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Curcumin: A Multi-dimensional Approach to Pancreatic Cancer Targeting Cell Death and Exosomes

Carlos J. Diaz Osterman

LOMA LINDA UNIVERSITY
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Curcumin: A Multi-dimensional Approach to Pancreatic Cancer Targeting Cell Death and Exosomes

by

Carlos J. Díaz Osterman

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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.

, Chairperson

Nathan R. Wall, Assistant Professor of Biochemistry

Carlos A. Casiano, Professor of Microbiology

William H. R. Langridge, Professor of Biochemistry

Ubaldo Soto, Assistant Research Professor of Microbiology

David J. Weldon, Associate Professor, Pharmaceutical and Administrative Sciences, School of Pharmacy

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ABBREVIATIONS

IAP	Inhibitor of apoptosis
cIAP1	Cellular inhibitor of apoptosis 1
cIAP2	Cellular inhibitor of apoptosis 2
XIAP	X-chromosome linked inhibitor of apoptosis
ATM	ataxia-telangiectasia-mutated
Chk1	checkpoint kinase-1
CDK-1	cyclin dependent kinase-1
STAT	signal transducers and activators of transcription
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Sp1	specificity protein 1
MMP-9	matrix metalloproteinase-9
VEGF miRNA	vascular endothelial growth factor microRNA
ESR1	estrogen receptor 1
SET8	SET domain containing lysine methyltransferase 8
CELF2	CUG triple repeat RNA binding protein Elav-like family member 2
COX-2	cyclooxygenase-2
MRP5	multidrug resistance protein-5
5-FU	5-fluorourasil
LHRH	luteinizing hormone releasing hormone
LHRHR	luteinizing hormone releasing hormone receptor
PEGylated curcumin	polyethylene glycol-conjugated curcumin
Jab1	c-Jun activation domain-binding protein-1

DNMT	DNA methyltransferase
HIF-1α	hypoxia-inducible factor 1alpha subunit
CDF	difluorinated curcumin
EZH2	enhancer of zeste homolog 2
CD44	cluster of differentiation 44
EpCAM	epithelial cell adhesion molecule
EGFR	epidermal growth factor receptor
PTEN	phosphatase and tensin homolog
PI3K	phosphoinositide 3-kinase
mTOR	mammalian target of rapamycin
$p27^{kip1}$	cyclin-dependent kinase inhibitor 1B
$p57^{kip2}$	cyclin-dependent kinase inhibitor 1C
PUMA	p53 upregulated modulator of apoptosis
DMPC	1,2-dimyristoyl-sn-glycero-e-phosphocholine
DMPG	1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol) sodium salt], IL-8, interleukin-8; IL-6, interleukin-6
TNF-α	tumor necrosis factor
BCL-XL	B-cell lymphoma-extra large
MCL-1	myeloid leukemia cell differentiation protein 1
PCNA	proliferating cell nuclear antigen; CHA, cholesteryl-hyaluronic acid

ABSTRACT OF THE DISSERTATION

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by

Carlos J. Díaz Osterman

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Dr. Nathan R. Wall, Chairperson

Pancreatic cancer is currently one of the most difficult diseases to treat due to difficulty of detection and the aggressive nature of the disease. In addition, pancreatic cancer has the highest mortality rates compared to other cancer types. These mortality rates are attributable in part to increasing resistance to cancer therapy. Cancer therapy resistance is caused by adaptations that favor survival within cancer cells and their environment, termed the tumor microenvironment. Intracellular adaptations include the overexpression of resistance-linked genes, such as the inhibitor of apoptosis (IAP) family of proteins and overall resistance to cell death. Adaptations in the tumor microenvironment include altered intercellular vesicular signaling through exosomes, resulting in tumor growth and progression. However, recent studies have shown that exosomes can also be used as a delivery mechanism for drugs with poor bioavailability, thus providing a therapeutic advantage for these compounds. Currently, researchers are moving toward a multi-dimensional approach to pancreatic cancer therapy that incorporates compounds that target crucial players in chemotherapy resistance and in the tumor microenvironment, such as exosomes. Our studies are centered on the anti-cancer properties of curcumin, a turmeric derivative, on these intracellular and intercellular

resistance mechanisms. The long term goal of this research is to determine the mechanisms by which curcumin modulates intracellular pathways related to pancreatic cancer survival and therapy resistance and exosome composition and release to improve the understanding of pancreatic cancer pathology and support the development of novel therapeutic approaches for pancreatic cancer patients. The specific objective of this research was to determine curcumin's role in modulating intracellular proteins imperative for pancreatic cancer chemotherapy resistance such as the IAP proteins. Moreover, this research addressed the effects of curcumin on exosome release and function, specifically in the context of delivery to recipient pancreatic cancer cells. We have established that curcumin reduces expression of the IAPs in pancreatic cancer cells, inhibiting their survival and growth. Furthermore, curcumin not only attenuates pro-survival signaling through exosomes, but also itself carried within the nanovesicles and delivered to recipient pancreatic cancer cells, resulting in pancreatic cancer cell death.

CHAPTER ONE

CURCUMIN AND PANCREATIC CANCER: A RESEARCH AND CLINICAL UPDATE

Carlos J. Díaz Osterman and Nathan R. Wall

Center for Health Disparities and Molecular Medicine

Department of Basic Sciences

Division of Biochemistry

Loma Linda University

Loma Linda, California, 92350

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Abstract

Pancreatic cancer is currently one of the most deadly types of cancer. Poor patient prognosis is linked to the lack of effective early detection techniques and emerging resistance to current therapeutic strategies. Thus, current research efforts are focusing on overcoming drug resistance. One branch of this field of study is the use of natural compounds to combat pancreatic cancer and drug resistance. Curcumin, a turmeric derivative, is one such compound that has been shown to have potent anti-cancer properties in the context of pancreatic cancer. However, curcumin's poor bioavailability limits its clinical utility. Multiple approaches have been taken to overcome this problem, including curcumin modifications, curcumin combination therapy with other natural and synthetic therapeutic agents and the use of nano-formulations. This review is intended to provide a compendium of the cutting-edge investigations related to preclinical and clinical uses of curcumin, including its analogues and nano-formulations, in the context of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most devastating types of cancer. The American Cancer Society has estimated that 48,960 individuals will be diagnosed with pancreatic cancer in the United States this year. Furthermore, approximately 40,560 of these patients (83%) will perish from this disease this year and only 7% of patients will survive at least five years [1]. Inadequate detection techniques, the aggressive nature of the disease, and the emerging resistance to the current treatment approaches have contributed to these unacceptably high mortality rates [2]. Tumor removal in combination with post-surgical chemotherapy treatment is considered the most effective treatment approach to date. Unfortunately, the early detection of pancreatic cancer, which is an eligibility requirement for surgery, is challenging due to the lack of accurate and reliable detection methods. However, patients that are ineligible for surgery still have the option to receive a battery of chemotherapeutic agents in combination with radiation therapy. Most combinational therapies consist of using anti-metabolites, DNA damage inducers, tyrosine kinase inhibitors and topoisomerase inhibitors, with the gold standard chemotherapeutic agent for pancreatic cancer being Gemcitabine, an anti-metabolite and inducer of DNA damage. However, despite clinical responses in some cases, overall patient survival and quality of life are not improved by these therapies [3-8]. The chemo- or radiation-therapy resistance developed by most patients with pancreatic cancer has hindered the efficacy of combinational chemotherapeutic and radiation-based treatment strategies [9]. Thus, a better understanding of the mechanisms of therapeutic resistance and the development of novel therapeutic approaches are of paramount importance to the eradication of this disease.

A low incidence of cancer has been documented in countries that incorporate high consumption of turmeric root into their diets [10, 11]. This association has been extensively investigated, particularly in the context of the biological roles of turmeric root derivatives. Curcumin is a derivative of the turmeric root that has been extensively investigated because of its low toxicity in normal tissues and capacity to hinder multiple signaling pathways that are crucial for the initiation and progression of various cancers, including pancreatic cancer [12]. Additionally, curcumin has been shown to synergistically enhance the anti-cancer effects of standard chemotherapeutic agents, including Gemcitabine, in pancreatic cancer [13]. Due to curcumin's poor bioavailability in clinical trials involving pancreatic cancer patients [14-17], novel approaches such as curcumin analogues and nano-particle formulations of curcumin have been developed [14, 18, 19] and are currently under investigation in various cancers [20-25], including pancreatic cancer [18, 26-28]. Currently, the literature concerning the pre-clinical and clinical anti-cancer properties of curcumin in various cancer types has been eloquently reviewed [12, 29, 30]; however, a comprehensive review addressing the current state of curcumin research in the context of pancreatic cancer is lacking. Therefore, the purpose of this review is to provide a compilation of cutting-edge studies involving the pre-clinical and clinical utility of curcumin and its analogues and nano-formulations in pancreatic cancer.

Curcumin: Molecular Mechanisms in Pancreatic Cancer

Vogel and Pelletier were the pioneers in obtaining the yellow crystalline form of curcumin from the turmeric root in 1818 [31]. Nevertheless, it was not until 1870 that

Daube elucidated the structure of curcumin as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloylmethane (Fig. 1) [31]. The discovery of the structure of curcumin paved the way for the investigation of its remarkable anticancer properties. In particular, curcumin's effects in pancreatic cancer have been widely studied. It has been shown to influence a range of cellular functions including proliferation, survival, angiogenesis, and invasion and metastasis. For instance, curcumin causes G2/M cell cycle arrest in pancreatic cancer cells following DNA damage and ATM-Chk1-dependent inhibition of CDK-1 and Cyclin B1 activity [32]. Interestingly, this study also demonstrated that curcumin showed lower cytotoxic effects and no DNA damage or cell cycle arrest in normal human pancreatic ductal epithelial cells [32]. Curcumin also has potent effects on cell survival, most notably through regulation of the inhibitor of apoptosis (IAP) family, which includes Survivin, cellular IAPs 1 and 2 (cIAP1 and cIAP2) and X-chromosome linked IAP (XIAP). One of the key roles of the IAP proteins in normal cells is to modulate the balance between cell survival and cell death. The unbalanced regulation of IAP proteins can drive cells to a more pro-survival phenotype. Indeed, pancreatic cancer cells overexpress IAP proteins and the overexpression of these proteins is associated with poor patient outcomes in pancreatic cancer [33-36]. In addition, studies have demonstrated that resistance to radiation and chemotherapy are also related to the overexpression of the IAP proteins [37-44]. Studies from our laboratory have demonstrated that although other standard chemotherapeutic agents are ineffective at reducing these IAP family members [45], curcumin abolishes Survivin, cIAPs 1 and 2, and XIAP protein and mRNA expression in pancreatic cancer cells [46]. This is consistent with previous reports that Survivin expression is reduced

following curcumin treatment in pancreatic cancer cells as a result of inhibition of upstream STAT3 signaling [47] or reduction in NF-κB activity [48]. The importance of STAT3 and NF-κB signaling in pancreatic cancer has been well established [49, 50]. Interestingly, Jutooru et al. also demonstrated that curcumin reduces Sp1 and NF-κB activity, resulting in downregulation of downstream genes including Survivin, MMP-9, VEGF and Cyclin D1. Furthermore, these studies demonstrated that reduction in NF-κB activity was dependent on Sp1 reduction following curcumin treatment, suggesting that curcumin's effects on Sp1 represent an upstream event in pancreatic cancer cells [51]. Studies by Sun et al. complement these findings by demonstrating that curcumin modulates microRNA (miRNA) expression profiles in pancreatic cancer cells [52]. MiRNAs are small non-coding RNAs [53-55] that have recently gained increased attention in the literature due to their involvement in important biological processes via post-transcriptional modulation of gene expression [54-57]. Altered post-transcriptional gene regulation conferred by miRNAs has been linked to pancreatic cancer [58]. One miRNA that is altered after curcumin treatment is miRNA-22, whose increased expression after treatment results in downregulation of target transcripts for Sp1 and estrogen receptor 1 (ESR1) [52]. Another study concluded that curcumin inhibits pancreatic cancer cell growth and invasion via upregulation of miRNA-7 and subsequent downregulation of SET8, which is known to promote tumorigenesis [59], and activation of p53 [60]. This is consistent with studies demonstrating the essential role of miRNA-7 in the regulation of tumor growth and metastasis in chemoresistant cancer cells [61].

In addition to miRNA-mediated regulation, curcumin has also been shown to modulate gene expression at the post-transcriptional level by altering the expression RNA binding protein CELF2 (CUGBP, Elav-like family member 2), a known regulator of RNA splicing, editing, and translation [62-65]. Curcumin induces CELF2 expression in pancreatic cancer cells, resulting in decreased translation of VEGF and COX-2 and overall reduction in tumor growth and angiogenesis in a pancreatic cancer xenograft model [66].

Finally, curcumin has been shown to combat chemotherapeutic resistance through reduction IAP expression, as described above, as well as via inhibition of the ATP-dependent multidrug resistance protein-5 (MRP5), an efflux pump notorious for promoting the export of chemotherapeutic drugs [67]. Interestingly, curcumin has been shown to suppress the activity of MRP5 and enhance the effects of 5-fluorouracil (5-FU) in pancreatic cancer [68]. In summary, curcumin's effects in pancreatic cancer are truly multi-dimensional, regulating miRNA expression, transcription factor activity and gene expression resulting in increased cell death and reduced pro-angiogenic, metastatic, and chemoresistant signaling. It is important to note that although several STAT3 inhibitors (NCT00955812, NCT02058017, NCT01423903, NCT01867073, NCT01563302, NCT01839604) and IAP antisense compounds (NCT000882869, NCT00557596, NCT00558545, NCT01018069, NCT00372736, NCT01186328, NCT00620321) have been studied in various phase I/II clinical trials, the majority of curcumin's targets have yet to be addressed in clinical trials. Furthermore, no compound has been demonstrated to possess the vast arsenal of anti-cancer mechanisms displayed by curcumin.

Curcumin: Analogues and Nano-formulations in Pancreatic Cancer

Curcumin Analogues in Pancreatic Cancer

The metabolism of curcumin involves hepatic enzymes such as glucuronidases and sulfotransferases, which are responsible for conjugating glucuronic acid or sulfate, respectively, to the hydroxyl groups on the ketone or enol forms of curcumin [69, 70]. These reactions enhance the hydrophilic potential of curcumin, facilitating its elimination [69, 70]. Unfortunately, these systemic clearance mechanisms are responsible for hindering curcumin's delivery to tumors [69]. Thus, the development of analogues of curcumin has focused on modifying the hydroxyl and other functional groups on the ketone or enol forms of curcumin to ensure greater stability and longevity of the compound in circulation (Fig. 1).

Early studies by Aggarwal et al. investigated the anti-cancer activity of a [Dlys^{6}]-LHRH-curcumin conjugate. This compound was developed with the rationale that luteinizing hormone releasing hormone (LHRH) and its corresponding receptor, LHRHR, are important regulators of cellular proliferation in human tumors in an autocrine and paracrine fashion [71]. Pancreatic cancer cells express higher levels of LHRH receptors than normal cells and the importance of these receptors is highlighted by the finding that the proliferation of cancerous cells may be interrupted *in vitro* using antagonists of LHRH receptors [71-74]. The [Dlys^{6}]-LHRH-curcumin conjugate was found to suppress pancreatic cancer growth in xenograft mice with higher efficacy compared to unmodified curcumin [73].

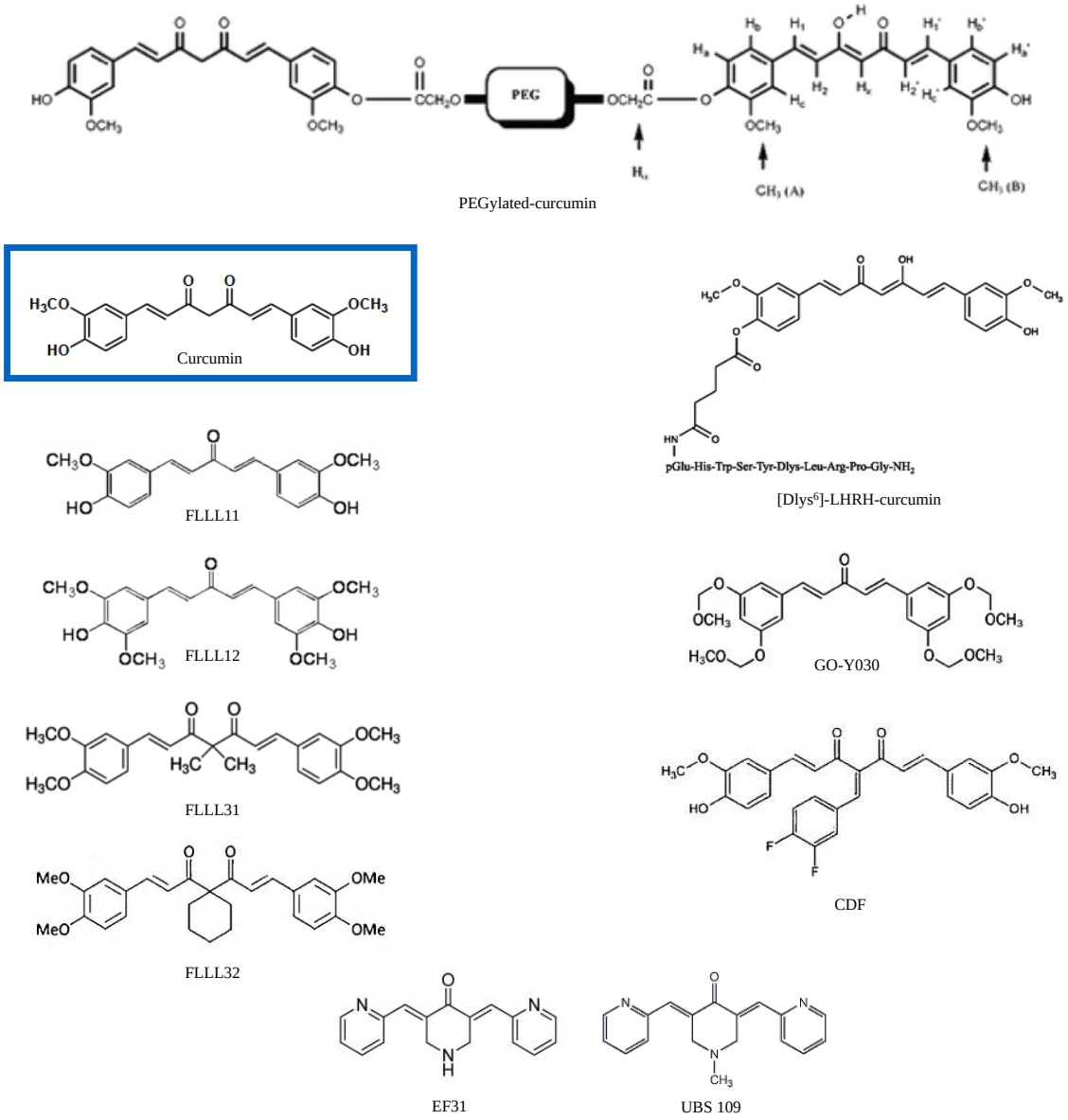

Figure 1. Curcumin and its analogues. Chemical structures of curcumin (blue box) and its analogues are shown. PEGylated curcumin = polyethylene glycol-conjugated curcumin, [Dlys^{6}]-LHRH-curcumin = luteinizing hormone releasing hormone-conjugated curcumin, CDF = difluorinated curcumin.

Curcumin has also been conjugated to a water-soluble polyethylene glycol compound (PEGylated curcumin), which was found to enhance curcumin's ability to suppress pancreatic cancer growth via inactivation of Jab1 [75], a protein that has been shown to promote pancreatic cancer cell proliferation and survival [76, 77]. In addition, PEGylated curcumin was able to sensitize pancreatic cancer cells to Gemcitabine, inducing apoptosis [75].

Curcumin has been modified through the addition of methyl or cyclohexyl functional groups, yielding analogues FLLL31 and FLLL32. These compounds have been demonstrated to have anti-proliferative and anti-angiogenic effects in addition to their ability to attenuate STAT3 activity *in vitro.* Interestingly, FLLL32 also exerts anti-tumor effects *in vivo* [78]. Analogues FLLL11 and FLLL12, characterized by ketone elimination and ether modifications, have also been investigated in the context of STAT3 and pancreatic cancer; these compounds are more effective than curcumin at inducing apoptosis via inhibition of STAT3 and AKT phosphorylation [18]. A similar analogue, GO-Y030, also inhibits phosphorylation and activation of STAT3 in pancreatic cancer cells, inducing apoptosis [79].

