei T. Cellular pharmacology of gemcitabine. *Annals of Oncology.* 2006;17:7-12.

13. Giovannetti E, Mey V, Danesi R, Mosca I, Del Tacca M. Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell line. *Clin Cancer Res.* 2004;10:2936-2943.

14. Poplin E, Feng Y, Berlin J, et al. Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with

gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group. *J Clin Oncol.* 2009;27(23):3778-3785.

15. Louvet C, Labianca R, Hammel P, et al. Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol.* 2005;23(15):3509-3516.

16. Louvet C, Andre T, Lledo G, et al. Gemcitabine combined with oxaliplatin in advanced pancreatic adenocarcinoma: final results of a GERCOR multicenter phase II study. *J Clin Oncol.* 2002;20:1512-1518.

17. Hung SW, Mody HR, Govindarajan R. Overcoming nucleoside analog chemoresistance of pancreatic cancer: a therapeutic challenge. *Cancer letters.* Jul 28 2012;320(2):138-149.

18. Srinivasula SM, Ashwell JD. IAPs: what's in a name? *Mol Cell.* 2008;30:123-135.

19. Altieri DC. Survivin and IAP proteins in cell-death mechanisms. *Biochem J.* 2010;430:199-205.

20. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. *Genes Dev.* 1999;13:239-252.

21. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol.* 1999;9:323-328.

22. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature.* 1997;388:300-304.

23. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* 1997;16:6914-6925.

24. Eckelman BP, Salvesen GS. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *The Journal of biological chemistry.* Feb 10 2006;281(6):3254-3260.

25. Hu S, Yang X. Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *The Journal of biological chemistry.* Mar 21 2003;278(12):10055-10060.

26. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, JP. N. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat Struc Biol.* 2000;7:602-608.

27. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer.* 2008;8:61-70.

28. Altieri DC. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer.* 2003;3:46-54.

29. Li F. Survivin study: what is the next wave? *J Cell Physiol.* 2003;197:8-29.

30. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol.* 1999;1:461-466.

31. Khan S, Aspe JR, Asumen MG, et al. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential *Br J Cancer.* 2009;100:1073-1086.

32. Khan S, Jutzy JMS, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. *Apoptosis.* 2011;16(1):1-12.

33. Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med.* 2001;7(3):297-303.

34. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount of proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res.* 1998;18(5A):3433-3437.

35. Ginestra A, Miceli D, Dolo V, Romano FM, Vittorelli ML. Membrane vesicles in ovarian cancer fluids: a new potential marker. *Anticancer Res.* 1999;19(4C):3439-3445.

36. Andre F, Schartz NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. *2002.* 2002;360(9329):295-305.

37. Wieckowski E, TL W. Human tumor-derived vs dendritic cell-derived exosomes have distinct biologic roles and molecular profiles. *Immunologic Research.* 2006;36(1-3):247-254.

38. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med.* 1998;4(5):594-600.

39. Khan S, Jutzy JMS, Aspe JR, et al. The application of membrane vesicles for cancer therapy. 2011.

40. Fulda S, Vucic D. Targeting IAP proteins for therapeutic intervention in cancer. *Nature reviews. Drug discovery.* Feb 2012;11(2):109-124.

41. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulators of mitosis and apoptosis and novel targets for cancer therapeutics. *Clin Cancer Res.* 2008;14:5000-5005.

42. Galloway NR, Aspe JR, Sellers C, Wall NR. Enhanced antitumor effect of combined gemcitabine and proton radiation in the treatment of pancreatic cancer. *Pancreas.* 2009;38(7):782-790.

43. Lau R, Pratt MA. The opposing roles of cellular inhibitor of apoptosis proteins in cancer. *ISRN oncology.* 2012;2012:928120.

44. Lopes RB, Gangeswaran R, McNeish IA, Wang Y, Lemoine NR. Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy. *International journal of cancer. Journal international du cancer.* Jun 1 2007;120(11):2344-2352.

45. Shrikhande SV, Kleeff J, Kayed H, et al. Silencing of X-linked inhibitor of apoptosis (XIAP) decreases gemcitabine resistance of pancreatic cancer cells. *Anticancer Res.* Sep-Oct 2006;26(5A):3265-3273.

46. Yoon DH, Shin JS, Jin DH, et al. The survivin suppressant YM155 potentiates chemosensitivity to gemcitabine in the human pancreatic cancer cell line MiaPaCa-2. *Anticancer Res.* May 2012;32(5):1681-1688.

47. Chromik J, Safferthal C, Serve H, Fulda S. Smac mimetic primes apoptosis-resistant acute myeloid leukaemia cells for cytarabine-induced cell death by triggering necroptosis. *Cancer letters.* Mar 1 2014;344(1):101-109.

48. Miura K, Karasawa H, Sasaki I. cIAP2 as a therapeutic target in colorectal cancer and other malignancies. *Expert opinion on therapeutic targets.* Nov 2009;13(11):1333-1345.

49. Karasawa H, Miura K, Fujibuchi W, et al. Down-regulation of cIAP2 enhances 5-FU sensitivity through the apoptotic pathway in human colon cancer cells. *Cancer science.* May 2009;100(5):903-913.

50. Nieuwland R, Sturk A. Why do cells release vesicles? *Thromb Res.* 2010;125.

51. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res.* 2007;67(7):2912-2915.

52. Anderson HC, Mulhall D, Garimella R. Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. *Lan Invest.* 2010;90(11):1549-1557.

53. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. *J Biochem.* 2006;140(1):13-21.

54. Mears R, Craven RA, Hanrahan S, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics.* 2004;4(12):4019-4031.

55. Hegmans JP, Bard MP, Hemmes A, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am J Pathol.* 2004;164(5):1807-1815.