The addition of pyridine and piperidine moieties has yielded novel curcumin analogues UBS109 and EF31. These compounds have also been tested in pancreatic cancer and have been shown to influence DNA methylation patterns through modulation of DNA methyltransferase (DNMT)-1 expression [80]. Furthermore, these analogues have increased anti-angiogenic effects in *in vitro* and *in vivo* models of pancreatic cancer through inhibition of NF-κB activity and reduction of HIF-1α and VEGF expression [26].

Difluorinated curcumin (CDF) has been shown to sensitize pancreatic cancer cell lines to Gemcitabine through inhibition of NF-κB and COX-2 [81]. CDF is also a suppressor of tumor growth via the reduction of histone methyltransferase EZH2, Notch-1, CD44, EpCAM and Nanog in an orthotropic xenograft pancreatic cancer model [82]. Furthermore, CDF has been demonstrated to influence miRNA expression profiles in pancreatic cancer. In particular, CDF upregulates miRNA-146a and downregulates miRNAs -21 and -221 in pancreatic cancer. MiRNA-146a has gained attention in pancreatic cancer because its downregulation is associated with high levels of EGFR and induction of NF-κB [83]. Recently, CDF has been shown to increase the expression of miRNA-146a resulting in a reduction in EGFR protein levels in a pancreatic cancer xenograft mouse model [84]. It has been previously demonstrated that miRNA-21 upregulation in pancreatic cancer cells is linked to inactivation of the tumor suppressor gene PTEN following the induction of the PI3K/Akt/mTOR pathway [85, 86]. Interestingly, CDF has been reported to decrease miRNA-21 expression, resulting in restoration of PTEN activity *in vitro* and *in vivo* [81, 82]. Moreover, this study concluded that CDF is able to stall tumor progression in a pancreatic cancer xenograft mouse model via modulation of COX-2, PTEN, miRNA-21, miRNA-200 and NF-κB [82, 87]. MiRNA-221 is present in high levels in pancreatic cancer cell lines and tumor tissues in contrast to normal pancreatic duct epithelial cells and normal pancreas tissues [88, 89]. CDF decreases the expression of miR-221 and activates PTEN, $p27^{kip1}$, $p57^{kip2}$ and PUMA leading to suppression of pancreatic cancer cell proliferation and migration [89].

Taken together, these studies demonstrate that curcumin analogues have significant anti-tumor effects in pancreatic cancer, suggesting that they are promising

candidates for evaluation in clinical trials. To date, however, there have been no clinical trials involving these compounds, though most curcumin analogues have shown clear benefits in preclinical modes of pancreatic cancer. Thus, these analogues represent a promising “next step” in the incorporation of curcumin into the clinical treatment regiment for pancreatic cancer patients.

Table 1. Enhancement of curcumin bioavailability and stability: curcumin analogues.

Name	Preclinical Data	Ref.
[Dlys^{6}]-LHRH-curcumin	Decreases tumor growth *in vivo*	[73]
GO-Y030	Inhibits STAT3 activation and induces apoptosis *in vitro*	[79]
FLLL11 and FLLL12	Inhibit STAT3 and AKT phosphorylation and activation and induce apoptosis *in vitro*	[18]
FLLL31 and FLLL32	Anti-proliferative and anti-angiogenic effects	[78]
	Attenuate STAT3 activity *in vitro* (both compounds)	
	Anti-tumor effects *in vivo* (only FLLL32)	
PEGylated-curcumin	Suppresses cancer growth via inactivation of Jab1 and enhances Gemcitabine *in vitro*	[75]
EF31 and UBS109	Anti-angiogenic	[26, 80]
	Enhance oxiplatin and 5FU efficacy	
CDF	Modulate DNMT-1 expression Decreases the expression of miR-21 leading to PTEN activation *in vitro* and *in vivo*	[81, 82, 84, 87, 89, 90]
	Stop tumor progression *in vivo* via modulation of COX-2, PTEN, miR-21, miR-200 and NF-κB	
	Enhances Gemcitabine efficacy *in vitro*	
	Increases miR-146a leading to a decrease of EGFR expression *in vivo*	
	Decreases miR-221 and increases expression of PTEN, p22, p57 and PUMA leading to a decrease in cell proliferation ad migration *in vitro*	
	Decreases tumor growth via down-regulation of EZH2, Notch-1, CD44, EpCAM and Nanog *in vivo*	
	Increases the expression of miR-26a, miR-143 and miR-101 and decreases let-7 levels *in vitro*	
	Down-regulates Ras and reduces tumor growth	

Curcumin Nano-formulations in Pancreatic Cancer

Early studies by Li et al. demonstrated that liposomal preparations of curcumin involving lipids 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt (DMPG) reduced cell viability in six pancreatic cancer cell lines as well as tumor volume in a pancreatic cancer xenograft model. Like curcumin, liposomal curcumin abolishes NF-κB binding in pancreatic cell lines, resulting in reduced expression of downstream target genes such as COX-2, IL-8 and VEGF *in vitro* and *in vivo* [91]. Mach et al. built on this work by establishing a putative dosing regimen for preclinical and clinical trials based on the minimum effective dose of liposomal curcumin in a MIA PaCa-2-derived xenograft model [28]. Another study involving lipid-based curcumin found that combination with nano-encapsulated aspirin and free sulforaphane was more efficient than curcumin alone at inducing apoptosis in human pancreatic cancer cell lines [92]. Curcumin nano-formulations can be also prepared using sugar molecules instead of lipids arranged in a cyclic fashion [93]. A recent example is rubusoside-solubilized curcumin, thought to form nanomicelles in water, that has improved water solubility and equivalent cytotoxicity in various cancer cell lines, including the pancreatic cancer cell line PANC-1, compared to free curcumin [94]. A separate study showed that the curcumin analogue CDF may be packaged within sugar structures termed cyclodextrins and retain its anti-cancer properties upon delivery to pancreatic cancer cells [93]. Furthermore, this study reported that the serum levels of nano-CDF were 10 times higher than CDF alone [93].

In 2007, Bisht et al. developed a micelle-based polymeric nanoparticle encapsulation of curcumin called nanocurcumin, or NanoCurc. In early studies, the *in*

vitro cytotoxicity of nanocurcumin was tested against eight pancreatic cancer cell lines, demonstrating comparable efficacy to free curcumin in most cell lines. Like free curcumin, nanocurcumin blocks NF-κB activity and reduces the expression of NF-κB-regulated genes including IL-6, IL-8, and TNF-α, which are known pro-inflammatory cytokines [95]. These studies were later expanded to include combination therapy involving NanoCurc and Gemcitabine in a preclinical xenograft pancreatic cancer model, resulting in suppression of tumor growth via reduction of NF-κB activity as well as MMP-9 and Cyclin D1 expression [96].

Recently, magnetic particles have been used to encapsulate curcumin to prolong curcumin's delivery to tumor tissues in a pancreatic cancer xenograft model. These curcumin-loaded magnetic particles were found to suppress pancreatic tumor growth, improving survival by downregulating pro-survival proteins such as BCL-XL, MCL-1 and PCNA. Importantly, increased β-catenin and reduced collagen I expression were detected following treatment with magnetic nanoparticle-based curcumin, suggesting possible effects on metastatic activity in this model [97].

Another recent nano-formulation was developed by Wei et al., involving the ester-mediated conjugation of curcumin to cholesteryl-hyaluronic acid (CHA) nanogel that targets curcumin to CD44 (a cell surface receptor for hyaluronic acid)-expressing cells. This formulation was tested in a MIA PaCa-2-based xenograft model, demonstrating significant reduction in tumor growth compared to free curcumin. In addition, CHA-nanogel curcumin also reduced the mRNA expression of NF-κB and target genes TNF-α and COX-2 compared to control [98].

Although these nano-formulations of curcumin have shown strong promise for integration into therapeutic approaches to pancreatic cancer, they have yet to be tested in clinical trials. Of the nano-formulations developed to date, Theracurmin, a colloidal nano-formulation using gum ghatti derived from ghatti trees, has shown the most progress and translatability. First described in 2011 by Sasaki et al., Theracurmin was found to significantly increase curcumin's bioavailability in healthy human volunteers [99]. In 2014, Kanai reported the results of a phase I clinical trial of Theracurmin in patients with pancreatic cancer in combination with Gemcitabine. This study reported that curcumin did not cause any unexpected adverse effects aside from abdominal pain in a subset of patients with peritonitis carcinomatosa. Furthermore, quality of life scores were significantly improved following Theracurmin incorporation as part of the therapeutic strategy [100].

Most recently, nano-scale extracellular vesicles called exosomes have been shown to target intercellular transporters of bioactive molecules that can modulate cancer growth [101-106]. In addition, studies have shown that exosomal transport between cancer cells can be used as a therapeutic advantage. Specifically, Aspe et al. have shown that exosomes isolated from melanoma cell lines overexpressing the pro-apoptotic Survivin-T34A mutant carry the mutant Survivin into the pancreatic adenocarcinoma cell line MIA PaCa-2, inducing apoptotic cell death [107]. Furthermore, the exosomal uptake of Survivin-T34A mutant by pancreatic cancer cells enhanced their sensitivity to Gemcitabine [107]. In the context of curcumin, our studies have shown that exosomes extend the anti-cancer properties of curcumin from treated to naïve pancreatic adenocarcinoma cells [108]. Specifically, exosomes isolated from PANC-1 cells treated

with curcumin were found to contain curcumin. Furthermore, these curcumin-containing exosomes reduced the viability of naïve PANC-1 cells [108]. The exosomal ability to carry functional curcumin between cells represents an exciting new direction in curcumin research: the extension of curcumin's effects to other cellular components of tumors via exosomal delivery. In summary, nano- and microvesicular formulations of curcumin may represent a gateway to translating the known anti-cancer properties of curcumin to patient care by overcoming key obstacles such as biodistribution and compound stability.

Table 2. Enhancement of curcumin using nano-formulations.

Nano-formulation	Preclinical or Clinical Data	Ref.
Rubusoside-based	Enhances curcumin's water solubility and anti-cancer abilities *in vitro*	[94]
Liposome-based	Decreases tumor volume with no toxicity at a dose of 20 mg/kg *in vivo*	[28, 91]
	Exhibits anti-angiogenic properties and decreases CD31 and IL-8 *in vivo*	
NanoCurc	In combination with Gemcitabine, suppresses tumor growth substantially via reduction of NF-κB, MMP-9 and Cyclin D1 expression *in vivo*	[96]
Magnetic-based	Promotes enhancement of curcumin delivery for up to 2-3 weeks and suppression of tumor growth via down-regulation of BCL-XL, MCL-1, PCNA and collagen 1	[97]
CHA-Nanogel	Provides superior curcumin biodistribution and slower elimination and suppresses tumor growth *in vivo*	[98]
Theracurmin	Increases curcumin's plasma levels in patients with minimal toxicity	[14, 109]

Curcumin: Clinical Utility Against Pancreatic Cancer

Currently, there are several studies with the impetus to apply curcumin's anti-cancer properties in pancreatic cancer patients. Phase I studies of curcumin in pancreatic cancer patients have demonstrated low toxicity and high consumption tolerance, up to 12 grams per day, with minimal secondary effects (diarrhea and nausea) [14, 15, 17, 110, 111]. Phase II clinical trials involving curcumin treatment in patients with advanced pancreatic cancer consisted of 25 patients, of which 21 could be evaluated. One patient presented 18 months of disease without progression, two other patients showed partial response and another patient exhibited brief tumor regression [112]. Consistent with preclinical data, decreased expression of NF-κB, COX-2 and phosphorylated STAT3 was detected in the peripheral blood mononuclear cells of patients treated with curcumin in this study [112]. Furthermore, peak plasma delivery of curcumin in these patients was 22 to 41 ng/mL with no significant toxicity observed in patients [112]. Another phase I/II clinical trial was designed to treat pancreatic cancer patients resistant to Gemcitabine with curcumin [16]. This study involved 21 patients and concluded that a dose of 8 grams of curcumin per day was appropriate since it did not induce toxicity after co-treatment with Gemcitabine. Curcumin levels detected in the plasma of five tested patients ranged from 29 to 412 ng/mL [16]. Furthermore, the median survival time was approximately 5.4 months and the one-year survival rate was 19% [16]. The combinational therapy presented in this study was non-toxic and feasible; however, its efficacy remains to be elucidated. In a similar phase II study, the feasibility and efficacy of 8 grams of curcumin per day in combination with 1,000 mg/m^2 of Gemcitabine was investigated in 17 patients with advanced pancreatic cancer [113]. In this clinical trial the efficacy of curcumin and

Gemcitabine treatment could be only analyzed in 11 patients because the individual and combinational treatments were toxic to five patients and another unfortunately succumbed to their disease [113]. At the end of the study one patient exhibited partial response, four patients had no disease progression and six patients had disease progression [113]. Based on these data, the study concluded that the time of disease progression was 1-12 months and the overall survival was 1-24 months [113]. The authors discussed that in their study 8 grams per day of curcumin is not well tolerated in combination with Gemcitabine; however, in the light of the other studies mentioned previously, further studies are needed to validate this conclusion [113]. In clinical trials, curcumin has been shown to have relatively low toxicity. However, its use is still challenged by poor biodistribution in spite of administration at high doses [14-17]. As described above, a recent study that evaluated the bioavailability of Theracurmin, a colloidal nano-formulation of curcumin [99], in human subjects concluded that curcumin plasma levels were higher in patients treated with Theracurmin compared with the patients treated with free curcumin [14]. For this reason Theracurmin has been considered a promising treatment agent for cancer clinical trials. A phase I study involving treatment of 14 pancreatic cancer patients with Theracurmin containing 200 and 400 mg of curcumin in combination with Gemcitabine demonstrated that the curcumin peak plasma levels ranged between 324 and 440 ng/mL, respectively [109]. It is note-worthy to mention that the plasma curcumin levels using Theracurmin are higher than the curcumin peak plasma levels, approximately 85 ng/mL, reported after 8 grams per day of free curcumin [16]. This study showed that the efficacy of curcumin after Theracurmin delivery in pancreatic cancer patients induced no response in general, a median survival

time of 4.4 months for 14 patients and more than 12 months survival in three patients [109].

Conclusion

This review summarizes a wide range of preclinical studies that have demonstrated that curcumin is a potent anti-cancer agent alone and in combination with standard chemotherapeutic agents. In addition, curcumin analogues and nano-formulations have shown promising effects against pancreatic cancer growth and survival while improving curcumin bioavailability. Interestingly, curcumin's anti-cancer effects have recently been demonstrated to extend beyond direct intracellular effects to other cancer cells through exosomes, suggesting that these nano-scale vesicular transporters may play a key role in curcumin's effects within the tumor microenvironment. In clinical trials, curcumin alone and in combination with Gemcitabine have exhibited general tolerability with low toxicity in pancreatic cancer patients. However, the efficacy of curcumin in patients with pancreatic cancer requires further investigation due to curcumin's poor bioavailability. Several attempts using Theracurmin have been performed to overcome this treatment obstacle, demonstrating promising results in pancreatic cancer patients. Nevertheless, the anti-cancer effects of Theracurmin with Gemcitabine or other chemotherapeutic agents still require attention. With significant advances in curcumin modulation at the cellular and preclinical levels, it is the hope of the authors that these findings will soon be translated to clinical trials, potentially improving pancreatic cancer patient outcomes and quality of life.

CHAPTER TWO

CURCUMIN INDUCES PANCREATIC ADENOCARCINOMA CELL DEATH VIA REDUCTION OF THE INHIBITORS OF APOPTOSIS

Carlos J. Díaz Osterman, Amber Gonda, Tessa Rae Stiff, Ulysses Sigaran, Malyn May Asuncion, Valenzuela, Heather R. Ferguson Bennit, Ron B. Moyron, Salma Khan and Nathan R. Wall

Center for Health Disparities and Molecular Medicine

Department of Basic Sciences

Division of Biochemistry

Loma Linda University

Loma Linda, California, 92350

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Abstract

The inhibitor of apoptosis (IAP) proteins are critical modulators of chemotherapeutic resistance in various cancers. To address the alarming emergence of chemotherapeutic resistance in pancreatic cancer, we investigated the efficacy of the turmeric derivative curcumin at reducing IAP protein and mRNA expression resulting in pancreatic cancer cell death. The pancreatic adenocarcinoma cell line PANC-1 was used to assess curcumin's effects in pancreatic cancer. Curcumin uptake was measured by spectral analysis and fluorescence microscopy. AlamarBlue and Trypan blue exclusion assays were used to determine PANC-1 cell viability following curcumin treatment. Visualization of PANC-1 cell death was performed using Hoffman modulation contrast microscopy. Western blot and PCR analyses were used to evaluate curcumin's effects on IAP protein and mRNA expression. Curcumin enters PANC-1 cells and is ubiquitously present within the cell following treatment. Furthermore, curcumin reduces cell viability and induces morphological changes characteristic of cell death. Additionally, curcumin decreases IAP protein and mRNA expression in PANC-1 cells. These data demonstrate that PANC-1 cells are sensitive to curcumin treatment. Furthermore, this work supports a role for curcumin as part of the therapeutic approach for overcoming chemotherapeutic resistance mediated by the IAPs in pancreatic cancer.

Introduction

Pancreatic cancer is an aggressive and devastating disease responsible for the highest mortality rates among cancer types with 94% of patients dying within five years of diagnosis. Pancreatic tumor resection remains the most efficacious treatment with 20 to 25% of the patients surviving five years post-surgery. However, early diagnosis, a prerequisite for the surgery, is made difficult by the absence of early signs or symptoms [2, 3, 114]. As a result, most patients are ineligible for the surgery at the time of diagnosis and are offered chemotherapy [3, 114, 115]. The gold standard chemotherapeutic for pancreatic cancer is Gemcitabine, which has shown significant clinical benefits, with survival rates ranging from six to fifteen months when resection is not an option and the patients exhibit either non-metastatic or metastatic disease [8, 116-118]. Unfortunately, emerging resistance to chemotherapy has hindered the efficacy of chemotherapeutics including Gemcitabine [9], highlighting the need for novel therapeutic approaches that address this rising resistance to therapy.

The inhibitor of apoptosis (IAP) proteins, including Survivin, cellular IAP 1 and 2 (cIAP1 and cIAP2), and X-chromosome linked IAP (XIAP), belong to a family of anti-apoptotic proteins known to confer resistance to treatment modalities such as radiation therapy and chemotherapy [33-36]. Additionally, the overexpression of these IAP proteins has been positively correlated with the progression of a variety of cancer types, including pancreatic cancer, resulting in a decline in patient survival after chemotherapeutic treatment [37-44]. Pre-clinical and clinical trials aimed at reducing IAP expression via antisense oligonucleotides and/or second mitochondria-derived activator of caspases (Smac) mimetics have yielded promising results in various cancers [37, 119-

124]; however, such studies have remained inconclusive in pancreatic cancer [125]. This may be due to a compensatory effect of IAPs to targeted therapies. Further studies involving agents that cause simultaneous reduction in multiple IAPs are needed to investigate this notion.

Curcumin, a turmeric derivative, has been considered as a potential anti-cancer therapy due to its capacity to interrupt signaling pathways that are crucial for the initiation and progression of cancer [12]. For instance, studies have demonstrated that curcumin inhibits the progression of various cancers by modulating the expression of anti-apoptotic factors [126-131]. Furthermore, curcumin induces apoptosis in pancreatic cancer [18] and regulates IAP expression in a variety of other cancer types [132, 133]. Preclinical studies involving curcumin in pancreatic cancer have shown that curcumin enhances Gemcitabine sensitivity *in vitro* and *in vivo* [48, 134, 135]. Moreover, Phase I and II clinical trials have yielded promising results on the use of curcumin as part of pancreatic cancer therapeutic strategies [16, 109, 112, 113, 136, 137]. This recent progress emphasizes the need for a better understanding of the mechanisms by which curcumin counteracts chemotherapeutic resistance. Therefore, the objective of this study was to determine curcumin's impact alone on IAP expression in pancreatic cancer cells.

Materials and Methods

Cells and Culture Conditions

The pancreatic adenocarcinoma cell line PANC-1 was acquired from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM; ATCC, Manassas, VA) supplemented with

Normocin at a final concentration of 100 µg/mL (InvivoGen, San Diego, CA), 100 units of penicillin, 100 µg/mL of streptomycin, 300 µg/mL of L-glutamine and 10% USDA-sourced heat-inactivated fetal bovine serum (Mediatech, Manassas, VA). In all experiments, cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 to 70-80% confluency prior to use.

Preparation of Curcumin Solutions

Curcumin (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared using DMSO and ethanol as solvents. Subsequent dilutions were made from this stock solution in DMEM. The final concentrations of DMSO and ethanol did not exceed 0.04% and 0.6%, respectively, and cell viability was not affected at these concentrations (data not shown).

Cell Viability Assays

Cell viability following curcumin treatment was estimated using AlamarBlue and Trypan blue exclusion assays. For AlamarBlue assays, PANC-1 cells were cultured in 96-well plates at 1.0×10^4 cells per well and treated with 10, 50 and 100 µM curcumin for 24, 48 and 72 hours. Subsequently, the AlamarBlue reagent (Life Technologies, Grand Island, NY) was added to each sample (10% final concentration) and incubated at 37°C/5% CO_2 for two hours. Viability was analyzed by detection of absorbance at 570 nm using 600 nm as a reference wavelength in a µQuant spectrophotometer (Bio-Tek, Winooski, VT). For Trypan blue exclusion assays, cells were cultured in 6-well plates at 3.0×10^5 cells per well and treated with 10, 50 and 100 µM curcumin for 24, 48 and 72

hours. Cells were then trypsinized and combined with the Trypan blue reagent (Life Technologies, Grand Island, NY) to calculate viability by counting the cells on a hemacytometer. The results presented are representative of three independent experiments. Data from curcumin-treated samples are normalized to the untreated control.

Hoffman Modulation Contrast Microscopy

PANC-1 cells were cultured in T25 flasks at 7.0 x 10^5 cells per flask and treated with 10, 50 and 100 µM curcumin for 24 and 48 hours. Cells were then imaged using an Olympus IX70 microscope with Hoffman modulation and an Insight Spot 2 Mega Sample camera and software. Three independent experiments were performed and within each experiment three images were captured in different sections of the T25 flasks to obtain a representative image.

Spectral Studies and Fluorescence Imaging

Spectral analysis of PANC-1 cell curcumin content post-treatment was performed as previously described [138-140]. Briefly, cells were cultured in T75 flasks at 1.5 x 10^6 cells per flask and treated with 50 µM curcumin for 24 hours. Cells were then trypsinized, washed three times with 1X PBS, and resuspended in 1mL of 100% methanol and sonicated to disrupt the cell membrane integrity. The samples were then centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant fraction collected for absorbance analysis at 420 nm using a µQuant spectrophotometer and KC Junior software (Bio-Tek, Winooski, VT). Untreated cells were also lysed in methanol and subjected to spectral

analysis to determine baseline auto-fluorescence of PANC-1 cells. Methanol-only, cell-free samples were used as blank controls. Data are representative of three independent experiments. For fluorescence microscopy of PANC-1 cell curcumin content post-treatment, PANC-1 cells were cultured in 6-well plates containing sterile cover slips at 3.0×10^5 cells per well and treated with 50 μM curcumin for 24 hours. Subsequently, cells were fixed with 4% paraformaldehyde and incubated at -20°C overnight. The following day, the samples were permeabilized using 0.1% Igepal in 1X PBS for 10 minutes at room temperature. The cover slips were then washed three times with 1X PBS and placed onto slides with the nuclear stain DAPI in mounting media for 5 minutes. Stained slides were imaged using a BZ-9000 BIOREVO fluorescence microscope (Keyence, Itasca, IL) with a 40X magnification objective. Results are representative of three independent experiments; within each experiment, three images were acquired from different portions of each slide to obtain a representative image.

Western Blot Analysis

PANC-1 cells were cultured in T25 flasks at 7.0×10^5 cells per flask and treated with 10, 50 and 100 μM curcumin for 24 and 48 hours. After treatment, lysates were prepared using a lysis buffer composed of 50 mM Tris-HCl pH 7.5, 1% Triton-X, 0.25% DOC, 150 mM sodium chloride, 1mM sodium orthovanadate, 20 mM sodium fluoride, 0.2 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1X protease inhibitor cocktail (Roche Life Science, Indianapolis, IN) and sonicated. To remove cell debris, samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. Protein concentration was determined using the Pierce BCA protein assay (Thermo Scientific, Waltham, MA) according to the

manufacturer's protocol. Proteins (50 µg) were heated to 95°C for 5 minutes and fractionated using 10, 12 and 15% Bis-Tris polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA) and blocked for 1 hour at room temperature in 5% milk (w/v in 1X PBS-0.1% Tween). Subsequently, membranes were incubated overnight at 4°C in the following primary antibody solutions (final dilution 1:1000): rabbit polyclonal anti-Survivin (Novus, Littleton, CO), rabbit monoclonal anti-XIAP, rabbit monocloncal anti-CIAP1, rabbit monoclonal anti-cIAP2, and rabbit monoclonal anti-β-actin (Cell Signaling Technology, Beverly, MA). Membranes were washed with 1X PBST three times for 15 minutes each then probed for 1 hour at room temperature with goat anti-rabbit secondary antibodies labeled with IRDye680 LT (LI-COR Biosciences, Lincoln, Nebraska), followed by three 15-minute washes in 1X PBST and imaging using the ODYSSEY infrared imaging system (LI-COR, Biosciences, Lincoln, Nebraska). β-actin was utilized as a loading control. Data are representative of 3-4 independent experiments. Densitometry analyses were performed using ImageJ software (http://imagej.nih.gov/ij/).

PCR Analysis

PANC-1 cells were cultured in T25 flasks at 7.0×10^5 cells per flask and treated with 10, 50 and 100 µM for 24 and 36 hours. Cells were then trypsinized and RNA isolation and purification was performed using the Tri-Reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. cDNA conversion was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). These cDNA products were used in combination with forward and reverse

primers (IDT Technologies, San Diego, CA) designated for IAP mRNA (Table 1) and Platinum Taq DNA Polymerase (Life Technologies, Grand Island, NY) for PCR in a MasterCycler Gradient Thermo Cycler (Eppendorf, Hamburg, Germany). The PCR products were detected using a 1% agarose gel containing ethidium bromide and visualized using an Alpha Innotech imager (Protein Simple, Santa Clara, CA). Data are representative of three independent experiments. Densitometry analyses were performed using ImageJ software.

Statistical Analysis

All statistical analyses in this study were performed using one-way ANOVA analysis and a probability of less than an 85% confidence limit ($p < 0.05$) was considered to be significant. Data are presented as mean $\pm$ SEM. Statistical analyses were performed using the Prism (Graphpad, La Jolla, CA) software.

Results

Effect of Curcumin on PANC-1 Cell Viability

PANC-1 cells were cultured in increasing concentrations of curcumin for 24-72 hours and analyzed by AlamarBlue (Fig. 2A) and Trypan blue exclusion (Fig. 2B) viability assays. Curcumin demonstrated a significant dose- and time-dependent inhibitory effect on PANC-1 cell viability when compared with untreated controls. Curcumin concentrations (10 and 50 μM) flanking the IC50 were chosen for further experiments.

Table 3. Primer sequences targeting IAPs

Name	Primer Sequence
Survivin Fwd	5’ – GCA TGG GTG CCC CGA CGT TG – 3’
Survivin Rev	5’ – GCT CCG GCC AGA GGC CTC AA – 3’
cIAP1 Fwd	5’ – ATT GTG TCA GCA CTT CTT AAT G – 3’
cIAP1 Rev	5’ – TTA AGA GAG AAA TGT ACG AAC AGT – 3’
cIAP2 Fwd	5’ – TGG AGA AGA CCA TTC AGA AGA T – 3’
cIAP2 Rev	5’ – TCA TGA AAG AAA TGT ACG AAC TGT – 3’
XIAP Fwd	5’ – ATG ACT TTT AAC AGT TTT GAA GGA – 3’
XIAP Rev	5’ – TTA AGA CAT AAA AAT TTT TTG CTT – 3’
GAPDH Fwd	5’ – ACG GAT TTG GTC GTA TTG GGC G – 3’
GAPDH Rev	5’ – CTC CTG GAA GAT GGT GAT GG – 3’

Primer sequences targeting IAPs. IAP = inhibitor of apoptosis, cIAP1 = cellular inhibitor of apoptosis 1, cIAP2 = cellular inhibitor of apoptosis 2, XIAP = X-chromosome linked inhibitor of apoptosis, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, Fwd = forward primer, Rev = reverse primer.

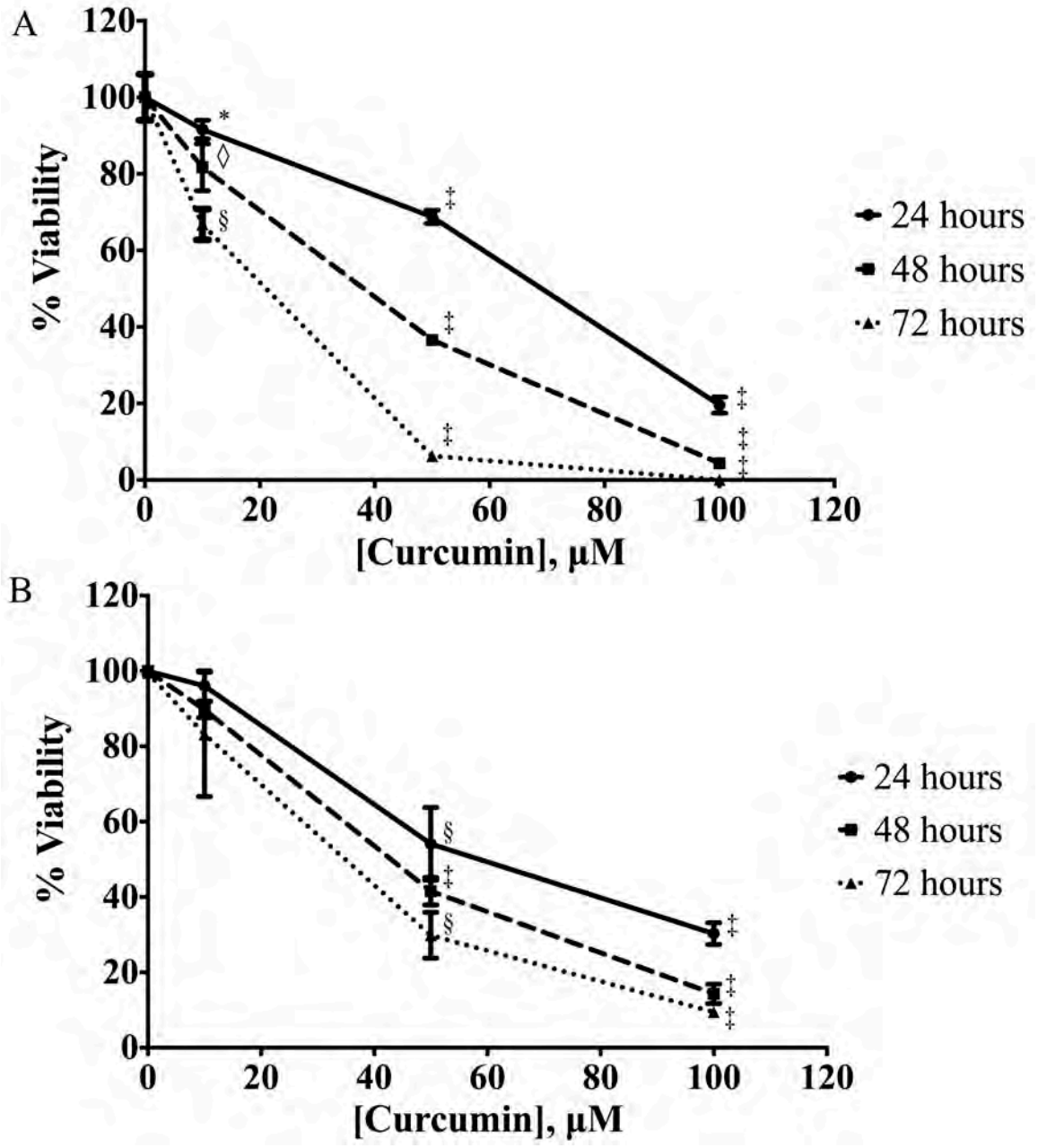


Figure 2. Curcumin induces PANC-1 cell death, analyzed by AlamarBlue and Trypan blue exclusion assays. PANC-1 cells were cultured in medium supplemented with curcumin at increasing doses for 24, 48, and 72 hours followed by (A) AlamarBlue and (B) Trypan blue exclusion viability assays. Data are presented as mean (±SEM). * $p \leq 0.05$, ◊ $p \leq 0.01$, § $p \leq 0.001$, ‡ $p \leq 0.0001$, curcumin treatment versus untreated control.

Effect of Curcumin on PANC-1 Cell Morphology

Apoptosis is a type of cell death with distinctive morphological features. To qualitatively evaluate the effects of curcumin treatment on PANC-1 cellular morphology, images were acquired using Hoffmann modulation contrast microscopy (Fig. 3). These images illustrate an increase in cell death in a dose- and time-dependent manner. The presence of membrane blebs and cell shrinkage, which are main morphological hallmarks of apoptotic cells, were observed following 50 and 100 μM curcumin treatment for 24 and 48 hours.

Curcumin Detection within Treated PANC-1 Cells

It has been reported previously that curcumin has excitation and emission spectra of 420 and 470 nm, respectively [138-140]. These spectral properties of curcumin were used to detect its presence within PANC-1 cells using spectrophotometric studies and fluorescence microscopy. PANC-1 cells were cultured in the presence of 50 μM curcumin for 24 hours and subsequently washed, trypsinized, and lysed in 100% methanol. The emission peak of curcumin-treated cell lysates at 420 nm (red) was detected compared to cells not treated with curcumin (green) or blank/methanol-only samples (blue) (Fig. 4A). This peak is identical to the emission spectrum of 50 μM curcumin in methanol (data not shown). The intrinsic fluorescence of curcumin was also exploited using fluorescence microscopy to visualize curcumin content in treated versus untreated PANC-1 cells (Fig. 4B). The DNA dye DAPI was used to visualize nuclei (blue). Curcumin demonstrates a pan-cellular staining pattern (green), within

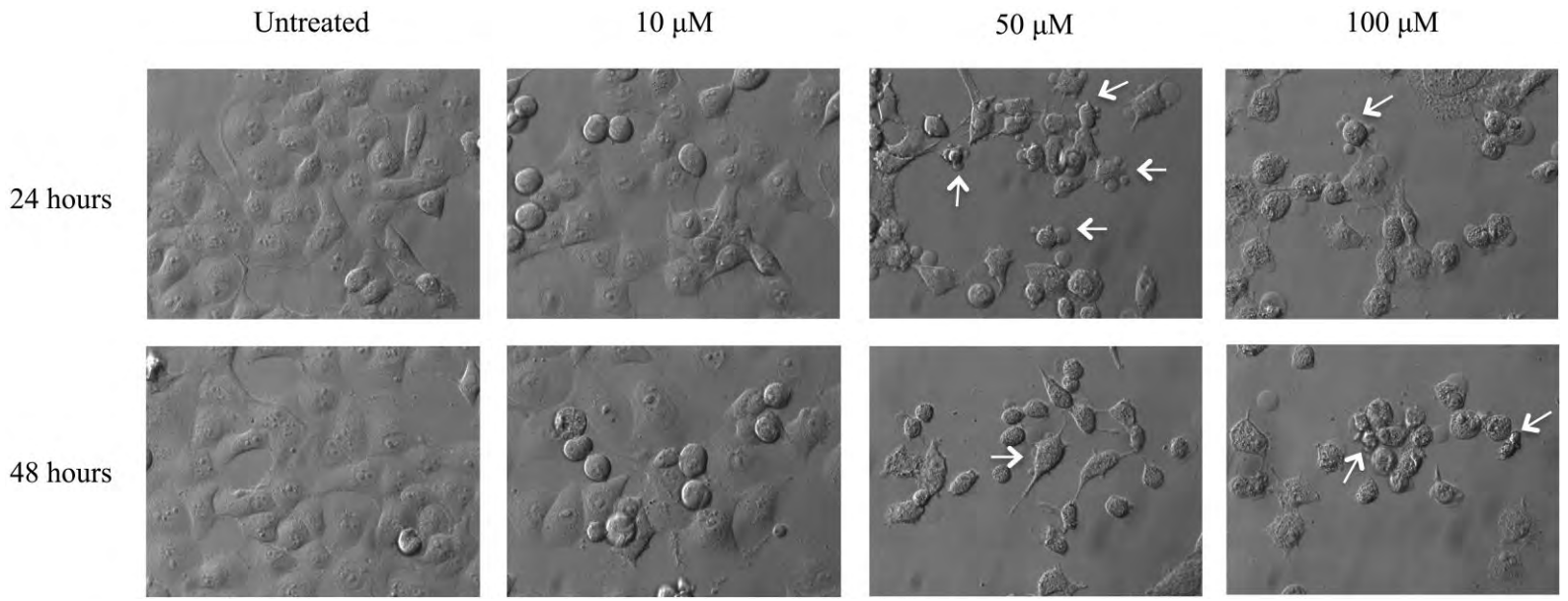
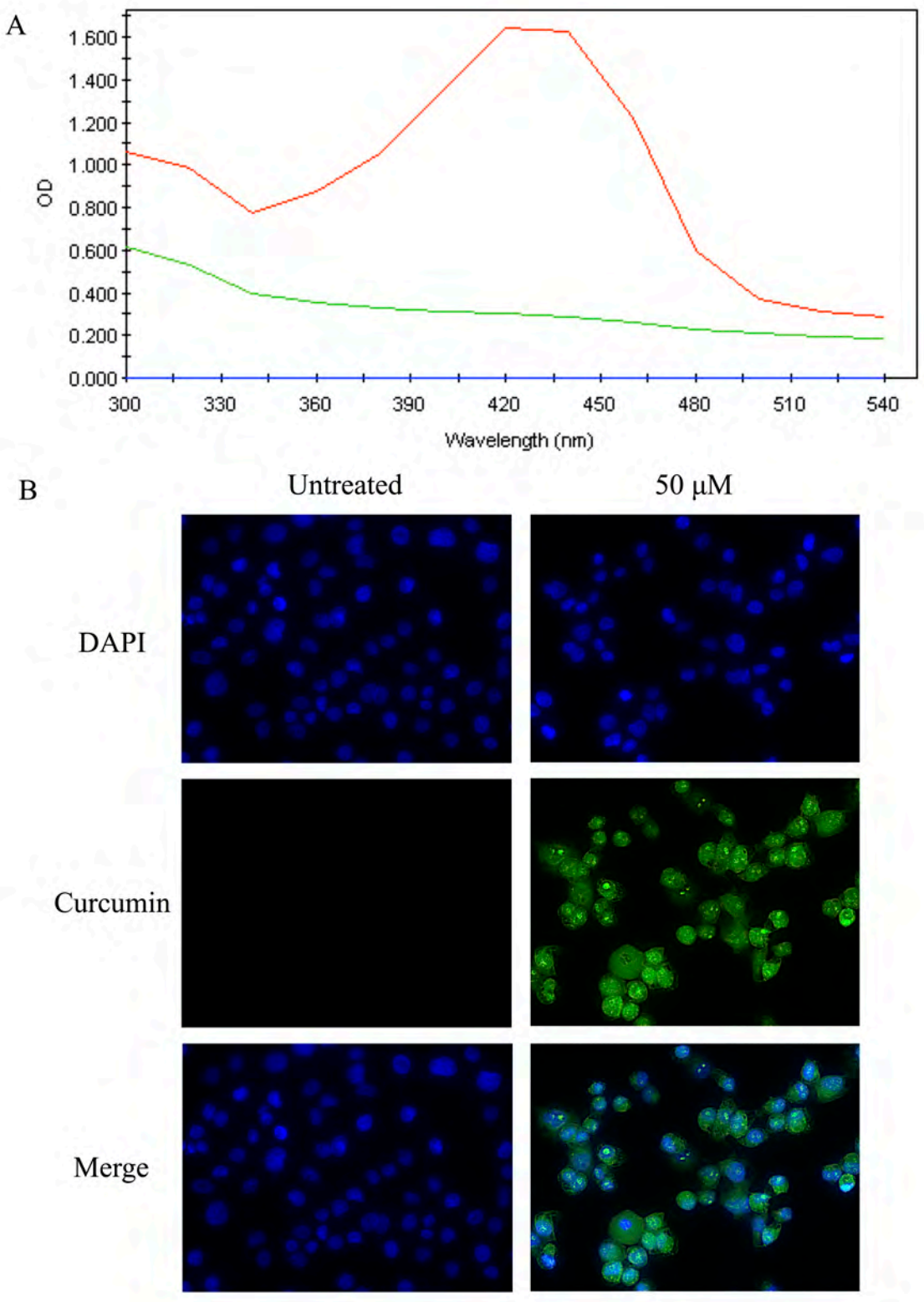


Figure 3. Morphology of curcumin-treated PANC-1 cells, visualized by Hoffman modulation contrast microscopy. PANC-1 cells were cultured in medium supplemented with curcumin at increasing doses for 24 and 48 hours followed by imaging via Hoffman modulation contrast microscopy. White arrows indicate membrane blebs and cell shrinkage, morphological hallmarks of apoptosis. Results depicted in the figure are representative of findings from 3 independent experiments.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540
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B
Untreated
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Curcumin
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Figure 4. Curcumin in pancreatic cancer cells, analyzed by spectral analysis and fluorescent microscopy. (A) PANC-1 cells were cultured in medium supplemented with 50 μM curcumin for 24 hours, trypsinized, lysed in 100% methanol and subjected to spectral analysis on a μQuant spectrophotometer at a 300-540 nm wavelength range. Red = curcumin-treated cells; green = untreated cells; blue = methanol as a vehicle control. (B) PANC-1 cells were cultured in medium supplemented with 50 μM curcumin (green) for 24 hours and stained with the DNA dye DAPI to show nuclei (blue) followed by imaging via fluorescence microscopy at 40X magnification. Results depicted in the figure are representative of findings from 3 independent experiments.

cytoplasmic, nuclear, and nucleolar pools visible by fluorescence microscopy. These results indicate that curcumin is capable of entering PANC-1 cells *in vitro* and is non-specific in its localization, suggesting multiple mechanisms of cytotoxicity.