56. Iero M, Valenti R, huber V, et al. Tumour-released exosomes and their implications in cancer immunity. *Cell Death Differ.* 2008;15(1):80-88.

CHAPTER FOUR

EXOSOMES SECRETED FROM HUMAN CANCER CELL LINES CONTAIN INHIBITORS OF APOPTOSIS (IAPS)

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Running Title: Existence of Exosomal IAPs

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Abstract

Exosomes are endosomal-derived nanovesicles released by normal and tumor cells which have been shown to transfer functionally active protein, lipids, mRNAs and miRNAs between cells. Varying in molecular profiles, biological roles, functional roles and protein contents, exosomes have been described as "multi-purpose carriers" playing a role in supporting the survival and growth of tumor cells. The IAP Survivin has been found to be present in tumor exosomes. However, the existence of other IAPs in tumor exosomes is still unknown. Survivin, cIAP1, cIAP2 and XIAP mRNA and protein are differently expressed in a panel of tumor cell lines: DLCL2, HeLa, MCF-7, PANC-1, and PC3. Exosomes were isolated from conditioned media collected from the cells from which RNA and protein were extracted. Our results provide evidence that like Survivin, XIAP, cIAP1 and cIAP2 proteins are found in tumor exosomes. The mRNA expression, however, is differentially expressed across the tumor cell lines. The presence of these bioactive molecules in exosomes may not only serve as warning signals, but also play a role in providing protection to the cancer cells against changes that are constantly occurring in the tumor microenvironment.

Keywords: Exosomes, IAPs, cancer cells, tumor microenvironment

Introduction

Exosomes are small membrane vesicles, ranging from 40-150nm in diameter, that are shed from various cell types such as B- and T-lymphocytes, neurons, intestinal epithelial cells, dendritic cells and tumor cells [1-3]. Tumor exosomes, which are constitutively released into the extracellular space, have different molecular profiles, biological roles and molecular contents, giving an indication of the cell of origin, as well as their functional role [4-6]. Diverse tumor antigens expressed on and or in that are secreted from neoplastic cells give an indication of the type of tumor cells from which tumor exosomes originated [7-9]. This has also been shown *in vivo*, where membrane vesicles isolated from cancer patients' plasma and neoplastic effusions are characterized by the expression of tumor- specific markers reflecting tumor origin [8, 10-12]. Tumor exosomes have a role in supporting the tumor cells' survival and growth [13]. The specific roles include, and not limited to, evasion of host immunity [14], tissue invasion [15] and neoangiogenesis [16, 17]. Not only do tumor exosomes contain proteins and tumor antigens, but functional mRNA has also been shown to be contained within these microvesicles [10].

The inhibitor of apoptosis (IAP) family of proteins are known to be endogenous caspase inhibitors, where cIAP1, cIAP2 and XIAP directly binds to activated caspase-3, -7, -9 using their baculorvirus IAP repeat (BIR) domains [18-21]. Survivin, a unique member of the IAP family, contains a BIR domain, but has a multifunctional role in various cellular activities, including regulating mitosis, inhibiting cells from undergoing apoptosis and adapting to stressful environments [22-24]. Survivin's multifunctional role depends on its subcellular location, where it is found to be localized in the nucleus,

mitochondria and cytoplasm [25]. We have shown that an extracellular pool of Survivin also exists, released from cancer cells in exosomes [26]. Upon release and resorption by neighboring cancer cells, these cells become resistant to therapy, rapidly proliferate and acquire an increased potential to be invasive [27]. In addition to Survivin, we also have recently shown that cIAP1, cIAP2 and XIAP are found in exosomes collected from PANC-1 conditioned media [28].

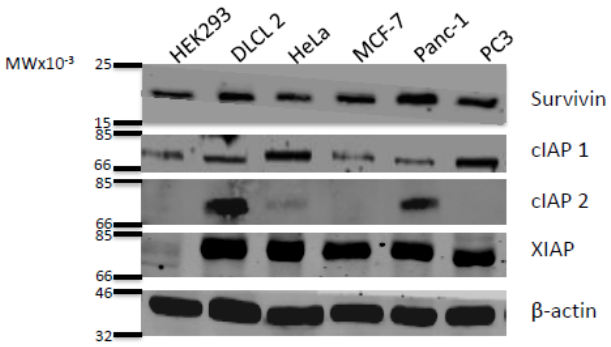
Here, we evaluate across a panel of cell lines representing five different cancer types and one non-cancer, whether like Survivin, cIAP1, cIAP2 and XIAP are released into the extracellular space via exosomes. We show that cIAP1, cIAP2 and XIAP and Survivin protein and mRNA are released by exosomes.

Results

Intracellular IAP mRNA and Protein is Differently Expressed in Cancer Cell Lines

IAPs play an important role in the cancer cell's ability to resist apoptosis (29). In this study, we used five different cancer cell lines from various cancer types. All cell lines, including a non- cancer cell line HEK293 displayed a range of IAP expression levels at protein level (Fig. 1A). In contrast, DLCL2 expressed an increased level of cIAP1. In comparison to protein expression, IAP mRNA is equally expressed in all the tumor cell lines. HEK293 cells highly expressed XIAP mRNA and showed low Survivin mRNA expression levels. In contrast, cIAP1 and cIAP2 expression levels were deficient in HEK293 cells (Fig. 1B).

Figure 1A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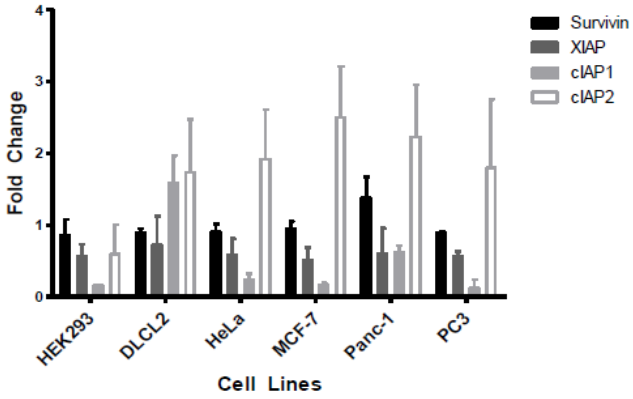
Survivin
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cIAP1
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HEK293
DLCL2
HeLa
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Panc-1
PC3
Cell Lines

Figure 1B

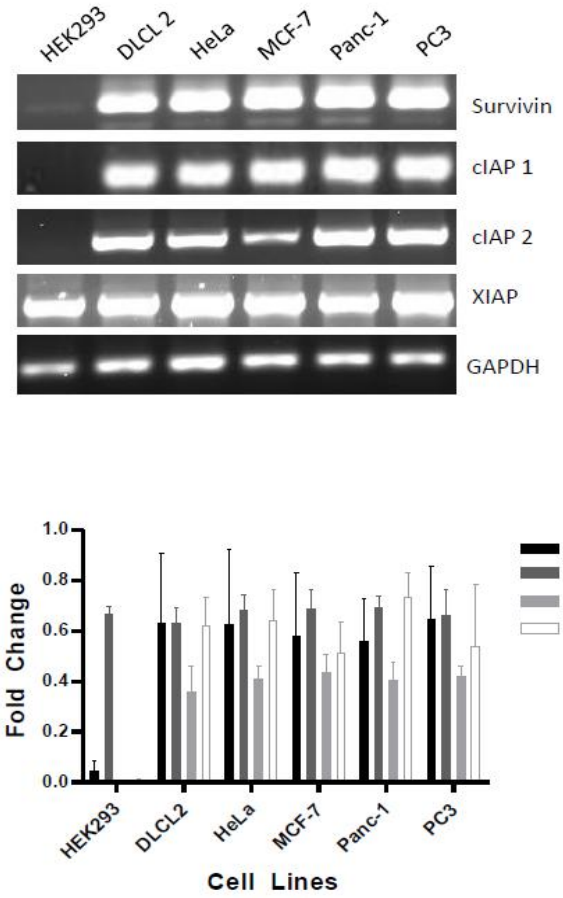


Figure 1 - Western Blot Analysis of Survivin, cIAP1, cIAP2, XIAP and β-actin taken from non-cancer cell line and nontreated cancer cell lines: Human embryonic kidney cell line (HEK293), Diffuse Large Cell Cleaved (DLCL), cervical (HeLa), breast (MCF-7), pancreatic (PANC-1), and prostate (PC3). **A**. Antibodies for Survivin, cIAP1, cIAP2, XIAP and β-actin were used for Western blotting cell line-purified protein. **B**. mRNA was also acquired from the same nontreated non-cancer and cancer cell lines and the varying IAP targets were amplified using PCR. Both Western blots as well as PCR are representative of 2-4 independent experiments.

Amount of Exosome Released Depends on Cell Line

Tumor cells have been shown to constitutively release TEX into the extracellular space [4]. To determine whether the type of cancer influences the amount of exosomes released, we collected conditioned media from different cancer cell lines. The presence and amount of purified exosomes were determined by NanoSight. Among the cancer cell lines, HeLa, MCF-7 and PC3 released the least amount into the media (Fig. 2). To verify that the vesicles collected were indeed exosomes, the vesicles' mode average sizes were analyzed using NanoSight's nanoparticle tracking analysis software. Although a range of vesicle sizes were detected, the majority of the collected vesicles lie within the size range of exosomes (Fig. 3).

IAPs are Present in Exosomes

We have previously shown that Survivin, cIAP1, cIAP2 and XIAP are trafficked into the extracellular space via exosomes [26, 28]. We therefore hypothesized that IAPs would also be exported out of a variety of tumor cells in the same manner. We evaluated the presence of Survivin, cIAP1, cIAP2 and XIAP from isolated exosomes collected from conditioned media by Western blot to determine if these IAPs would be present in exosomes. Across all cell lines, Survivin, along with cIAP1, cIAP2 and XIAP were found in the exosomes of the cell lines evaluated (Fig. 4A). The quantity of IAPs released in the exosomes depends on the cell line.