Curcumin Decreases IAP protein and mRNA Expression

To determine the effects of curcumin on Survivin, cIAP1, cIAP2 and XIAP expression, Western blot (Fig. 5A) and reverse-transcription PCR (Fig. 6A) were performed following treatment with 10, 50 and 100 μM curcumin. Protein expression of cIAP1, cIAP2 and XIAP were significantly decreased at 50 and 100 μM curcumin treatment compared to untreated controls at 24 and 48 hours (Fig. 5B). In addition, Survivin protein expression was significantly decreased after 50 μM curcumin treatment for 24 hours and after 50 or 100 μM curcumin treatment for 48 hours. Moreover, 10 μM curcumin was sufficient to significantly reduce cIAP2 expression after 48 hours of treatment. Similarly, significant reduction in Survivin, cIAP1, cIAP2 and XIAP mRNA expression was observed at 50 and 100 μM curcumin compared to untreated controls at 24 and 36 hours (Fig. 6B).

Discussion

Pancreatic cancer is a deadly disease that causes higher mortality rates annually than other cancer types. Currently, surgical resection is one of the most effective therapeutic approaches for pancreatic cancer. However, pancreatic cancer does not exhibit notable signs or symptoms during early stages of development, making an early

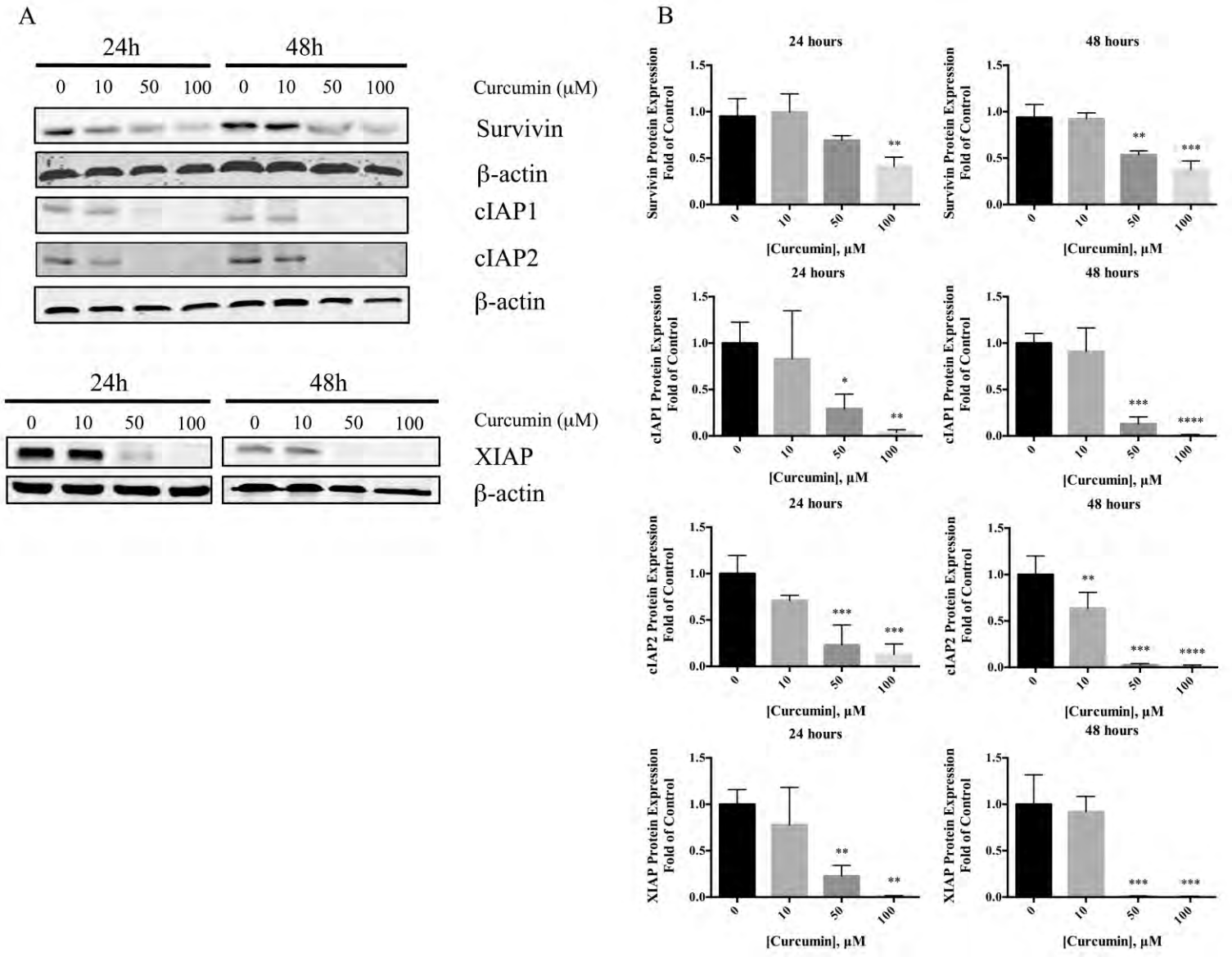
A
24h
48h
0 10 50 100 0 10 50 100
Curcumin (μM)
Survivin
β-actin
cIAP1
cIAP2
β-actin
24h
48h
0 10 50 100 0 10 50 100
Curcumin (μM)
XIAP
β-actin
B
24 hours
48 hours
Survivin Protein Expression Fold of Control
cIAP1 Protein Expression Fold of Control
cIAP2 Protein Expression Fold of Control
XIAP Protein Expression Fold of Control
[Curcumin], μM

Figure 5. Expression of IAP proteins in PANC-1 cells following curcumin treatment. (A) Cells were cultured in medium supplemented with 10-100 μM curcumin for 24 and 48 hours. Whole-cell lysates were prepared and expression levels of Survivin, cIAP1, cIAP2, XIAP and actin proteins were analyzed by Western blot as described in the Materials and Methods. Data are representative of 3-4 independent experiments. (B) Protein levels of IAPs are presented as relative ratios to control cells without curcumin treatment after normalization to β-actin levels. Data are presented as mean (± SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, curcumin treatment versus untreated control.

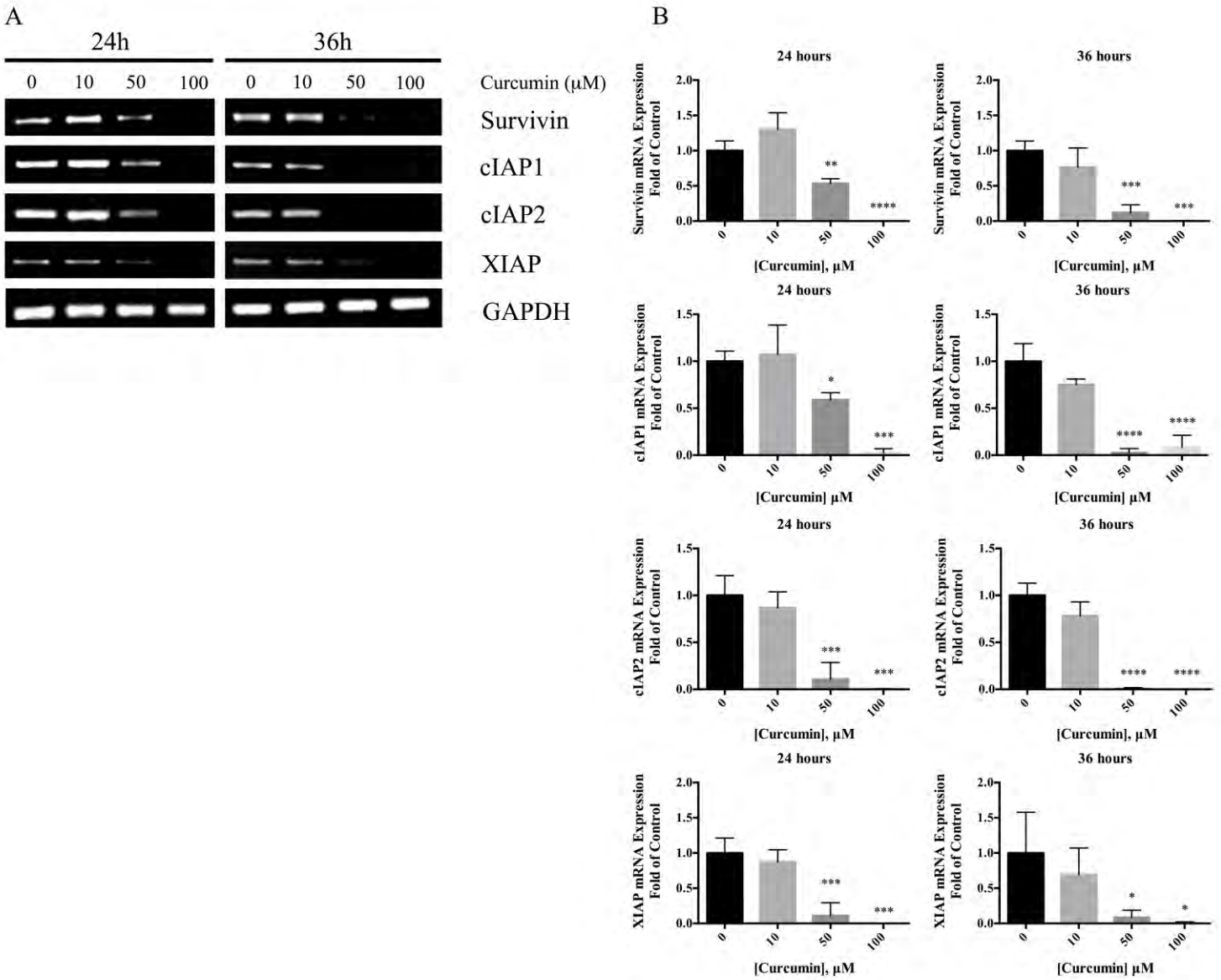
A
24h
36h
0 10 50 100 0 10 50 100
Curcumin (μM)
Survivin
cIAP1
cIAP2
XIAP
GAPDH
B
24 hours
36 hours
Survivin mRNA Expression Fold of Control
cIAP1 mRNA Expression Fold of Control
cIAP2 mRNA Expression Fold of Control
XIAP mRNA Expression Fold of Control
[Curcumin], μM
[Curcumin] μM

Figure 6. Expression of IAP mRNA following curcumin treatment in PANC-1 cells. (A) Cells were cultured in medium supplemented with 10-100 μM curcumin for 24 and 36 hours. RNA was extracted using the Trizol-chloroform extraction method then converted to cDNA and probed using primers for Survivin, cIAP1, cIAP2, XIAP and GAPDH (Table 1). Data are representative of 3 independent experiments. (B) mRNA levels of IAPs are presented as relative ratios to control cells without curcumin treatment after normalization to GAPDH levels. Data are presented as mean (± SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, curcumin treatment versus untreated control.

diagnosis difficult. For this reason, most patients with advanced non-metastatic or metastatic disease are ineligible for surgery and receive chemotherapy. Unfortunately, the efficacy of chemotherapeutic agents is limited by emerging drug resistance [2, 3, 114, 115]. Therefore, the pancreatic cancer field is moving toward investigating therapeutic approaches that target crucial mediators of chemoresistance. It has been well established that evasion of cell death is a required event in the development of resistance to chemotherapy [141, 142], with this resistance linked to the upregulation of anti-apoptotic proteins such as the inhibitor of apoptosis (IAP) family members [143, 144].

Apoptosis is a type of cell death highly dependent on the activation of caspases (cysteine-aspartic proteases) that cause endonuclease-mediated fragmentation of DNA and cellular demise. This series of events is a prerequisite for progressive cellular disassembly into apoptotic bodies that are subsequently consumed by phagocytic cells [145]. The IAP family, particularly Survivin, cIAP1, cIAP2 and XIAP, are proteins that have substantial roles in modulating the inactivation of apoptosis [120]. While the IAPs have been shown to bind caspases, only XIAP directly inhibits caspases [146]. In addition to its role in cell cycle regulation, Survivin is thought to bind active caspases through cofactor proteins such as hepatitis B X-interacting protein (HBXIP) to prevent amplification by cleavage of other pro-caspase isoforms [147, 148]. cIAP1 and cIAP2, though capable of binding caspases, have been shown to inhibit apoptosis by interrupting caspase activation through their E3 ubiquitin ligase function [149-151].

The nuclear factor-kappa B (NF-κB) transcription factor has been found to be constitutively activated in pancreatic cancer [152, 153] and is known to regulate key mediators of cancer cell survival, proliferation, angiogenesis, and metastasis [154-157].

NF-κB has been shown to regulate the production of certain IAPs, including Survivin, cIAP1, and XIAP [13, 154-157]. Furthermore, NF-κB activity and IAP expression have been implicated in resistance to Gemcitabine [13, 158]. Gemcitabine induces an increase in IAP expression in pancreatic cancer cells, particularly Survivin and XIAP, as well as cIAP1 in lung cancer cells [159-162].

Indeed, studies targeting NF-κB [158, 160, 163, 164] or IAPs [161, 165, 166] have demonstrated increased sensitivity to Gemcitabine. The increased sensitivity to Gemcitabine following IAP reduction is the rationale for the use of second mitochondria-derived activator of caspases (Smac) mimetics. Smac is an endogenous pro-apoptotic protein transcribed by the *DIABLO* gene. This protein promotes apoptosis by direct interaction and inhibition of XIAP and Survivin proteins. Several Smac mimetics are currently under investigation in clinical trials [37]. While these Smac mimetics have shown promising results in preclinical trials *in vitro* and *in vivo*, both in the reduction of IAP expression and in re-sensitization to Gemcitabine [167], they have no known effects on NF-κB expression or activity. Recent studies have demonstrated that dual inhibition of NF-κB activity and IAP expression may have superior benefits than reducing IAP expression alone. Indeed, dual targeting of NF-κB and XIAP was more effective in re-sensitizing pancreatic adenocarcinoma cells to Gemcitabine therapy than XIAP knockdown alone [161]. Thus, the optimal “next step” in the development of a therapeutic strategy for pancreatic cancer involves compounds that target upstream mediators of IAP expression, such as NF-κB, as well as multiple IAPs simultaneously.

Curcumin, a turmeric derivative, is a candidate for such a therapeutic agent. It has been shown to inhibit pancreatic adenocarcinoma cell proliferation, survival, invasion

and angiogenesis *in vitro* and *in vivo* [48, 168]. In addition, studies by Kunnumakkara et al. have demonstrated that curcumin attenuates NF-κB activation, resulting in decreased production of anti-apoptotic factors, including Survivin and cIAP1, as well as pro-angiogenic and metastatic factors, in MIA PaCa-2-derived xenograft tumors [13]. Multiple studies have demonstrated synergistic activity between curcumin and Gemcitabine in pancreatic adenocarcinoma cells [48, 134, 135]. Interestingly, while XIAP is considered to be the most potent regulator of apoptosis in humans, its levels following curcumin treatment remain to be elucidated. Furthermore, the effect of curcumin on mRNA expression of the IAPs remains to be investigated. This information is essential to understanding whether curcumin's effects on IAP expression are due to transcriptional regulation or post-translational mechanisms. In this study, we explore curcumin's effects on protein and mRNA expression of a panel of key IAPs, including Survivin, cIAP1, cIAP2 and XIAP in the pancreatic adenocarcinoma cell line PANC-1. Phase I and II clinical trials have been conducted to evaluate the safety and efficacy of curcumin alone and in combination with standard Gemcitabine-based chemotherapy [16, 109, 112, 113]. The major challenge to curcumin's clinical use is poor bioavailability. A recent Phase I clinical trial was conducted using a novel microparticle-based form of curcumin called Theracurmin in combination with standard Gemcitabine-based chemotherapy [109]. This study reported promising results, increasing plasma levels over those reported in previous clinical trials, despite using approximately 5% of the dose of curcumin used in earlier studies (400mg vs. 8g/day) while inducing minimal toxicity in patients.

While some controversy exists as to the Gemcitabine-sensitivity of the pancreatic adenocarcinoma cell line MIA PaCa-2 [161, 163], PANC-1 cells are generally considered to be resistant to Gemcitabine. Therefore, we investigated the sensitivity of these cells to curcumin *in vitro* using AlamarBlue and Trypan blue exclusion viability assays. Our results are consistent with those published using other viability assays in PANC-1 cells [169-171], demonstrating dose- and time-dependent reduction in cell viability following curcumin treatment (Fig. 2). In addition, Hoffmann modulation contrast microscopy illustrates the morphology of PANC-1 cells following curcumin treatment (Fig. 3). Cells treated with curcumin exhibit features characteristic of apoptotic cell death, including cell shrinkage and membrane blebbing.

To further elucidate the possible mechanisms of action of curcumin in PANC-1 cells, the spectral properties of curcumin (Fig. 4A) were used to determine the intracellular localization of the compound as analyzed by fluorescence microscopy (Fig. 4B). Consistent with the notion that curcumin exerts effects on multiple intracellular signaling pathways [172-175], our results demonstrate that curcumin displays a pan-cellular localization.

To determine the effects of curcumin on IAP protein and mRNA expression, we performed Western blot and RT-PCR analyses on curcumin-treated PANC-1 cells. Our data demonstrate that curcumin reduces protein and mRNA levels of Survivin, cIAP1, cIAP2 and XIAP (Fig. 5 and 6), with the most marked effects on IAP expression demonstrated by cIAPS 1 and 2 and XIAP. Interestingly, while mRNA expression of Survivin diminishes with increasing curcumin concentrations and incubation times, Survivin's protein level appears to be relatively stable at the highest dose (100 μM) and

time (48 hours) evaluated, remaining at approximately 50% of the level in untreated PANC-1 cells. These data suggest that mechanisms exist to stabilize Survivin protein under conditions of curcumin treatment, despite a reduction in Survivin mRNA production. This finding carries heavy implications for resistance to therapy, since Survivin itself has been found to bind and stabilize XIAP, enhancing the latter's caspase-9 inhibiting activity [176]. Thus, while curcumin exerts potent effects on upstream (NF-κB-mediated) signaling leading to reduced IAP expression, residual Survivin may represent a key mechanisms for evasion of cell death in the context of this therapeutic strategy.

In summary, our data demonstrate for the first time that curcumin is effective in reducing expression of multiple IAPs critical to chemoresistance, both at the mRNA and protein level, resulting in increased cell death *in vitro*. The ability to modulate multiple members of the IAP family may prove to be a key factor in selecting compounds for further study as selection of individual IAP-specific targeting has been less than effective. Furthermore, we demonstrate that, of these IAPs, Survivin shows the least sensitivity to curcumin-mediated downregulation, suggesting a possible role in resistance to curcumin treatment, a phenomenon that we are currently investigating in our laboratory.

Acknowledgments

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CHAPTER THREE

CURCUMIN MODULATES PANCREATIC ADENOCARCINOMA CELL-DERIVED EXOSOMAL FUNCTION

Carlos J. Díaz Osterman, James C. Lynch, Patrick Leaf, Duncan Griffiths and Nathan R. Wall

Center for Health Disparities and Molecular Medicine

Department of Basic Sciences

Division of Biochemistry

Loma Linda University

Loma Linda, California, 92350

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Abstract

Pancreatic cancer has the highest mortality rates of all cancer types. One potential explanation for the aggressiveness of this disease is that cancer cells have been found to communicate with one another using membrane-bound vesicles known as exosomes. These exosomes carry pro-survival molecules and increase the proliferation, survival, and metastatic potential of recipient cells, suggesting that tumor-derived exosomes are powerful drivers of tumor progression. Thus, to successfully address and eradicate pancreatic cancer, it is imperative to develop therapeutic strategies that neutralize cancer cells and exosomes simultaneously. Curcumin, a turmeric root derivative, has been shown to have potent anti-cancer and anti-inflammatory effects *in vitro* and *in vivo*. Recent studies have suggested that exosomal curcumin exerts anti-inflammatory properties on recipient cells. However, curcumin's effects on exosomal pro-tumor function have yet to be determined. We hypothesized that curcumin alters the pro-survival role of exosomes from pancreatic cancer cells toward a pro-death role, resulting in reduced cell viability of recipient pancreatic cancer cells. The main objective of this study was to determine the functional alterations of exosomes released by pancreatic cancer cells exposed to curcumin compared to exosomes from untreated pancreatic cancer cells. We demonstrate, using an *in vitro* cell culture model, that curcumin is incorporated into exosomes derived from curcumin-treated pancreatic cancer cells, and that these exosomes reduce the viability of recipient pancreatic cancer cells. Collectively, these data suggest that the efficacy of curcumin may be enhanced in pancreatic cancer cells through exosomal facilitation.