Figure 2

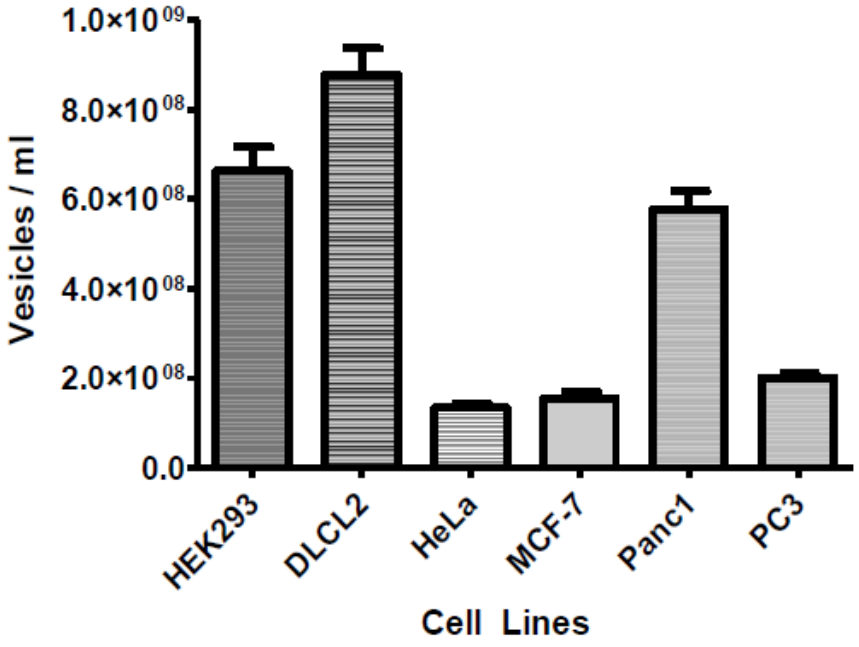


Figure 2 - Histogram representing concentration of vesicles per ml to quantify exosome numbers. Exosomal contents in conditioned medium from HEK293, DLCL2, HeLa, MCF7, PANC-1, and PC3 cell lines. Data are the mean ±SD of 3 independent experiments in triplicate.

Figure 3

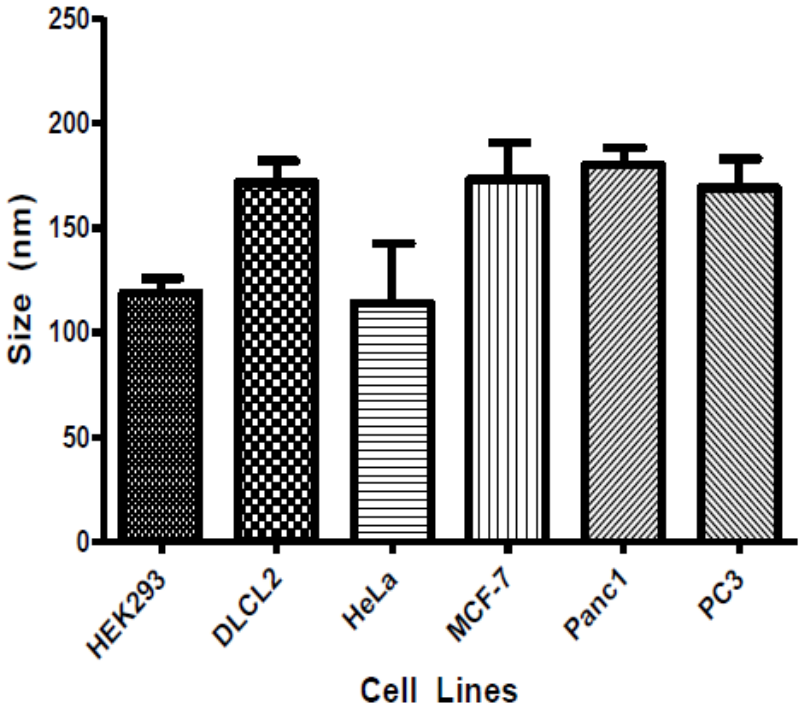


Figure 3 - Mode average size of exosomes isolated using ExoQuick TC™. While there were a range of sizes of vesicles isolated, the mode average size of vesicles falls in the size range of exosomes.

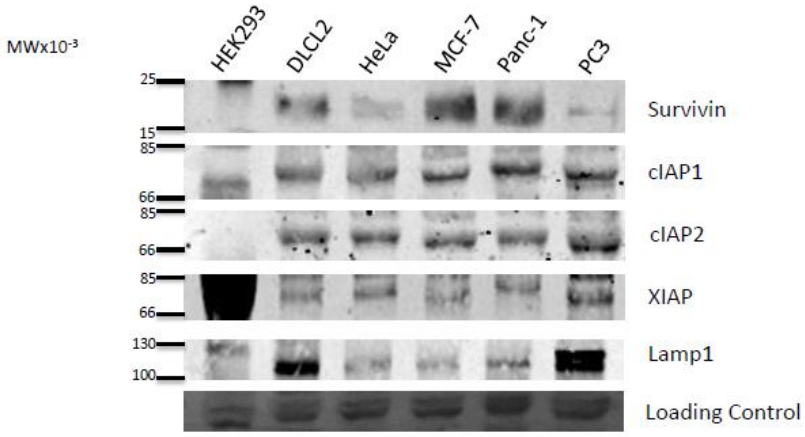
IAP mRNA are Released into the Extracellular Space by Exosomes

Exosomes serve as vesicles for not only proteins, but also for genetic materials [30]. In addition to verifying the presence of IAP protein in the exosomes, the presence of exosomal IAP mRNA was also investigated in the panel of tumor cell lines. To examine this possibility, mRNA was extracted from isolated exosomes and PCR was performed. Not all of the cancer cells showed representative abundance of all four IAPs. Survivin mRNA was found more abundantly than all other IAPs in the cell lines evaluated (Fig. 4B).

Discussion

IAPs are characterized by an ~70 amino acid baculovirus IAP repeat (BIR) domain and a RING domain in the C-terminus of each family member [29, 31]. IAPs are known to be endogenous caspase inhibitors [32] directly binding to caspase-3, -7 and -9 using their BIR domains [18-21]. The IAP family regulates cell survival and members of this family are often deregulated in cancer, which may be a factor for chemoresistance and treatment failure [33]. In most normal adult tissues, Survivin expression is very low or undetectable [23, 34, 35]. The high levels of Survivin expression in cancer cells have been associated with grim prognosis, disease progression, metastatic dissemination, therapy resistance and overall dismal disease outcome [23, 34]. The biological characteristics of the tumor, as well as the way the host responds to the tumor also plays a major role on the growth and spread of cancer [27]. Here we show that though there is a consistent cellular expression of IAP mRNA in all cell lines we evaluated, there is

Figure 4A


MWx10-3
HEK293
DLCL2
HeLa
MCF-7
Panc-1
PC3
25
15
85
66
85
66
85
66
130
100
Survivin
cIAP1
cIAP2
XIAP
Lamp1
Loading Control

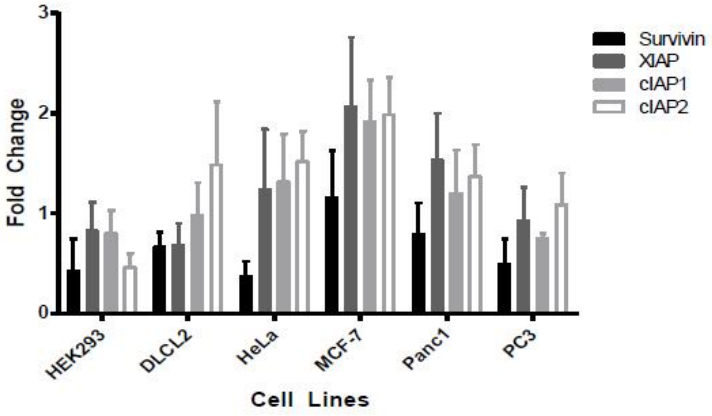
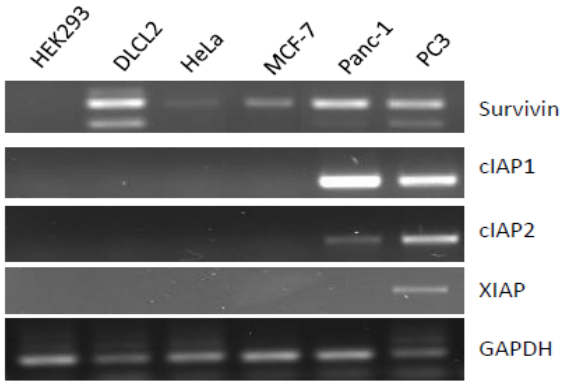

Survivin
XIAP
cIAP1
cIAP2
Fold Change
3
2
1
0
HEK293
DLCL2
HeLa
MCF-7
Panc1
PC3
Cell Lines

Figure 4B



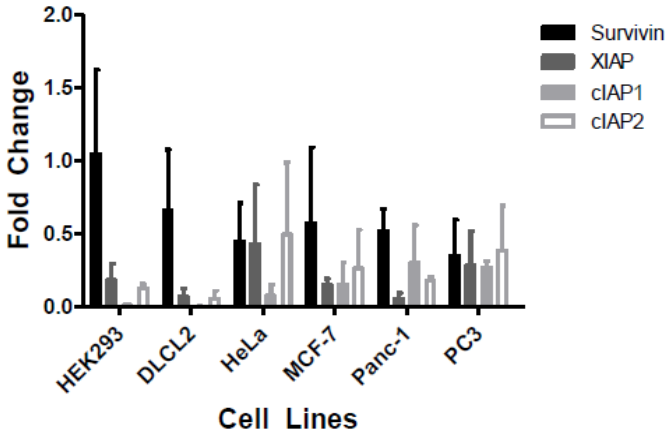


Figure 4 - Western Blot Analysis of Survivin, cIAP1, cIAP2, XIAP and Lamp-1 taken from the conditioned medium off of nontreated non-cancer and cancer cell lines: Human embryonic kidney cell line (HEK293), Diffuse Large Cell Cleaved (DLCL), cervical (HeLa), breast (MCF-7), pancreatic (PANC-1), and prostate (PC3). **A**. Antibodies for Survivin, cIAP1, cIAP2, XIAP and Lamp-1 were used for Western blotting exosome-purified protein. **B**. mRNA was also acquired from the same nontreated non-cancer and cancer cell lines and the varying IAP targets were amplified using PCR. Both Western blots as well as PCR are representative of 2-4 independent experiments.

a distinct cell-type specific expression of IAP protein expression (Fig. 1). IAP protein expression varies, perhaps reflecting the cell line's level of therapy resistance and aggressiveness.

The tumor microenvironment is composed of a variety of cell types which make up the invasive carcinoma, its stromal elements and the immune cells. Communication between these components by secretion of various proteins, such as growth factors, ECM-degrading proteinases and chemokines is crucial for the progression, development and maintenance of the tumor [36]. Small membrane vesicles are known to be secreted from tumors [37] and increasing interest and studies to define their role are underway to elucidate the role of these vesicles or TEX play in cancer development and progression. TEX have been described as "multi-purpose carriers" having vital roles in the communication, protection, progression as well as genetic information exchange with neighboring cells in the microenvironment [38]. Various bioactive molecules have been found packaged within as well as on the TEX, strongly influencing the surrounding environment [7, 39-41] through direct signaling interaction or through trafficking of these molecules into a recipient cell(s). Survivin has a multifunctional role in various cellular activities depending on its subcellular location. We have recently established that Survivin is also found in the extracellular space [27] and exported out of the cancer cells via exosomes [26]. The work described here was to establish whether other IAPs were also exported from cancer cells in a similar fashion.

Exosomes were isolated from condition media collected from the panel of cell lines. These samples were analyzed using the NanoSight to determine the presence and amount of purified exosomes (Figs. 2 & 3). Release of TEX can be affected by various

changes taking place in the microenvironment, such as chemo-, and radiation stress, as well as the biome they contain [42-44]. Interestingly, chemoresistant cells that have been treated with chemotherapeutic agents show a significant increase secretion of vesicles compared to chemosensitive cells [45, 46]. The difference in the amount of exosomes collected between cell lines could be due to stress, such as overconfluency of the cells.