Introduction

Currently, pancreatic cancer is one of the most devastating diagnoses to receive. It is responsible for one of the highest mortality rates among cancer types [2]. It is anticipated that 48,960 people will be diagnosed with pancreatic cancer and 40,560 people will die from pancreatic cancer in the United States this year [1]. These unacceptably high mortality rates are linked to inadequate screening tools and therapeutic options, as well as the aggressive nature of the disease [4, 177]. This aggressiveness is correlated with the influence of the tumor microenvironment, which is composed of blood vessels, immune cells, fibroblasts, extracellular matrix and cancer cells [178]. The signaling networks between the components of the tumor microenvironment are important drivers of tumor growth. Investigations have shown that tumor-derived extracellular vesicles such as exosomes are key modulators of this communication due to their capacity to transport cancer-promoting material [103-106]. In order to successfully treat pancreatic cancer, it is crucial to develop novel therapeutic strategies that concomitantly target tumor cells and important mediators of the tumor microenvironment such as exosomes.

Curcumin is a turmeric root constituent that has been considered as a potential pancreatic cancer therapeutic agent. Studies have demonstrated lower cancer incidence in countries with high curcumin consumption [10, 11] and preclinical studies have shown that curcumin exhibits anti-cancer [12, 32, 47, 51, 133, 168, 179-186] and anti-inflammatory [187-191] properties in different cancer types *in vitro* and *in vivo*. Additionally, curcumin has synergistic effects with Gemcitabine, the gold standard treatment for pancreatic cancer [48, 134, 135]. These encouraging results have prompted

researchers to assess the efficacy of curcumin in the treatment of pancreatic cancer. Phase I and II clinical trials have yielded promising results on the use of curcumin as part of pancreatic cancer therapeutic strategies [16, 109, 112, 113, 136, 137]. However, curcumin has low bioavailability and this is one of the major obstacles to its application in the clinical setting. To overcome this, investigations have moved toward the development of innovative delivery approaches for curcumin, such as liposomes and nanoparticles, to enhance its bioavailability and efficacy [14, 91, 95, 97, 99, 100, 109, 192-208]. It has been demonstrated in various cancer types, including pancreatic cancer, that curcumin's solubility and efficacy is enhanced by liposomal delivery *in vitro* and *in vivo* with minimal toxicity, providing promising evidence for clinical application [206-209]. Studies performed with nanoparticle-based curcumin, Theracurmin, have indicated that membrane encapsulation can improve the bioavailability of hydrophobic compounds such as curcumin by increasing water solubility [14, 99]. Furthermore, phase I studies with Theracurmin demonstrated that this treatment approach is non-toxic and results in higher curcumin bio-distribution compared to non-encapsulated curcumin in patients with pancreatic cancer [14, 100, 109]. In addition to being encapsulated in synthetic nanoparticles, curcumin is able to be packaged in lymphoma-derived exosomes and retain its anti-inflammatory function after delivery to recipient cells [210].

Collectively, these data suggest that curcumin is a suitable candidate for pancreatic cancer therapy due to its anti-cancer and anti-inflammatory properties. Moreover, curcumin has the potential to influence the role of exosomes in the tumor microenvironment. Thus, the objective of this study was to evaluate the impact of exosomal curcumin on recipient pancreatic cancer cells. The present study demonstrates,

for the first time, that exosomes from curcumin-treated pancreatic cancer cells carry curcumin and that these curcumin-containing exosomes reduce the viability of recipient pancreatic cancer cells. These findings suggest that the effects of curcumin may extend to other components of the tumor microenvironment through exosomes.

Materials and Methods

Cells and Culture Conditions

The pancreatic adenocarcinoma cell line PANC-1 was acquired from the American Type Culture Collection (ATCC, catalog no. CRL-1469, Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM; ATCC, Manassas, VA) supplemented with Normocin at a final concentration of 100 µg/mL (InvivoGen, San Diego, CA), 100 units of penicillin, 100 µg/mL of streptomycin, 300 µg/mL of L-glutamine and 10% USDA-sourced heat-inactivated fetal bovine serum (Mediatech, Manassas, VA). In all the experiments, cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 to 70-80% confluency prior to use.

Preparation of Solutions

Curcumin (Sigma Aldrich, St. Louis, MO) stock solutions (13.5 mM) were prepared using DMSO and ethanol as solvents. Subsequent dilutions were made from this stock solution in fully supplemented DMEM to a final concentration of 50 µM. Heparin sodium salt (Sigma Aldrich, St. Louis, MO) was used to prepare a 50 mg/mL stock solution in sterile water and used at a final concentration of 10 µg/mL. Recipient PANC-1 cells were pre-treated with 10 µg/mL heparin in fully supplemented DMEM for 30

minutes at 37°C, 5% CO_2 prior to incubation with exosomes. Of note, 10 µg/mL heparin was also added during the subsequent incubation with exosomes as well.

Exosome Isolation

Exosomes were isolated from conditioned media as previously described by Savina et al. [211, 212] with minor modifications. Briefly, PANC-1 cells were cultured in fifteen T75 flasks at 1.5 x 10^6 cells per flask and conditioned media (CM) was collected following 24 hours of treatment with fully supplemented DMEM (for isolation of curcumin-negative exosomes) or 50 µM of curcumin (for curcumin-positive exosome isolation). The cellular debris and other impurities in the CM were eliminated by three consecutive cycles of centrifugation. First, the CM was centrifuged in a Beckman Coulter Allegra X-15R centrifuge (SX475OA rotor) at 400 x g for 10 minutes, then at 2000 x g for 20 minutes, followed by centrifugation in a Thermo Scientific Sorvall Legend X1R centrifuge (F15-8X50Y rotor) at 10,000 x g for 30 minutes. Subsequently exosomes were isolated from the CM by ultracentrifugation in a Beckman XL-90 centrifuge equipped with a SW-27 rotor at 24,000rpm for 16 hours at 4°C over a 30% sucrose cushion. The exosomes within the sucrose cushion were washed with 1X PBS and centrifuged in a Beckman XL-90 centrifuge equipped with a 70-Ti rotor at 31,000 rpm for 2 hours at 4°C. The remaining pellet, the exosomal fraction, was resuspended in 500 µL of 1X PBS, transferred to a microcentrifuge tube, and immediately used in subsequent assays.

Exosome Detection and Validation

Acetylcholinesterase activity assays were performed to detect exosome presence based on the protocols described by Savina et al. and Lancaster and Febbrario [211, 213] with minor modifications. Briefly, 37.5 µL of the exosomal fraction were transferred into each of three wells of a 96-well flat-bottom plate. 112.5 µL of 1.25 mM acetylthiocholine (Sigma) and 150 µL of 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (Sigma) were then added to each well. The samples were immediately analyzed using a µQuant spectrophotometer (Bio-Tek Inc., Winooski, VT) and changes in absorbance at 412 nm were monitored every 5 minutes for 30 minutes. The exosome absorbance data were analyzed using the KC Junior software (Bio-Tek Inc.). The results presented represent acetylcholinesterase enzymatic activity after 30 minutes compared to control (reagents in assay diluent, 1X PBS).

Nicomp 380 ZLS analysis (Particle Sizing Systems, Port Richey, FL) was used to assess the size of the particles present in the exosome isolation fraction. Briefly, the exosomal fraction was diluted 1:30 with 1X PBS in a 4 mL (1 cm x 1 cm) plastic cuvette at 23°C and the size dispersion was measured using Nicomp 380 ZLS dynamic light scattering (DLS) with a display range of 0.6 to 6000 nm. The sample was exposed to a HeNe 5 mW laser using a wavelength of 639 nm. The data were analyzed using the Nicomp Fit Model Type and PSS zpw 388 Nicomp software. Finally, real-time detection, sizing, and quantification were performed using a NanoSight NS300 following the manufacturer's protocols (Malvern Instruments, Malvern, UK). Briefly, exosome isolates were diluted 1:1000 in 1X PBS and sonicated in a water bath sonicator for 30 seconds to prevent exosome aggregation. Samples were then loaded into the NS300 instrument and

subjected to nanoparticle tracking analysis (NTA) yielding size distribution and concentration (particles/mL). Acetylcholinesterase activity assays and NanoSight NTA were performed in three independent experiments, while Nicomp DLS analysis was performed in two independent experiments.

Spectral Studies

The spectral properties of curcumin, particularly a characteristic absorbance peak at 420 nm, have been utilized in detecting the compound under experimental conditions. These spectral analyses of exosomal curcumin were designed, with minor modifications, based on studies detecting curcumin within cells [138-140]. Briefly, exosomes were isolated as described above. To determine whether curcumin was coating the exterior surface of exosomes, exosomal fractions were subjected to spectral analysis prior to methanol-sonication disruption of exosomal membranes. To determine whether curcumin was located in the interior of the exosomes, the exosomal fraction was resuspended in 1 mL of 100% methanol and sonicated to disrupt exosome membrane integrity. The samples were then centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was collected for absorbance analysis at 420 nm using a µQuant spectrophotometer equipped with KC Junior software. Exosomes from untreated cells (curcumin-negative exosomes) were also isolated and lysed in methanol and subjected to spectral analysis to determine baseline auto-fluorescence of exosomes without curcumin. 1X PBS (for non-lysed exosome samples) or methanol (for lysed exosome samples) were used as negative controls for this assay. Data are representative of three independent experiments.

Fluorescence Imaging

Fluorescence microscopy was performed to detect the entry of exosomal curcumin into recipient PANC-1 cells. Briefly, PANC-1 cells were cultured in 6-well plates containing sterile cover slips at 3.0 x 10^5 cells per well and exposed for 24 hours to curcumin-negative or curcumin-positive exosomes. Subsequently, cells were washed three times with 1X PBS, followed by fixation with 4% paraformaldehyde overnight at -20°C and permeabilization using 0.1% Igepal in 1X PBS for 10 minutes at room temperature. The cover slips were then washed three times with 1X PBS and placed onto slides with the nuclear stain DAPI in mounting medium for 5 minutes. Stained slides were imaged using a BZ-9000 BIOREVO fluorescence microscope (Keyence, Itasca, IL) with a 40X magnification objective. In a separate experiment, PANC-1 cells were incubated with 10 µg/mL of heparin for 30 minutes prior to exposure to curcumin-positive exosomes to determine exosomal curcumin delivery in the presence of heparin, an inhibitor of exosomal uptake by recipient cells [214-216]. Results are representative of three independent experiments; within each experiment, three images were acquired from different portions of each slide to obtain a representative image. Quantification of curcumin fluorescence per cell was performed using the BZ II analyzer software (Keyence, Itasca, IL). For each image acquired (three per independent experiment, three independent experiments), the hybrid cell count and adjust extension area software features were used to calculate cell number based on DAPI staining, followed by quantification of curcumin fluorescence. For each image acquired, curcumin fluorescence was divided by cell number and multiplied by 100 and the averages of these values were

obtained for three independent experiments. Graphs were generated using the Prism (Graphpad, La Jolla, CA) software.

Cell Viability

Cell viability following exosomal curcumin entry was detected using the AlamarBlue assay. Briefly, PANC-1 cells were cultured in 96-well flat-bottom plates at 1.0×10^4 cells per well in the presence of fully supplemented DMEM (untreated), curcumin-negative exosomes, or curcumin-positive exosomes for 24, 48 and 72 hours. Subsequently, the AlamarBlue reagent (Life Technologies, Grand Island, NY) was added to each sample at a 10% final concentration and incubated at 37°C/5% CO_2 for 2 hours. Viability was analyzed by detection of absorbance at 570 nm using 600 nm as a reference wavelength in a µQuant spectrophotometer. To determine whether any observed changes in viability were due to exosome-mediated effects, a separate sample was pre-treated with 10 µg/mL heparin for 30 minutes prior to addition of curcumin-positive exosomes; heparin was maintained during subsequent treatment with curcumin-positive exosomes. Results presented are representative of three independent experiments and each treatment (curcumin-negative exosomes, curcumin-positive exosomes, and heparin + curcumin-positive exosomes) is normalized to the untreated control.

Statistical Analysis

All statistical analyses in this study were performed using either one-way ANOVA or Students' t-test analyses using the Prism (Graphpad) software. Statistical analysis of acetylcholinesterase activity assays was performed using Kruskal-Wallis one-

way ANOVA with a post-hoc Dunn's multiple comparison test. Analysis of curcumin fluorescence was performed using an unpaired one-tailed Students' t-test. Statistical analysis of viability assays was performed using one-way ANOVA with a post-hoc uncorrected Fisher's LSD test. A probability of less than a 95% confidence limit ($p < 0.05$) was considered to be significant. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Detection, Size Distribution and Quantification of Exosomal Particles

To confirm exosome isolation, known methods of exosome detection, sizing and quantification were employed. Since it has previously been established that the acetylcholinesterase enzyme is enriched in exosomes [217], an acetylcholinesterase activity assay was used to detect the presence of exosomes in our isolates. Our data demonstrate significantly increased acetylcholinesterase activity in exosome isolates compared to control (assay reagents in 1X PBS) (Fig. 7A). No significant difference was observed in acetylcholinesterase activity between exosomes derived from untreated PANC-1 cells (curcumin-negative exosomes) and exosomes derived from PANC-1 cells treated with 50 μM of curcumin (curcumin-positive exosomes). To further validate exosome isolation, particle size was measured using two separate methods: Nicomp dynamic light scattering (DLS) analysis and NanoSight nanoparticle tracking analysis (NTA). Our data demonstrate that both exosome isolates (curcumin-negative exosomes and curcumin-positive exosomes) contain particles within the established size range for exosomes, 40-150 nm [218] (Fig. 7B-C). Finally, NanoSight NTA was utilized to

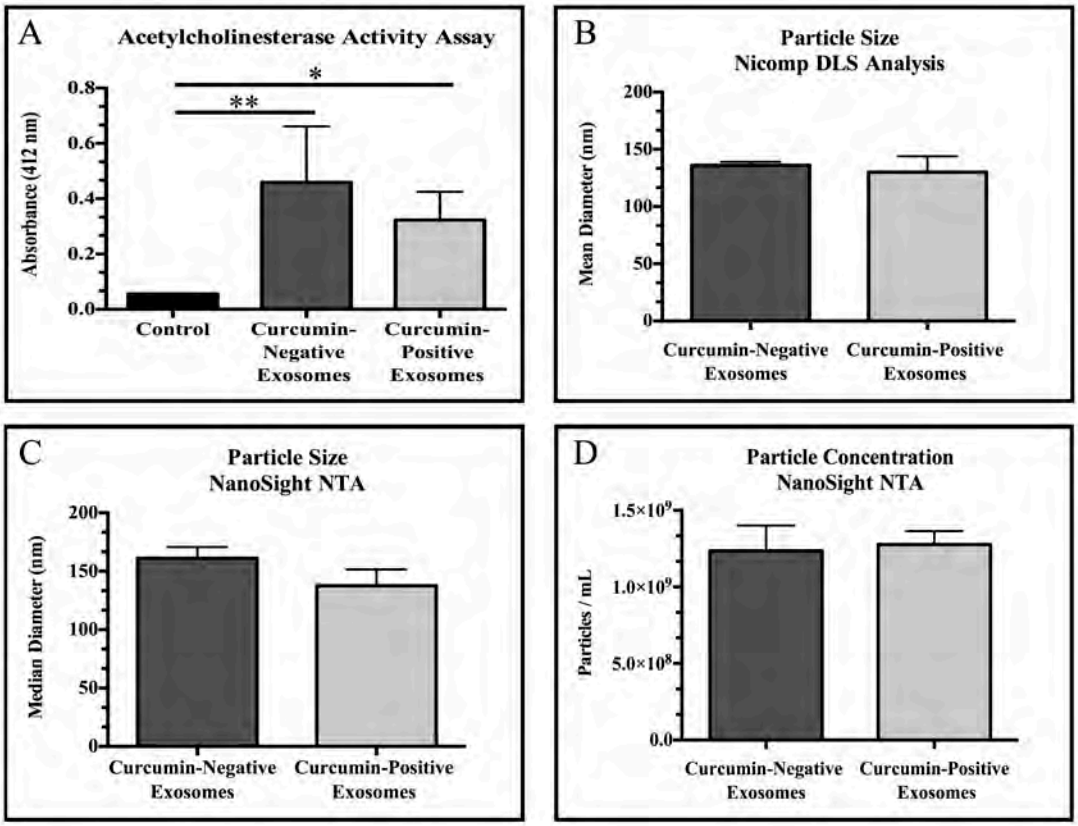
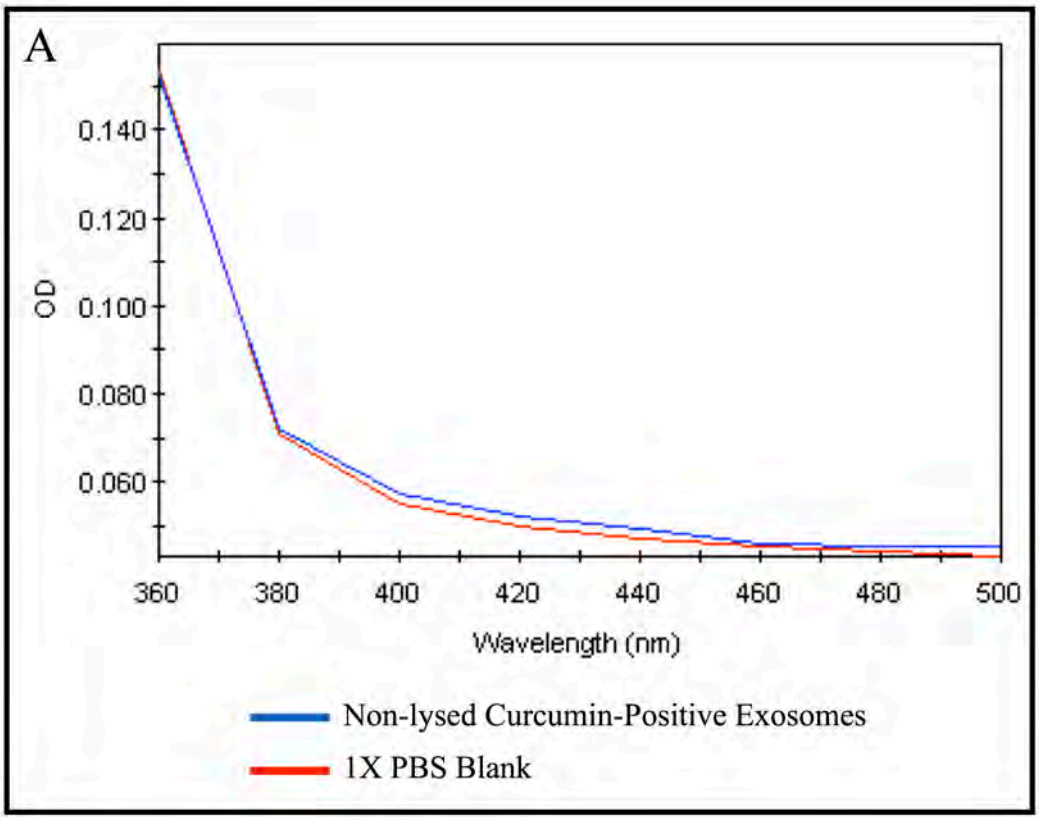


Figure 7. Validation of exosome isolation. (A) Acetylcholinesterase activity assays were used to detect exosomes in isolates from untreated PANC-1 cells (curcumin-negative exosomes) or PANC-1 cells treated with 50 μM of curcumin for 24 hours (curcumin-positive exosomes) and compared to assay diluent, 1X PBS (control). (B) Nicomp dynamic light scattering (DLS) analysis was used to measure size distribution of particles in exosome isolates. (C) NanoSight nanoparticle tracking analysis (NTA) was used to confirm size distribution of particles in exosome isolates. (D) Particle concentration (particles/mL) was measured using NanoSight NTA. No significant differences were observed in acetylcholinesterase activity, size distribution, or particle concentration between curcumin-negative exosomes and curcumin-positive exosomes. Data are represented as mean $\pm$ SEM of three independent experiments, * $p < 0.05$, ** $p < 0.01$, exosome fraction versus control.

quantify exosome isolates (particles/mL). The amount of exosomes released from curcumin-treated PANC-1 cells was not statistically different from the amount of exosomes released from untreated PANC-1 cells (Fig. 7D).