Here we show that Survivin, along with cIAP1, cIAP2 and XIAP, are secreted from tumor and non-tumor cells into the extracellular space via exosomes (Fig. 4A). Secretion of IAPs through exosomes and their subsequent uptake by neighboring cells of the tumor microenvironment can serve as a protective strategy from cell death. It could also be a mechanism for these IAPs and other exosomal biomolecules to travel long distances within the body, affecting, stabilizing or manipulating environments far from the primary tumor in order to aid secondary tumor growth and resistance. We have shown that Survivin, when released to the extracellular milieu has the ability to stimulate cellular proliferation, increase resistance and invasive potential [27], and modulate immune cells [47]. It may be that the tumor microenvironmental presence of exosome containing biomolecules could play a bigger role in antitumor protections than the cellular modulation of these IAPs, having significant reach beyond that possible for circulating tumor cells.

Genetic material, found in vesicles, has been implicated in furthering tumor growth (30). Our lab has recently shown that IAP mRNA was found in exosomes isolated from PANC-1 conditioned media [28]. We therefore hypothesized that IAP mRNA is also found in exosomes collected from different tumor cell lines. While Survivin mRNAs were secreted by all the cell lines investigated in this study, cIAP2 and XIAP mRNA are

more selectively found in the exosomes from the cell lines observed (Fig. 4B). Interestingly, there appeared from experiment to experiment some variation in the mRNAs found in these exosomes which was not the case with protein. We hypothesize that this variance may be the result of the type of RNA product and its status at the time of capture by the exosome. It may be possible that exosomes package truncated mRNAs as their RNA transcripts undergo a widespread post-transcriptional cleavage. As a result these truncated RNAs provide a more small RNA, regulatory role like a miRNA [48, 49]. Full length IAP mRNA transcripts were also found to be present in exosomes, which may be translated into functional proteins upon reabsorption into recipient cells, as shown by Skog et al [10]. In addition, the release of these bioactive molecules may not only serve as warning signals to the neighboring cells, but also provide protection against the constant environmental changes in the tumor microenvironment.

Materials and Methods

Cell Lines and Cultures

Cervical carcinoma (HeLa), prostate carcinoma (PC3), breast carcinoma (MCF-7), pancreatic carcinoma (PANC-1) and human embryonic kidney (HEK293) cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The non-Hodgkin's lymphoma cell line (DLCL2) was a kind gift from Dr. Ayad Al-Katib (Wayne State University, Detroit, MI). The cells were maintained in DMEM, McCoy's or RPMI (ATCC, CellGro; Manassas, VA) supplemented with 100U penicillin, 100µg/ml streptomycin, 10% fetal bovine serum (FBS: CellGro; Manassas, VA). The cells were

grown in a humidified atmosphere at 37°C of 95% O_2 and 5% CO_2 until 60% confluent and the medium was changed. The conditioned media was collected after 24 hours.

Exosome Isolation

The method for exosome isolation was performed using ExoQuick TC™ (Mountain View, CA). Briefly, CM was collected from the treated cells and centrifuged at 3,000 x g for 15 min. 1ml of ExoQuick TC™ was mixed with 5ml of CM and incubated at 4°C for 12h. Following incubation, the CM was centrifuged at 1,500 x g for 30 min to pellet exosomes. The pellet was resuspended in the appropriate buffer to isolate RNA or protein to be used for PCR or Western blot analysis. Exosome pellet resuspended in PBS was used for NanoSight analysis.

Verification of Exosome Presence and Exosome Quantification

To verify exosome presence and determine total exosome number, exosomes were diluted 1:10000 in PBS from the total isolated exosome sample and analyzed using a NanoSight LM10-HS microscope (Wiltshire, UK). Size distribution and total number of exosomes per milliliter were calculated by the nanoparticle tracking analysis software (Wiltshire, UK).

Western Blots

For total cell Western blot analysis, the cells were harvested and lysed in cell lysis buffer (0.5% Triton X-100, 300mM NaCl, 50mM Tris/HCl, 1mM PMSF) with sonication. The lysates were centrifuged at 10,000 rpm at 4°C for 20 min to remove cell

debris. For exosomes Western blot analysis, exosomes were solubilized in lysis buffer. Protein concentration was determined using the Micro BCA protein assay (Pierce Chemical; Rockford, IL). A total of 50µg cellular protein or 30µg exosome protein was separated using a 10-12% SDS polyacrylamide gels and transferred onto nitrocellulose membrane (BioRad; Hercules, CA). Blots were immunostained with antibodies against Survivin ((1:500-2000), NOVUS Biologicals, Littleton, CO), cIAP1, cIAP2 and XIAP ((1:500-1000), Cell Signaling, Danvers, MA). β-actin ((1:1000), Cell Signaling) was used as control for cell samples and Lamp-1 ((1:500, BioLegend, San Diego, CA) was used as a loading control for exosome samples. Goat anti-rabbit antibodies (LI-COR Biosciences, Lincoln, NE) were used as secondary antibody. The immunoreactive bands were visualized using the Odyssey imaging system (LI-COR Biosciences).

PCR

Harvested cells and isolated exosomes were resuspended in TRI Reagent® (Molecular Research Center, Cincinnati, OH) and stored at -80°C until needed. RNA was extracted per the manufacturer's directions. RNA quantification was performed using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). Reverse transcription of RNA was performed using the First Strand cDNA Synthesis kit (Syd Labs, Inc, Malden, MA). Genomic DNA is eliminated prior to reverse transcription of RNA into cDNA. A total concentration of 100ng/µl cDNA was utilized to perform PCR reactions using Phusion® Flash High-Fidelity PCR Master Mix (Finnzymes, Thermo Scientific; Pittsburgh, PA).The forward and reverse primers (IDT, San Diego, CA) were designed to detect Survivin, cIAP1, cIAP2 and XIAP genes.

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Conflict of Interest

Dr. Nathan Wall is the interim CEO of Canget BioTekpharma LLC for which he receives travel reimbursements for travel associated with this venture. All other authors declare no conflict of interest.

References

1. Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J Cell Sci. 2000;113(9):3365-74.

2. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunol Lett. 2006;107(2):102-8.

3. Simpson RJ, Lim JWE, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomic. 2009;6(3):267-83.

4. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001;7:297-303.

5. Wieckowski E, Whiteside TL. Human tumor-derived vs dendritic cell-derived exosomes have distinct biologic roles and molecular profiles. Immunol Res. 2006;36:247-54.

6. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998;4:594-600.

7. Iero M, Valenti R, Huber V, Filipazzi P, Parmiani G, Fais S, et al. Tumour-released exosomes and their implications in cancer immunity. Cell Death Differ. 2008;15:80-8.

8. Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. Lancet. 2002;360(9329):295-305. Epub 2002/07/31.

9. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. The Journal of experimental medicine. 2002;195(10):1303-16. Epub 2002/05/22.

10. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10(12):1470-6. Epub 2008/11/18.

11. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology. 2005;128(7):1796-804. Epub 2005/06/09.

12. Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA, et al. Proteomic analysis of exosomes isolated from human malignant pleural

effusions. American journal of respiratory cell and molecular biology. 2004;31(1):114-21. Epub 2004/02/21.

13. Anderson HC, Mulhall D, Garimella R. Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Lan Invest. 2010;90(11):1549-57.

14. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. Cancer Res. 2007;67(7):2912-5.

15. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer research. 1998;18(5A):3433-7. Epub 1998/12/22.

16. Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(10):3794-9. Epub 2009/02/24.

17. Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. International journal of cancer Journal international du cancer. 2005;113(5):752-60. Epub 2004/10/23.

18. Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. Genes Dev. 1999;13:239-52.

19. Miller LK. An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol. 1999;9:323-8.

20. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature. 1997;388:300-4.

21. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 1997;16:6914-25.

22. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat Struc Biol. 2000;7:602-8.

23. Altieri DC. Validating survivin as a cancer therapeutic target. Nat Rev Cancer. 2003;3:46-54.

24. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer. 2008;8:61-70.

25. Li F. Survivin study: what is the next wave? J Cell Physiol. 2003;197:8-29.

26. Khan S, Jutzy JMS, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. Apoptosis. 2011;16(1):1-12.

27. Khan S, Aspe JR, Asumen MG, Almaguel F, Odumosu O, Acevedo-Martinez S, et al. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential Br J Cancer. 2009;100:1073-86.

28. Valenzuela MMA, Castro IV, Gonda A, Osterman CD, Jutzy JMS, Aspe JR, et al. Cell death in response to antimetabolites directed at ribonucleotide reductase and thymidylate synthase. OncoTargets and Therapy. 2015;In Press.

29. Altieri DC. Survivin and IAP proteins in cell-death mechanisms. Biochem J. 2010;430:199-205.

30. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10:1470-6.

31. Srinivasula SM, Ashwell JD. IAPs: what's in a name? Mol Cell. 2008;30:123-35.

32. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. 2002;3(6):401-10.

33. Darding M, Meier P. IAPs: Guardians of RIPK1. Cell Death Differ. 2012;19(1):58-66.

34. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulators of mitosis and apoptosis and novel targets for cancer therapeutics. Clin Cancer Res. 2008;14:5000-5.

35. Ambrosini G, Adida C, Sirugo G, Altieri DC. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nat Med. 1997;3:917-21.

36. Mbeunkui F, Johann DJ Jr. Cancer and the Tumor Microenvironment: A Review of an Essential Relationship. Cancer Chemother Pharmacol. 2009;63(4):571-82.

37. Taylor DD, Gercel-Taylor C. Tumour-derived exosomes and their role in cancer-associated T-cell signalling defects. Br J Cancer. 2005;92:305-11.

38. Nieuwland R, Sturk A. Why do cells release vesicles? Thromb Res. 2010;125 Suppl 1:S49-51.

39. Hegmans JP, Bard MP, Hemmes A, Luider TM, Kleijmeer MJ, Prins JB, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. Am J Pathol. 2004;164:1807-15.

40. Mears R, Craven RA, Hanrahan S, Totty N, Upton C, Young SL, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics. 2004;4:4019-31.

41. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. J Biochem. 2006;140:13-21.

42. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009;9:581-93.

43. Yu X, Harris SL, Levine AJ. The regulation of exosome secretion: a novel function of the p53 protein. Cancer Res. 2006;66:4795-801.

44. Lehmann BD, Paine MS, Brooks AM, McCubrey JA, Renegar RH, Wang R, et al. Senescenceassociated exosome release from human prostate cancer cells. Cancer Res. 2008;68:7864-71.

45. Shedden K, Xie XT, Chandaroy P, Chang YT, Rosania GR. Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res. 2003;63:4331-7.

46. Safaei R, Larson BJ, Cheng TC, Gibson MA, Otani S, Naerdemann W, et al. Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol Cancer Ther. 2005;4:1595-604.

47. Jutzy JM, Khan S, Asuncion-Valenzuela MM, Milford TA, Payne KJ, Wall NR. Tumor-released survivin induces a type-2 t cell response and decreases cytotoxic T cell function, in vitro. Cancer microenvironment : official journal of the International Cancer Microenvironment Society. 2013;6(1):57-68.