Curcumin Detection within Exosomes

It has been previously reported that curcumin has an absorbance spectrum with a peak at 420 nm [138-140]. This was used to detect its presence within exosomes using spectrophotometric studies. To determine whether curcumin coats the exterior of exosomes, whole exosomes isolated from curcumin-treated PANC-1 cells were subjected to spectral analysis and compared to assay diluent (1X PBS) (Fig. 8A). These studies demonstrate a lack of the characteristic absorbance peak corresponding to curcumin at 420 nm. To determine whether curcumin is incorporated into exosomes, exosomes isolated from PANC-1 cells untreated or treated with 50 μM of curcumin for 24 hours were isolated and lysed in 100% methanol. The absorbance peak of exosomes isolated from curcumin-treated PANC-1 cells at 420 nm (lysed curcumin-positive exosomes; green) was compared to exosomes isolated form PANC-1 cells not treated with curcumin (lysed curcumin-negative exosomes; blue) or methanol-only blank samples (red) (Fig. 8B). Collectively, these data demonstrate that curcumin is not detectable in the exterior surface of the exosomes but is rather located within the lumen of the exosomes.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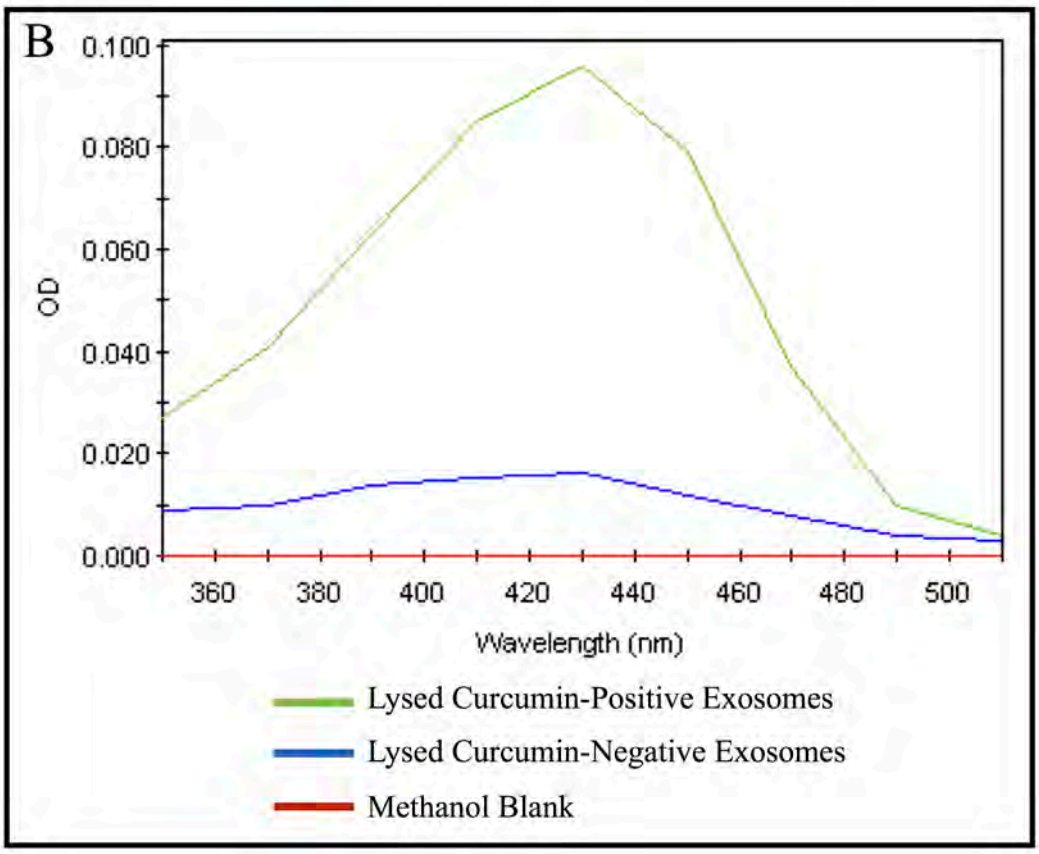
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Lysed Curcumin-Negative Exosomes
Methanol Blank

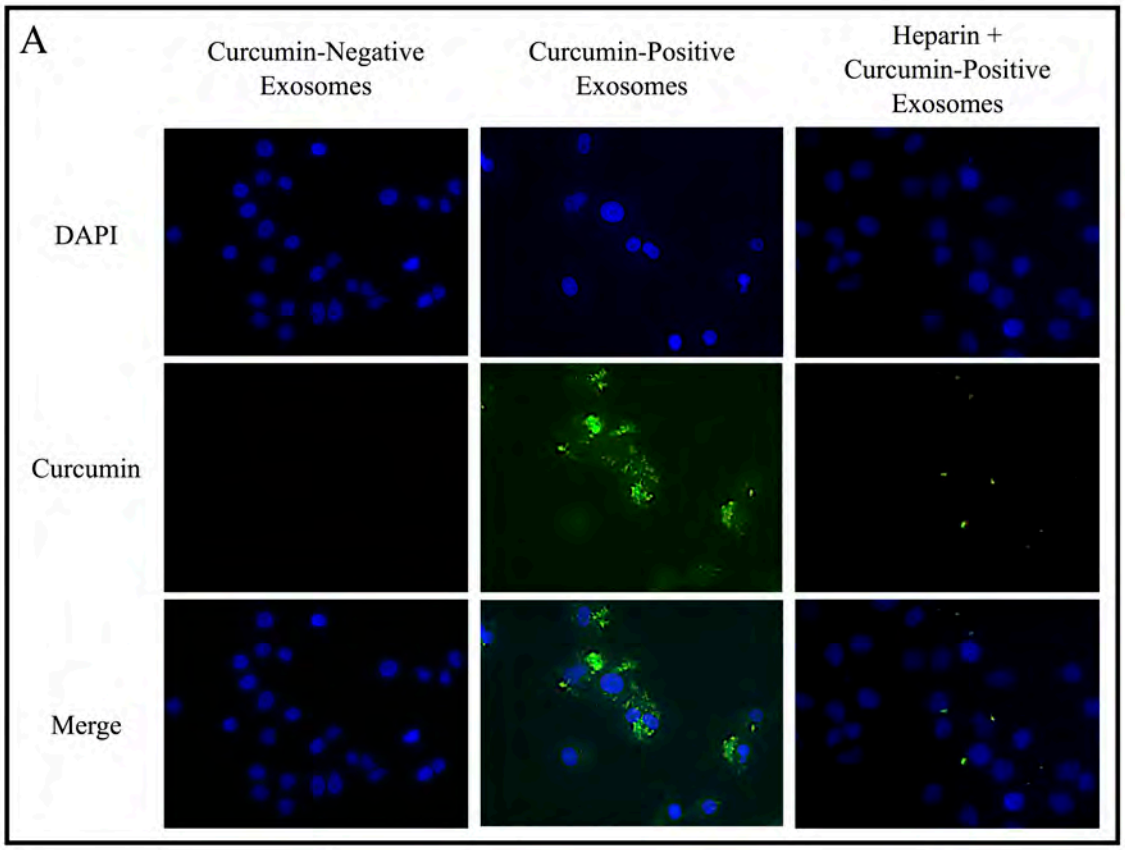
Figure 8. Spectrophotometric detection of curcumin within exosomes from PANC-1 cells. Exosomes were isolated from untreated PANC-1 cells (curcumin-negative exosomes) or PANC-1 cells treated with 50 μM of curcumin for 24 hours (curcumin-positive exosomes). (A). Whole (non-lysed, blue) exosomes from curcumin-treated PANC-1 cells were subjected to spectral analysis compared to vehicle (1X PBS blank, red), in which optical density (OD) at 420 nm was measured. No peak in absorbance was detected at 420 nm from whole (non-lysed) exosomes. (B) Methanol and sonication were used to lyse exosomes from curcumin-treated PANC-1 cells (lysed curcumin-positive exosomes, green) or exosomes from untreated PANC-1 cells (lysed curcumin-negative exosomes, blue). A methanol-only blank (red) was used as a negative control for this assay. A characteristic peak in OD at 420 nm was detected in lysed curcumin-positive exosomes, but not in lysed curcumin-negative exosomes or the methanol-only blank. Data are representative of three independent experiments.

Entry of Exosomal Curcumin into Recipient PANC-1 Cells

To determine whether exosomal curcumin can be delivered into recipient PANC-1 cells, the intrinsic fluorescence capacity of curcumin, with approximate excitation and emission spectra of 420 and 520 nm, respectively [139, 140], was exploited using fluorescence microscopy. Naïve PANC-1 cells were incubated with either curcumin-negative exosomes or curcumin-positive exosomes for 24 hours and subjected to fluorescence microscopy. The DNA dye DAPI was used to visualize cell nuclei (blue). Curcumin content (green) in recipient PANC-1 cells demonstrates a cytoplasmic pattern excluding the nucleus (Fig. 9A). Interestingly, if recipient PANC-1 cells were pre-treated with 10 μg/mL heparin, the cytoplasmic detection of curcumin is markedly reduced, validating exosomal transfer of curcumin in these studies (Fig. 9A-B).

Exosomal Curcumin Reduces Recipient PANC-1 Cell Viability

The effects of exosomal curcumin on recipient PANC-1 cell viability were assessed using AlamarBlue viability assays (Fig. 10). After exosome-mediated entry, curcumin demonstrates inhibitory effects on recipient PANC-1 cell viability compared to untreated controls. Furthermore, our data demonstrate that PANC-1 cell viability is restored upon pre-treatment with 10 μg/mL heparin, an inhibitor of exosome binding [214-216], implicating exosomal transfer of curcumin as a crucial mediator of reduced cell viability in this system. Interestingly, an increase in PANC-1 cell viability after exposure to curcumin-negative exosomes was noted. This is consistent with the notion that tumor-derived exosomes have been shown to deliver cancer-driving factors to recipient cells, promoting aggressive behavior [101, 102]. Our data indicate that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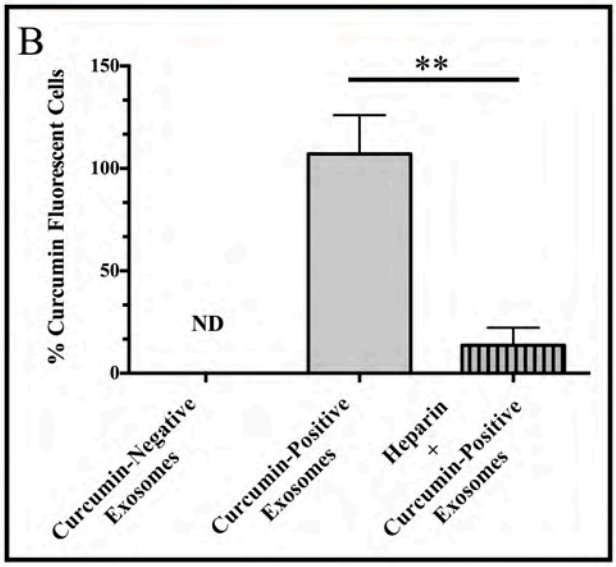
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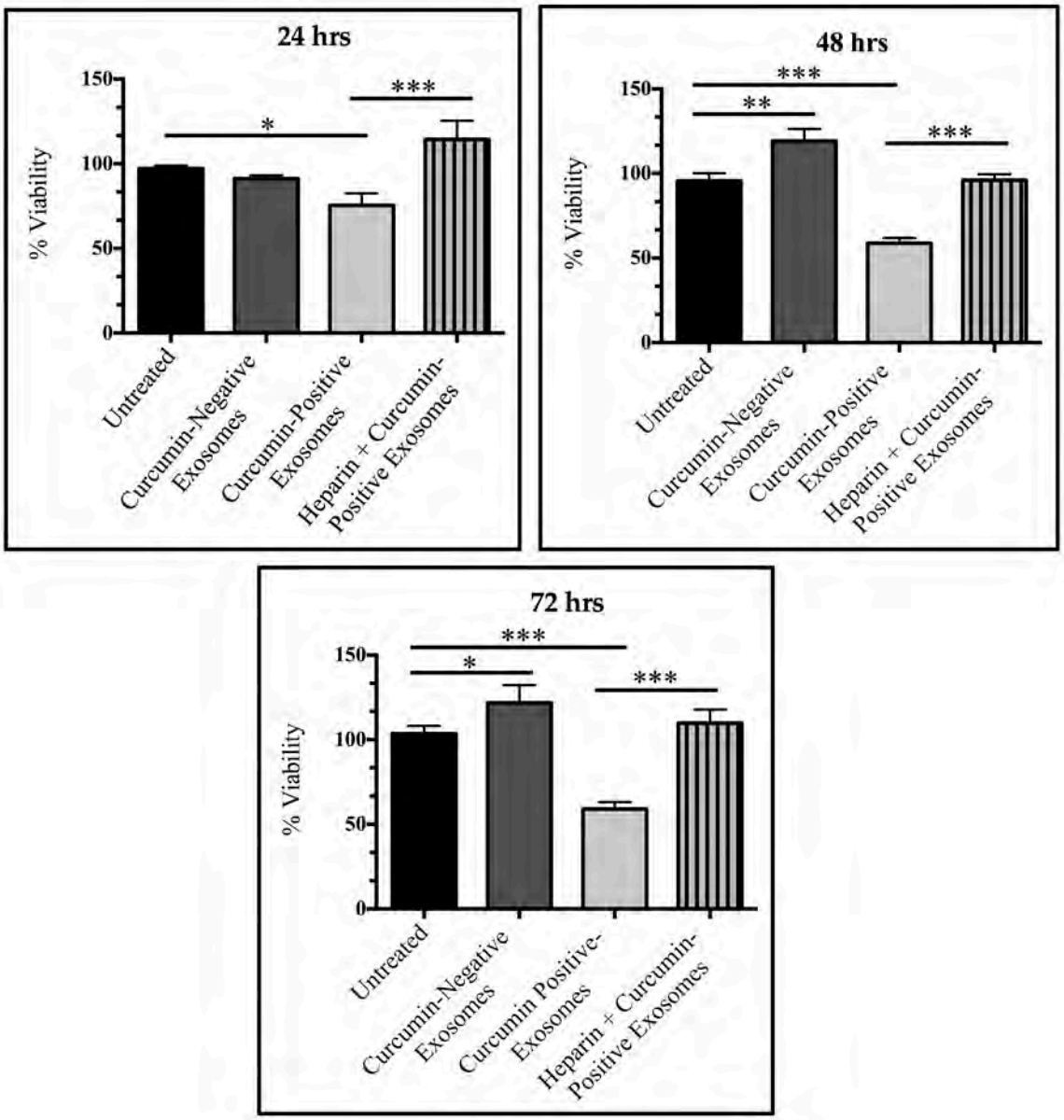
Curcumin-Positive Exosomes
Heparin + Curcumin-Positive Exosomes

Figure 9. Exosomal curcumin in recipient PANC-1 cells. Naïve PANC-1 cells were co-incubated with exosomes from untreated PANC-1 cells (curcumin-negative exosomes) or exosomes from PANC-1 cells treated with 50 μM of curcumin for 24 hours (curcumin-positive exosomes). In a separate culture, naïve recipient PANC-1 cells were treated with 10 μg/mL heparin to inhibit exosomal binding 30 minutes prior to and during co-incubation with curcumin-positive exosomes (heparin + curcumin-positive exosomes). After 24 hours, cells were washed and stained with DAPI for visualization of nuclei. (A) Curcumin fluorescence (green) and DAPI (blue) were detected by fluorescence microscopy at 40X magnification. (B) Quantification of curcumin fluorescence was performed using the BZ II analyzer software. Data were collected in three separate images per independent experiment, three independent experiments. ND = not detectable.


24 hrs
% Viability
150
100
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Untreated
Curcumin-Negative Exosomes
Curcumin-Positive Exosomes
Heparin + Curcumin-Positive Exosomes
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% Viability
150
100
50
0
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Untreated
Curcumin-Negative Exosomes
Curcumin-Positive Exosomes
Heparin + Curcumin-Positive Exosomes
72 hrs
% Viability
150
100
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Untreated
Curcumin-Negative Exosomes
Curcumin Positive-Exosomes
Heparin + Curcumin-Positive Exosomes

Figure 10. Exosomal curcumin reduces recipient PANC-1 cell viability. Naïve recipient PANC-1 cells were cultured for the indicated times with exosomes isolated from untreated PANC-1 cells (curcumin-negative exosomes) or exosomes isolated from PANC-1 cells treated with 50 μM of curcumin (curcumin-positive exosomes). In a separate experiment, naïve recipient cells were treated with 10 μg/mL heparin prior to and during incubation with curcumin-positive exosomes (heparin + curcumin-positive exosomes). Viability was determined via AlamarBlue viability assays and exosome treatments were compared to naïve PANC-1 cells not exposed to exosomes or heparin (untreated). Data are represented as mean $\pm$ SEM of three independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ treatment versus untreated control.

curcumin conserves its cytotoxic effects on recipient pancreatic cancer cells after exosomal trafficking.

Discussion

Patients diagnosed with pancreatic cancer have abysmal survival rates because the current treatment options are not sufficient to completely eradicate the disease. The most effective available therapeutic approach is to resect the pancreas; however, only a minimal percent of patients meet the criteria for surgery due to the lack of effective early detection tools. Additionally, most patients relapse despite intensive post-surgery treatment regimens [219]. The resistance to therapy and tumor aggressiveness observed in pancreatic cancer are related to the effects of the components of the tumor microenvironment [178]. Moreover, these effects are highly dependent on signaling networks driven in part by tumor-derived extracellular vesicles such as exosomes [103-106]. For instance, our group has previously shown that Survivin, a protein highly expressed in cancers and essential for carcinogenesis, is localized in intra-cellular an extracellular pools, and that extracellular Survivin enters cancer cells, increasing proliferation, resistance, and invasive potential [101]. These results are consistent with another study conducted by our laboratory that demonstrated that Survivin is transported out of cancer cells via exosomes [102]. Exosomes have also been shown to transport mutant KRAS proteins to colon cancer cells, increasing tumor growth [220, 221]. These results suggest that exosomes have the ability to modulate the components of the tumor microenvironment via the transfer of bioactive molecules that modulate cancer growth. In addition to transporting cancer-promoting material within the tumor microenvironment,

exosomes released from primary tumors have been demonstrated to aid in the formation of a suitable metastatic environment that promotes the transition of non-cancerous cells into pre-cancerous cells [222]. For instance, pancreatic cancer cell-derived exosomes have been shown to prepare pre-metastatic organs for population with cancer cells *in vivo* [223]. These results demonstrate the imperative role of exosomes in metastasis in various cancer types including pancreatic cancer.

Curcumin has been considered a promising therapeutic agent for cancer treatment due to its multi-dimensional anti-cancer properties. For instance, curcumin has been shown to modulate signaling molecules essential for the progression of most cancer types including pancreatic cancer [12, 48, 168]. Additionally, curcumin exhibits synergetic effects with Gemcitabine *in vitro* and *in vivo*. In the context of the clinic, curcumin has a tolerable consumption profile as demonstrated by phase I and II clinical trials [16, 109, 112]. One of the main obstacles to curcumin's utility in the clinic is low biodistribution [224]. In response to this, numerous investigations have developed alternative approaches to enhance curcumin delivery [14, 91, 95, 97, 99, 100, 109, 192-208]. These studies demonstrate the potential role of curcumin in pancreatic cancer therapy. However, it is imperative to determine the role of curcumin in the pancreatic cancer microenvironment, particularly in the context of exosomes.

Previous studies have shown that curcumin has a pan-cellular distribution in breast cancer cells [139]. This finding may offer an explanation for curcumin's multi-dimensional regulatory roles and its capacity to influence various cell signaling pathways. Remarkably, our results indicate that curcumin is able to be packaged into exosomes derived from pancreatic cancer cells treated with curcumin. Furthermore, our work shows

exosomal curcumin enters recipient pancreatic cancer and is able to cause significant cytotoxic effects on recipient pancreatic cancer cells. This is consistent with other studies that demonstrated that curcumin's cytotoxic effects on cancer cells are enhanced upon encapsulation of curcumin in synthetic nanoparticles and micelles *in vitro* and *in vivo* [91, 95, 97, 99, 100, 192, 193, 195-208]. Our results demonstrate an approximate 50% decrease in PANC-1 cell viability 72 hours after exosomal curcumin uptake. It is also important to note that PANC-1 cell-derived exosomes devoid of curcumin increased viability of recipient PANC-1 cells. This is consistent with the notion that tumor-derived exosomes have a cancer-supportive role in the tumor microenvironment [101, 102]. It is also noteworthy to mention that regardless of exosomal curcumin content, tumor-derived exosomes carry pro-cancerous material [103-106]. However, our results demonstrate that these exosomal components were not an impediment to curcumin's cytotoxic function after exosomal delivery into recipient pancreatic cancer cells.

In summary, our results provide new evidence of curcumin's ability to expand its anti-cancer functions from one pancreatic cancer cell to a recipient pancreatic cancer cell with the aid of exosome transportation. These findings reveal that curcumin's function may not be restricted to individual tumor cells, but may also be extended to components of the tumor microenvironment such as other tumor cells through exosomes. Exosomes represent a crucial mechanism of communication between the components of the tumor microenvironment and also for preparing future metastatic sites [222]. Thus, our results contribute to a better understanding of the role of curcumin in intercellular communication between pancreatic cancer cells and other components of the tumor microenvironment (vascular smooth muscle, stromal cells or fibroblasts, and immune

cells). Collectively, these discoveries highlight the promising role of curcumin as a therapeutic agent for the treatment of pancreatic cancer due to its multi-dimensional anti-cancer properties.

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CHAPTER FOUR

DISCUSSION

Due to the minimal possibility of survival and devastating effects of treatment on the quality of life of pancreatic cancer patients, it is essential to contribute scientific efforts to the development of therapeutic strategies that circumvent barriers to the eradication of this disease. This thesis is intended to provide insight into the mechanisms of action underlying curcumin's anti-cancer properties. It is our hope that, with the contributions of others, this work will promote further progress in the future eradication of pancreatic cancer.

This work began with the purpose of determining the mechanisms by which natural agents such as curcumin can cause pancreatic cancer cell death and perhaps be used to treat pancreatic cancer patients. Low incidence of cancer has been reported in countries with high consumption of natural agents with anti-cancer properties, such as curcumin, in contrast with those countries lacking such agents as part of the dietary regimen [11]. These findings paved the way for our laboratory to consider curcumin as a possible therapeutic agent for pancreatic cancer.

Thus, in conjunction with an established foundation of literature confirming curcumin's anti-cancer properties and its potential role in the modulation of the tumor microenvironment, we developed aims to investigate the mechanisms by which curcumin triggers cell death in pancreatic cancer. These studies were expanded to include curcumin's ability to be packaged in exosomes and carried to recipient pancreatic cancer cells to induce further cell death.

Our work began by demonstrating that curcumin is able to enter and cause cytotoxic effects in pancreatic cancer cells. Indeed, our fluorescence microscopy and spectral analyses demonstrated that curcumin enters PANC-1 cells and is distributed in a ubiquitous pattern within these cells. Furthermore, curcumin significantly reduced PANC-1 cell viability in a time- and dose-dependent manner. Interestingly, cellular morphology following curcumin treatment, as observed by Hoffman modulation contrast microscopy, demonstrated hallmarks of apoptosis including cell shrinkage and the formation of blebs.

To effectively address chemotherapy resistance in pancreatic cancer, known drivers of chemotherapy resistance were investigated in the context of curcumin treatment. The inhibitor of apoptosis (IAP) proteins, which include Survivin, cIAP1, cIAP2 and XIAP, are promising targets due to their key roles in cancer resistance [37-44]. Thus, we investigated the effects of curcumin treatment on IAP protein and mRNA expression in pancreatic cancer cells. Our data reveal that curcumin decreased both the protein and mRNA expression of Survivin, cIAP1, cIAP2 and XIAP in PANC-1 cells in a time- and dose-dependent manner. Therefore, together with our results from studies on cell viability, we conclude that curcumin reduces pancreatic cancer cell viability in part through reduction of pro-survival and chemo-resistance factors such as the IAP family.

Pancreatic cancer therapy resistance has also been strongly linked to the tumor microenvironment, which is composed of blood vessels, stromal cells, immune cells, and cancer cells [178]. For effective operation, these components require regulated communication via biological signals including soluble factors and exosomes. Exosomes are extracellular membrane-bound vesicles ranging from 40-150 nm that have been

linked with cancer progression and metastasis due to their ability to transport important oncoproteins that promote a suitable growth environment for cancer cells.

Due to the importance of exosomes in cancer [103-106], we chose to investigate curcumin's effects on this component of the tumor microenvironment. Exosomes were isolated via the sucrose cushion-ultracentrifugation technique from PANC-1 cells treated or untreated with curcumin. Exosome isolation was validated using acetylcholinesterase activity assays and nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) were used to determine particle size (NTA, DLS) and concentration (NTA). Results from these studies demonstrated that there was no significant difference between untreated and curcumin-treated conditions in terms of the size distribution or concentration of particles released from these cells. Using spectral studies, curcumin's presence was detected within exosomes, but not on the surface of the exosomes, providing valuable insight into the packaging of curcumin into exosomes from pancreatic cancer cells treated with curcumin. Further investigation of the functional role of curcumin within exosomes revealed that exosomal curcumin is delivered to recipient pancreatic cancer cells and retains its cytotoxic effects upon delivery. Exosomal curcumin delivery to recipient pancreatic cancer cells was demonstrated using fluorescence microscopy, while cellular outcomes were assessed using the AlamarBlue viability assay. Interestingly, a significant reduction in recipient PANC-1 cell viability was observed following exposure to exosomal curcumin; this effect was reversed with a blockade of exosomal binding to recipient cells using heparin.

The novelty and significance of these findings is highlighted by previous studies by our lab and others that demonstrate that tumor-derived exosomes carry oncoproteins

important for tumor progression, thus increasing viability and proliferation of recipient pancreatic cancer cells [101, 102]. These findings are supported by our results, which demonstrate that exosomes from untreated PANC-1 cells increase the viability of recipient PANC-1 cells. In contrast, however, our findings demonstrate that the presence of curcumin within tumor-derived exosomes is sufficient to reverse this pro-survival signaling, leading to a reduction in recipient cell viability. Finally, the essential role of exosomal delivery of curcumin in the reduction of recipient cell viability was demonstrated using heparin, an inhibitor of exosome to recipient cell binding. In the presence of heparin, the reduction of recipient cell viability was abolished, suggesting that the previously observed cytotoxic effects were dependent upon exosome binding and delivery into recipient cells.

In summary, the results presented in this thesis provide a significant contribution to pancreatic cancer research by elucidating curcumin's effects on IAP expression, thus addressing key mediators of chemoresistance, as well as exosome composition and function, expanding our knowledge of curcumin's multi-dimensional nature (Fig. 11 and Fig. 12). Collectively, these benefits pave the way for future research on multi-dimensional therapeutic approaches to pancreatic cancer including combinatorial strategies incorporating standard chemotherapies as well as natural compounds such as curcumin, offering the promise of overcoming resistance to chemotherapy and improving chemoresistant patient outcomes. Finally, these findings on curcumin's role in pancreatic cancer have the potential to be extended to other types of cancer, ultimately contributing to the eradication of cancer.

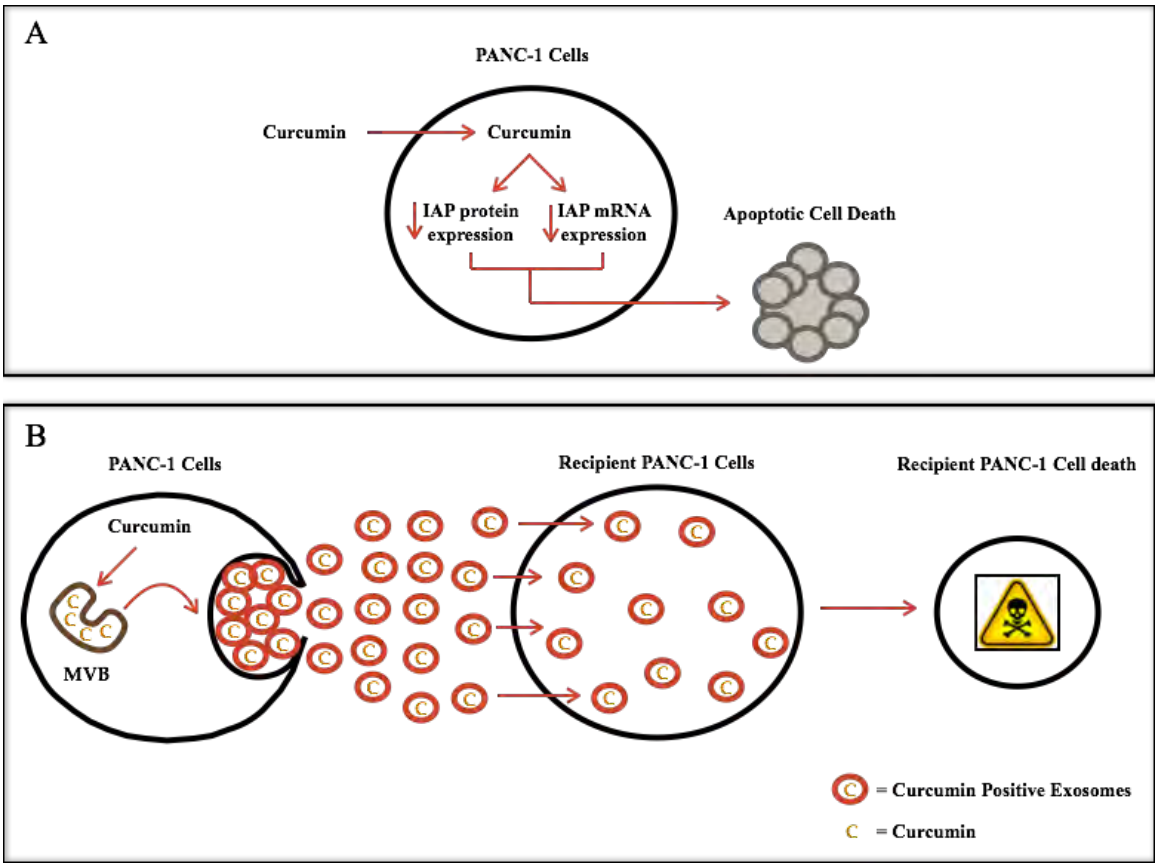

Figure 11. Curcumin's effects on IAP expression and exosomal function. (A) Curcumin enters PANC-1 cells and decreases the protein and mRNA expression of IAPs, culminating in apoptotic cell death. (B) Curcumin is packaged within exosomes and released from PANC-1 cells. These curcumin-containing exosomes induce cytotoxic effects in recipient PANC-1 cells. MVB = multi-vesicular bodies.

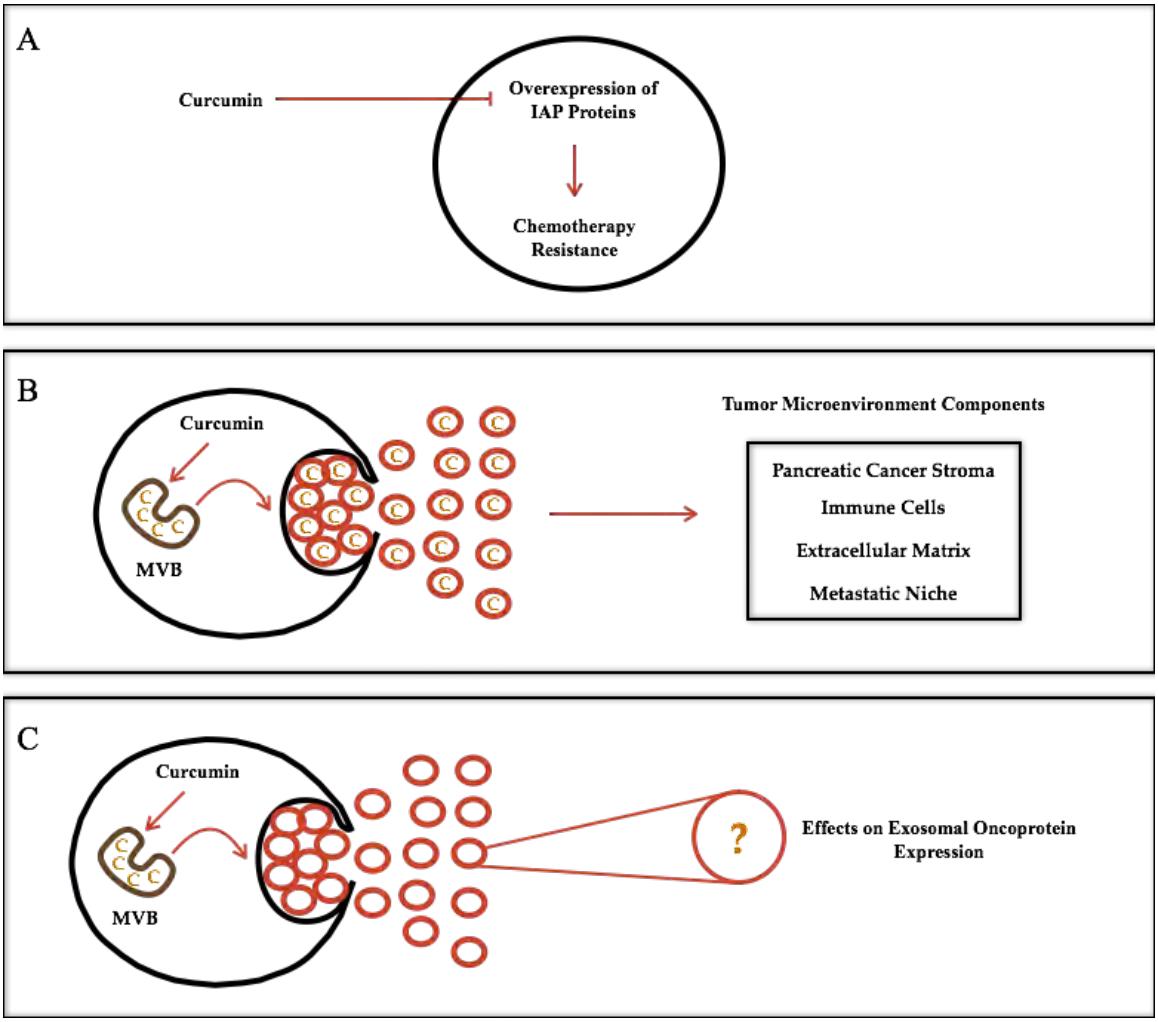

Figure 12. Possible roles of curcumin in chemoresistance, the modulation of the tumor microenvironment, and exosomal composition. (A) Curcumin has the potential to overcome pancreatic cancer chemotherapy resistance due to its capacity to decrease protein and mRNA expression of key mediators of resistance such as the IAPs. (B) Curcumin may have the ability to modulate different components of the tumor microenvironment via exosomal transport. (C) Curcumin's presence within pancreatic cancer cell-derived exosomes may interfere with the exosomal packaging of oncoproteins essential for metastatic niche formation. C = curcumin, MVB = multi-vesicular bodies.

REFERENCES

1. American Cancer Society. Cancer Facts & Figures 2015. Atlanta: American Cancer Society; 2015.

2. American Cancer Society. Cancer Facts & Figures 2013. Atlanta: American Cancer Society; 2013.

3. Huang ZQ, Saluja AK, Dudeja V, Vickers SM, Buchsbaum DJ. Molecular targeted approaches for treatment of pancreatic cancer. Current pharmaceutical design. 2011;17(21):2221-38.

4. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. The New England journal of medicine. 2014;371(22):2140-1.

5. Li HY, Cui ZM, Chen J, Guo XZ, Li YY. Pancreatic cancer: diagnosis and treatments. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015;36(3):1375-84.

6. Becker AE, Hernandez YG, Frucht H, Lucas AL. Pancreatic ductal adenocarcinoma: risk factors, screening, and early detection. World journal of gastroenterology : WJG. 2014;20(32):11182-98.

7. Loc WS, Smith JP, Matters G, Kester M, Adair JH. Novel strategies for managing pancreatic cancer. World journal of gastroenterology : WJG. 2014;20(40):14717-25.

8. Furuse J. [Current status and future directions of chemotherapy for pancreatic cancer]. Nihon Shokakibyo Gakkai zasshi = The Japanese journal of gastro-enterology. 2013;110(12):2060-5.

9. Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C, et al. Overcoming drug resistance in pancreatic cancer. Expert opinion on therapeutic targets. 2011;15(7):817-28.

10. Gupta SC, Sung B, Kim JH, Prasad S, Li S, Aggarwal BB. Multitargeting by turmeric, the golden spice: From kitchen to clinic. Molecular nutrition & food research. 2013;57(9):1510-28.

11. Ferrucci LM, Daniel CR, Kapur K, Chadha P, Shetty H, Graubard BI, et al. Measurement of spices and seasonings in India: opportunities for cancer epidemiology and prevention. Asian Pacific journal of cancer prevention : APJCP. 2010;11(6):1621-9.

12. Shehzad A, Lee J, Lee YS. Curcumin in various cancers. BioFactors (Oxford, England). 2013;39(1):56-68.

13. Kunnumakkara AB, Guha S, Krishnan S, Diagaradjane P, Gelovani J, Aggarwal BB. Curcumin potentiates antitumor activity of gemcitabine in an orthotopic model of pancreatic cancer through suppression of proliferation, angiogenesis, and inhibition of nuclear factor-kappaB-regulated gene products. Cancer research. 2007;67(8):3853-61.

14. Kanai M, Imaizumi A, Otsuka Y, Sasaki H, Hashiguchi M, Tsujiko K, et al. Dose-escalation and pharmacokinetic study of nanoparticle curcumin, a potential anticancer agent with improved bioavailability, in healthy human volunteers. Cancer chemotherapy and pharmacology. 2012;69(1):65-70.

15. Garcea G, Berry DP, Jones DJ, Singh R, Dennison AR, Farmer PB, et al. Consumption of the putative chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2005;14(1):120-5.

16. Kanai M, Yoshimura K, Asada M, Imaizumi A, Suzuki C, Matsumoto S, et al. A phase I/II study of gemcitabine-based chemotherapy plus curcumin for patients with gemcitabine-resistant pancreatic cancer. Cancer chemotherapy and pharmacology. 2011;68(1):157-64.

17. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(20):6847-54.

18. Friedman L, Lin L, Ball S, Bekaii-Saab T, Fuchs J, Li PK, et al. Curcumin analogues exhibit enhanced growth suppressive activity in human pancreatic cancer cells. Anti-cancer drugs. 2009;20(6):444-9.

19. Lee WH, Loo CY, Young PM, Traini D, Mason RS, Rohanizadeh R. Recent advances in curcumin nanoformulation for cancer therapy. Expert opinion on drug delivery. 2014;11(8):1183-201.

20. Ma D, Tremblay P, Mahngar K, Collins J, Hudlicky T, Pandey S. Selective cytotoxicity against human osteosarcoma cells by a novel synthetic C-1 analogue of 7-deoxypancratistatin is potentiated by curcumin. PloS one. 2011;6(12):e28780.

21. Dong Y, Yin S, Song X, Huo Y, Fan L, Ye M, et al. Involvement of ROS-p38-H2AX axis in novel curcumin analogues-induced apoptosis in breast cancer cells. Molecular carcinogenesis. 2015.

22. Terlikowska KM, Witkowska AM, Zujko ME, Dobrzycka B, Terlikowski SJ. Potential application of curcumin and its analogues in the treatment strategy of patients with primary epithelial ovarian cancer. International journal of molecular sciences. 2014;15(12):21703-22.

23. Xia YQ, Wei XY, Li WL, Kanchana K, Xu CC, Chen DH, et al. Curcumin analogue A501 induces G2/M arrest and apoptosis in non-small cell lung cancer cells. Asian Pacific journal of cancer prevention : APJCP. 2014;15(16):6893-8.

24. Zhou DY, Ding N, Van Doren J, Wei XC, Du ZY, Conney AH, et al. Effects of curcumin analogues for inhibiting human prostate cancer cells and the growth of human PC-3 prostate xenografts in immunodeficient mice. Biological & pharmaceutical bulletin. 2014;37(6):1029-34.

25. Alizadeh AM, Khaniki M, Azizian S, Mohaghgheghi MA, Sadeghizadeh M, Najafi F. Chemoprevention of azoxymethane-initiated colon cancer in rat by using a novel polymeric nanocarrier--curcumin. European journal of pharmacology. 2012;689(1-3):226-32.

26. Nagaraju GP, Zhu S, Ko JE, Ashritha N, Kandimalla R, Snyder JP, et al. Antiangiogenic effects of a novel synthetic curcumin analogue in pancreatic cancer. Cancer letters. 2015;357(2):557-65.