48. Mercer TR, Dinger ME, Bracken CP, Kolle G, Szubert JM, Korbie DJ, et al. Regulated post-transcriptional RNA cleavage diversifies the eukaryotic transcriptome. Genome research. 2010;20(12):1639-50. Epub 2010/11/04.

49. Batagov AO, Kurochkin IV. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. Biology direct. 2013;8:12. Epub 2013/06/14.

CHAPTER FIVE

CONCLUSION

Pancreatic cancer remains a devastating disease. Today, the standard of care for metastatic pancreatic adenocarcinoma is still gemcitabine monotherapy. Since 1996 when Gem was approved to be used against pancreatic cancer, there has been no significant advancement in the treatment for this deadly disease [1,2]. Additionally, there is a low patient response rate to Gem due to chemoresistance, innate and acquired, which continues to be a major problem [3]. Although Gem is the gold standard treatment, survival of patients is extended by only 5 weeks [4]. There have been numerous efforts and clinical trials to try to improve the efficacy of Gem in various chemotherapeutic combinations, but to no avail [5]. In addition to improving Gem's efficacy, the need to develop a novel agent with a different mechanism of action, would prove move effective in the treatment of pancreatic cancer. It is therefore important to study the different factors of chemoresistance to aid in the discovery of new chemotherapeutic agents to overcome this problem in the clinic.

One factor that may play a role in Gem chemoresistance is the release of bioactive proteins into the microenvironment via TEX, such as Survivin. Within the tumor microenvironment, constant changes are taking place such as cellular stressors by means of chemotherapy. This may induce the cancer cells and tissues to adapt to changes, which can modulate the release of TEX, as well as the packaging of bioactive molecules involved in communication, protection, even exchange of genetic information between cancer cells [6,7]. Initial observations of accelerated tumor growth in immunocompetent and nude mice pre-treated with murine mammary TEX led to a number of studies which

revealed the role of TEX [8]. TEX has now been described to have a supportive role in the survival and growth of the tumor cells and is involved in promoting host tissue invasion, the subsequent metastasis and facilitating immune response evasion [9-11]. In addition, exosomal Survivin has shown to cause neighboring cancer cells to proliferate rapidly, exhibit metastatic potential and become resistant to therapy [12]. It is speculated that the diverse function of TEX is due to the various bioactive molecules on and within the vesicles, which strongly influences the tumor microenvironment [13-16].

We have shown that not only is Survivin released in exosomes, but also other IAP family members, namely cIAP1, cIAP2 and XIAP. In addition to IAP proteins being exosomal, we also discovered that IAP mRNA is present, both full length and truncated. This phenomenon is not cell line dependent, as we have shown this to be the case in five cancer and one non-cancer cell lines.

Cellular and exosomal IAP protein and mRNA levels in PANC-1 cells were not decreased when treated with various anti-metabolite agents at sublethal and lethal doses. Instead, the protein and mRNA levels remained the same or showed an increase, which may contribute to pancreatic cancer's lack of response to these agents and eventual progression to chemoresistance against these anti-metabolites.

These findings can be used to design and develop novel compounds that can be used in combination with Gem or 5FU as a combination therapy to prevent the release of exosomal IAPs into the tumor microenvironment, decreasing proliferation rate, resistance to therapy and potential to metastasize. As the use of Gem and 5FU in combination with other chemotherapy agents did not show any significant benefit, the development of a

novel therapeutic agents designed to target exosomes, in particular IAP packaging, may make a vital impact in the treatment for metastatic pancreatic cancer.

References

1. Hertel L, Kroin J, Misner J, Tustin J. Synthesis of 2-deoxy-2, 2-difluoro-D-ribose and 2-deoxy-2,2′-difluoro-D-ribofuranosyl nucleosides. *The Journal of Organic Chemistry.* 1988;53:2406-2409.

2. Hung SW, Mody HR, Govindarajan R. Overcoming nucleoside analog chemoresistance of pancreatic cancer: a therapeutic challenge. *Cancer letters.* Jul 28 2012;320(2):138-149.

3. Burris HA, 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* Jun 1997;15(6):2403-2413.

4. Wang Z, Li Y, Ahmad A, et al. Pancreatic cancer: understanding and overcoming chemoresistance. *Nature reviews. Gastroenterology & hepatology.* Jan 2011;8(1):27-33.

5. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nature reviews. Clinical oncology.* Mar 2010;7(3):163-172.

6. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009;9(8):581-593.

7. Nieuwland R, Sturk A. Why do cells release vesicles? *Thrombosis research.* Apr 2010;125 Suppl 1:S49-51.

8. Liu C, Yu S, Zinn K, et al. Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol.* 2006;176(3):1375-1385.

9. Nieuwland R, Sturk A. Why do cells release vesicles? *Thromb Res.* 2010;125.

10. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res.* 2007;67(7):2912-2915.

11. Anderson HC, Mulhall D, Garimella R. Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. *Lan Invest.* 2010;90(11):1549-1557.

12. Khan S, Aspe JR, Asumen MG, et al. Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential *Br J Cancer.* 2009;100:1073-1086.

13. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. *J Biochem.* 2006;140(1):13-21.

14. Mears R, Craven RA, Hanrahan S, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics.* 2004;4(12):4019-4031.

15. Hegmans JP, Bard MP, Hemmes A, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am J Pathol.* 2004;164(5):1807-1815.

16. Iero M, Valenti R, huber V, et al. Tumour-released exosomes and their implications in cancer immunity. *Cell Death Differ.* 2008;15(1):80-88.