27. Shehzad A, Ul-Islam M, Wahid F, Lee YS. Multifunctional polymeric nanocurcumin for cancer therapy. Journal of nanoscience and nanotechnology. 2014;14(1):803-14.

28. Mach CM, Mathew L, Mosley SA, Kurzrock R, Smith JA. Determination of minimum effective dose and optimal dosing schedule for liposomal curcumin in a xenograft human pancreatic cancer model. Anticancer research. 2009;29(6):1895-9.

29. Vallianou NG, Evangelopoulos A, Schizas N, Kazazis C. Potential anticancer properties and mechanisms of action of curcumin. Anticancer research. 2015;35(2):645-51.

30. Shanmugam MK, Rane G, Kanchi MM, Arfuso F, Chinnathambi A, Zayed ME, et al. The multifaceted role of curcumin in cancer prevention and treatment. Molecules (Basel, Switzerland). 2015;20(2):2728-69.

31. Aggarwal B, Bhatt I, Ichikawa H, Ahn K, Sethi G, Sandur S, et al. Curcumin–biological and medicinal properties. Turmeric: the genus Curcuma. 2007;45.

32. Sahu RP, Batra S, Srivastava SK. Activation of ATM/Chk1 by curcumin causes cell cycle arrest and apoptosis in human pancreatic cancer cells. British journal of cancer. 2009;100(9):1425-33.

33. Dai Y, Lawrence TS, Xu L. Overcoming cancer therapy resistance by targeting inhibitors of apoptosis proteins and nuclear factor-kappa B. American journal of translational research. 2009;1(1):1-15.

34. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clinical cancer research : an

official journal of the American Association for Cancer Research. 2008;14(16):5000-5.

35. Kami K, Doi R, Koizumi M, Toyoda E, Mori T, Ito D, et al. Downregulation of survivin by siRNA diminishes radioresistance of pancreatic cancer cells. Surgery. 2005;138(2):299-305.

36. Shen X, Zheng JY, Shi H, Zhang Z, Wang WZ. Survivin knockdown enhances gastric cancer cell sensitivity to radiation and chemotherapy in vitro and in nude mice. The American journal of the medical sciences. 2012;344(1):52-8.

37. Dubrez L, Berthelet J, Glorian V. IAP proteins as targets for drug development in oncology. OncoTargets and therapy. 2013;9:1285-304.

38. Tanimoto T, Tsuda H, Imazeki N, Ohno Y, Imoto I, Inazawa J, et al. Nuclear expression of cIAP-1, an apoptosis inhibiting protein, predicts lymph node metastasis and poor patient prognosis in head and neck squamous cell carcinomas. Cancer letters. 2005;224(1):141-51.

39. Che X, Yang D, Zong H, Wang J, Li X, Chen F, et al. Nuclear cIAP1 overexpression is a tumor stage- and grade-independent predictor of poor prognosis in human bladder cancer patients. Urologic oncology. 2012;30(4):450-6.

40. Zhang Y, Zhu J, Tang Y, Li F, Zhou H, Peng B, et al. X-linked inhibitor of apoptosis positive nuclear labeling: a new independent prognostic biomarker of breast invasive ductal carcinoma. Diagnostic pathology. 2011;6:49.

41. Seligson DB, Hongo F, Huerta-Yepez S, Mizutani Y, Miki T, Yu H, et al. Expression of X-linked inhibitor of apoptosis protein is a strong predictor of human prostate cancer recurrence. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(20):6056-63.

42. Krajewska M, Krajewski S, Banares S, Huang X, Turner B, Bubendorf L, et al. Elevated expression of inhibitor of apoptosis proteins in prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(13):4914-25.

43. Ferreira CG, van der Valk P, Span SW, Jonker JM, Postmus PE, Kruyt FA, et al. Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2001;12(6):799-805.

44. Ferreira CG, van der Valk P, Span SW, Ludwig I, Smit EF, Kruyt FA, et al. Expression of X-linked inhibitor of apoptosis as a novel prognostic marker in radically resected non-small cell lung cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001;7(8):2468-74.

45. Asuncion Valenzuela MM, Castro I, Gonda A, Diaz Osterman CJ, Jutzy JM, Aspe JR, et al. Cell death in response to antimetabolites directed at ribonucleotide reductase and thymidylate synthase. OncoTargets and therapy. 2015;8:495-507.

46. Diaz Osterman CJ, Gonda A, Stiff TR, Sigaran U, Asuncion Valenzuela MM, Ferguson Bennit HR, et al. Curcumin induces pancreatic adenocarcinoma cell death via reduction of the inhibitors of apoptosis. Pancreas, In Press. 2015.

47. Glienke W, Maute L, Wicht J, Bergmann L. Curcumin inhibits constitutive STAT3 phosphorylation in human pancreatic cancer cell lines and downregulation of survivin/BIRC5 gene expression. Cancer investigation. 2010;28(2):166-71.

48. Bimonte S, Barbieri A, Palma G, Luciano A, Rea D, Arra C. Curcumin inhibits tumor growth and angiogenesis in an orthotopic mouse model of human pancreatic cancer. BioMed research international. 2013;2013:810423.

49. Srivastava R, Sharma N, Shankar S. STAT3 as an emerging molecular target in pancreatic cancer. Gastrointestinal Cancer: Targets and Therapy. 2014:115.

50. Zhang Z, Rigas B. NF-kappaB, inflammation and pancreatic carcinogenesis: NF-kappaB as a chemoprevention target (review). International journal of oncology. 2006;29(1):185-92.

51. Jutooru I, Chadalapaka G, Lei P, Safe S. Inhibition of NFkappaB and pancreatic cancer cell and tumor growth by curcumin is dependent on specificity protein down-regulation. The Journal of biological chemistry. 2010;285(33):25332-44.

52. Sun M, Estrov Z, Ji Y, Coombes KR, Harris DH, Kurzrock R. Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. Molecular cancer therapeutics. 2008;7(3):464-73.

53. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350-5.

54. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.

55. Pasquinelli AE, Hunter S, Bracht J. MicroRNAs: a developing story. Current opinion in genetics & development. 2005;15(2):200-5.

56. Harfe BD. MicroRNAs in vertebrate development. Current opinion in genetics & development. 2005;15(4):410-5.

57. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nature reviews Genetics. 2004;5(5):396-400.

58. Sun T, Kong X, Du Y. Aberrant MicroRNAs in Pancreatic Cancer: Researches and Clinical Implications. 2014;2014:386561.

59. Yu N, Huangyang P, Yang X, Han X, Yan R, Jia H, et al. microRNA-7 suppresses the invasive potential of breast cancer cells and sensitizes cells to DNA damages by targeting histone methyltransferase SET8. The Journal of biological chemistry. 2013;288(27):19633-42.

60. Ma J, Fang B, Zeng F, Pang H, Zhang J, Shi Y, et al. Curcumin inhibits cell growth and invasion through up-regulation of miR-7 in pancreatic cancer cells. Toxicology letters. 2014;231(1):82-91.

61. Kalinowski FC, Brown RA, Ganda C, Giles KM, Epis MR, Horsham J, et al. microRNA-7: a tumor suppressor miRNA with therapeutic potential. The international journal of biochemistry & cell biology. 2014;54:312-7.

62. Goo YH, Cooper TA. CUGBP2 directly interacts with U2 17S snRNP components and promotes U2 snRNA binding to cardiac troponin T pre-mRNA. Nucleic acids research. 2009;37(13):4275-86.

63. Anant S, Henderson JO, Mukhopadhyay D, Navaratnam N, Kennedy S, Min J, et al. Novel role for RNA-binding protein CUGBP2 in mammalian RNA editing. CUGBP2 modulates C to U editing of apolipoprotein B mRNA by interacting with apobec-1 and ACF, the apobec-1 complementation factor. The Journal of biological chemistry. 2001;276(50):47338-51.

64. Subramaniam D, Natarajan G, Ramalingam S, Ramachandran I, May R, Queimado L, et al. Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2. American journal of physiology Gastrointestinal and liver physiology. 2008;294(4):G1025-32.

65. Ramalingam S, Ramamoorthy P, Subramaniam D, Anant S. Reduced Expression of RNA Binding Protein CELF2, a Putative Tumor Suppressor Gene in Colon Cancer. Immuno-gastroenterology. 2012;1(1):27-33.

66. Subramaniam D, Ramalingam S, Linehan DC, Dieckgraefe BK, Postier RG, Houchen CW, et al. RNA binding protein CUGBP2/CELF2 mediates curcumin-induced mitotic catastrophe of pancreatic cancer cells. PloS one. 2011;6(2):e16958.

67. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nature reviews Drug discovery. 2006;5(3):219-34.

68. Li Y, Revalde JL, Reid G, Paxton JW. Modulatory effects of curcumin on multi-drug resistance-associated protein 5 in pancreatic cancer cells. Cancer chemotherapy and pharmacology. 2011;68(3):603-10.

69. Heger M, van Golen RF, Broekgaarden M, Michel MC. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. Pharmacological reviews. 2014;66(1):222-307.

70. Prasad S, Tyagi AK, Aggarwal BB. Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer research and treatment : official journal of Korean Cancer Association. 2014;46(1):2-18.

71. Grundker C, Gunthert AR, Westphalen S, Emons G. Biology of the gonadotropin-releasing hormone system in gynecological cancers. European journal of endocrinology / European Federation of Endocrine Societies. 2002;146(1):1-14.

72. Emons G, Ortmann O, Schulz KD, Schally AV. Growth-inhibitory actions of analogues of Luteinizing Hormone Releasing Hormone on tumor cells. Trends in endocrinology and metabolism: TEM. 1997;8(9):355-62.

73. Aggarwal S, Ndinguri MW, Solipuram R, Wakamatsu N, Hammer RP, Ingram D, et al. [DLys(6)]-luteinizing hormone releasing hormone-curcumin conjugate inhibits pancreatic cancer cell growth in vitro and in vivo. International journal of cancer Journal international du cancer. 2011;129(7):1611-23.

74. Liebow C, Lee MT, Kamer AR, Schally AV. Regulation of luteinizing hormone-releasing hormone receptor binding by heterologous and autologous receptor-stimulated tyrosine phosphorylation. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(6):2244-8.

75. Li J, Wang Y, Yang C, Wang P, Oelschlager DK, Zheng Y, et al. Polyethylene glycosylated curcumin conjugate inhibits pancreatic cancer cell growth through inactivation of Jab1. Molecular pharmacology. 2009;76(1):81-90.

76. Fukumoto A, Tomoda K, Yoneda-Kato N, Nakajima Y, Kato JY. Depletion of Jab1 inhibits proliferation of pancreatic cancer cell lines. FEBS letters. 2006;580(25):5836-44.

77. Shackleford TJ, Claret FX. JAB1/CSN5: a new player in cell cycle control and cancer. Cell division. 2010;5:26.

78. Lin L, Hutzen B, Zuo M, Ball S, Deangelis S, Foust E, et al. Novel STAT3 phosphorylation inhibitors exhibit potent growth-suppressive activity in pancreatic and breast cancer cells. Cancer research. 2010;70(6):2445-54.

79. Hutzen B, Friedman L, Sobo M, Lin L, Cen L, De Angelis S, et al. Curcumin analogue GO-Y030 inhibits STAT3 activity and cell growth in breast and pancreatic carcinomas. International journal of oncology. 2009;35(4):867-72.

80. Nagaraju GP, Zhu S, Wen J, Farris AB, Adsay VN, Diaz R, et al. Novel synthetic curcumin analogues EF31 and UBS109 are potent DNA hypomethylating agents in pancreatic cancer. Cancer letters. 2013;341(2):195-203.

81. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through

modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer research. 2010;70(9):3606-17.

82. Bao B, Ali S, Kong D, Sarkar SH, Wang Z, Banerjee S, et al. Anti-tumor activity of a novel compound-CDF is mediated by regulating miR-21, miR-200, and PTEN in pancreatic cancer. PloS one. 2011;6(3):e17850.

83. Li Y, Vandenboom TG, 2nd, Wang Z, Kong D, Ali S, Philip PA, et al. miR-146a suppresses invasion of pancreatic cancer cells. Cancer research. 2010;70(4):1486-95.

84. Ali S, Ahmad A, Aboukameel A, Ahmed A, Bao B, Banerjee S, et al. Deregulation of miR-146a expression in a mouse model of pancreatic cancer affecting EGFR signaling. Cancer letters. 2014;351(1):134-42.

85. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133(2):647-58.

86. Bunney TD, Katan M. Phosphoinositide signalling in cancer: beyond PI3K and PTEN. Nature reviews Cancer. 2010;10(5):342-52.

87. Soubani O, Ali AS, Logna F, Ali S, Philip PA, Sarkar FH. Re-expression of miR-200 by novel approaches regulates the expression of PTEN and MT1-MMP in pancreatic cancer. Carcinogenesis. 2012;33(8):1563-71.

88. Panarelli NC, Chen YT, Zhou XK, Kitabayashi N, Yantiss RK. MicroRNA expression aids the preoperative diagnosis of pancreatic ductal adenocarcinoma. Pancreas. 2012;41(5):685-90.

89. Sarkar S, Dubaybo H, Ali S, Goncalves P, Kollepara SL, Sethi S, et al. Down-regulation of miR-221 inhibits proliferation of pancreatic cancer cells through up-regulation of PTEN, p27(kip1), p57(kip2), and PUMA. American journal of cancer research. 2013;3(5):465-77.

90. Ali S, Ahmad A, Aboukameel A, Bao B, Padhye S, Philip PA, et al. Increased Ras GTPase activity is regulated by miRNAs that can be attenuated by CDF treatment in pancreatic cancer cells. Cancer letters. 2012;319(2):173-81.

91. Li L, Braiteh FS, Kurzrock R. Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis. Cancer. 2005;104(6):1322-31.

92. Sutaria D, Grandhi BK, Thakkar A, Wang J, Prabhu S. Chemoprevention of pancreatic cancer using solid-lipid nanoparticulate delivery of a novel aspirin, curcumin and sulforaphane drug combination regimen. International journal of oncology. 2012;41(6):2260-8.

93. Dandawate PR, Vyas A, Ahmad A, Banerjee S, Deshpande J, Swamy KV, et al. Inclusion complex of novel curcumin analogue CDF and beta-cyclodextrin (1:2) and its enhanced in vivo anticancer activity against pancreatic cancer. Pharmaceutical research. 2012;29(7):1775-86.

94. Zhang F, Koh GY, Jeansonne DP, Hollingsworth J, Russo PS, Vicente G, et al. A novel solubility-enhanced curcumin formulation showing stability and maintenance of anticancer activity. Journal of pharmaceutical sciences. 2011;100(7):2778-89.

95. Bisht S, Feldmann G, Soni S, Ravi R, Karikar C, Maitra A, et al. Polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"): a novel strategy for human cancer therapy. Journal of nanobiotechnology. 2007;5:3.

96. Bisht S, Mizuma M, Feldmann G, Ottenhof NA, Hong SM, Pramanik D, et al. Systemic administration of polymeric nanoparticle-encapsulated curcumin (NanoCurc) blocks tumor growth and metastases in preclinical models of pancreatic cancer. Molecular cancer therapeutics. 2010;9(8):2255-64.

97. Yallapu MM, Ebeling MC, Khan S, Sundram V, Chauhan N, Gupta BK, et al. Novel curcumin-loaded magnetic nanoparticles for pancreatic cancer treatment. Molecular cancer therapeutics. 2013;12(8):1471-80.

98. Wei X, Senanayake TH, Bohling A, Vinogradov SV. Targeted nanogel conjugate for improved stability and cellular permeability of curcumin: synthesis, pharmacokinetics, and tumor growth inhibition. Molecular pharmaceutics. 2014;11(9):3112-22.

99. Sasaki H, Sunagawa Y, Takahashi K, Imaizumi A, Fukuda H, Hashimoto T, et al. Innovative preparation of curcumin for improved oral bioavailability. Biological & pharmaceutical bulletin. 2011;34(5):660-5.

100. Kanai M. Therapeutic applications of curcumin for patients with pancreatic cancer. World journal of gastroenterology : WJG. 2014;20(28):9384-91.

101. 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. British journal of cancer. 2009;100(7):1073-86.

102. Khan S, Jutzy JM, Aspe JR, McGregor DW, Neidigh JW, Wall NR. Survivin is released from cancer cells via exosomes. Apoptosis : an international journal on programmed cell death. 2011;16(1):1-12.

103. Taylor DD, Black PH. Shedding of plasma membrane fragments. Neoplastic and developmental importance. Developmental biology (New York, NY : 1985). 1986;3:33-57.

104. Taylor DD, Lyons KS, Gercel-Taylor C. Shed membrane fragment-associated markers for endometrial and ovarian cancers. Gynecologic oncology. 2002;84(3):443-8.

105. Atay S, Godwin AK. Tumor-derived exosomes: A message delivery system for tumor progression. Communicative & integrative biology. 2014;7(1):e28231.

106. Kharaziha P, Ceder S, Li Q, Panaretakis T. Tumor cell-derived exosomes: a message in a bottle. Biochimica et biophysica acta. 2012;1826(1):103-11.

107. Aspe JR, Diaz Osterman CJ, Jutzy JM, Deshields S, Whang S, Wall NR. Enhancement of Gemcitabine sensitivity in pancreatic adenocarcinoma by novel exosome-mediated delivery of the Survivin-T34A mutant. Journal of extracellular vesicles. 2014;3.

108. Diaz Osterman CJ, Lynch JC, Leaf P, Griffiths D, Wall NR. Curcumin modulates pancreatic adenocarcinoma cell-derived exosomal function. PLoS One, In Press. 2015.

109. Kanai M, Otsuka Y, Otsuka K, Sato M, Nishimura T, Mori Y, et al. A phase I study investigating the safety and pharmacokinetics of highly bioavailable curcumin (Theracurmin) in cancer patients. Cancer chemotherapy and pharmacology. 2013;71(6):1521-30.

110. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, et al. Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2008;17(6):1411-7.

111. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer research. 2001;21(4b):2895-900.

112. Dhillon N, Aggarwal BB, Newman RA, Wolff RA, Kunnumakkara AB, Abbruzzese JL, et al. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(14):4491-9.

113. Epelbaum R, Schaffer M, Vizel B, Badmaev V, Bar-Sela G. Curcumin and gemcitabine in patients with advanced pancreatic cancer. Nutrition and cancer. 2010;62(8):1137-41.

114. Muller MW, Friess H, Koninger J, Martin D, Wente MN, Hinz U, et al. Factors influencing survival after bypass procedures in patients with advanced pancreatic adenocarcinomas. American journal of surgery. 2008;195(2):221-8.

115. Alexakis N, Halloran C, Raraty M, Ghaneh P, Sutton R, Neoptolemos JP. Current standards of surgery for pancreatic cancer. The British journal of surgery. 2004;91(11):1410-27.

116. Kim R. FOLFIRINOX: a new standard treatment for advanced pancreatic cancer? The Lancet Oncology. 2011;12(1):8-9.

117. Li J, Wientjes MG, Au JL. Pancreatic cancer: pathobiology, treatment options, and drug delivery. The AAPS journal. 2010;12(2):223-32.

118. Ueno H, Kiyosawa K, Kaniwa N. Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? British journal of cancer. 2007;97(2):145-51.

119. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. Oncogene. 2008;27(48):6252-75.

120. Saleem M, Qadir MI, Perveen N, Ahmad B, Saleem U, Irshad T, et al. Inhibitors of apoptotic proteins: new targets for anticancer therapy. Chemical biology & drug design. 2013;82(3):243-51.

121. Oberoi-Khanuja TK, Murali A, Rajalingam K. IAPs on the move: role of inhibitors of apoptosis proteins in cell migration. Cell death & disease. 2013;4:e784.

122. Liu JL, Wang Y, Jiang J, Kong R, Yang YM, Ji HF, et al. Inhibition of survivin expression and mechanisms of reversing drug-resistance of human lung adenocarcinoma cells by siRNA. Chinese medical journal. 2010;123(20):2901-7.

123. He SQ, Rehman H, Gong MG, Zhao YZ, Huang ZY, Li CH, et al. Inhibiting survivin expression enhances TRAIL-induced tumoricidal activity in human hepatocellular carcinoma via cell cycle arrest. Cancer biology & therapy. 2007;6(8):1247-57.

124. Rodel F, Frey B, Leitmann W, Capalbo G, Weiss C, Rodel C. Survivin antisense oligonucleotides effectively radiosensitize colorectal cancer cells in both tissue culture and murine xenograft models. International journal of radiation oncology, biology, physics. 2008;71(1):247-55.

125. Mahadevan D, Chalasani P, Rensvold D, Kurtin S, Pretzinger C, Jolivet J, et al. Phase I trial of AEG35156 an antisense oligonucleotide to XIAP plus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma. American journal of clinical oncology. 2013;36(3):239-43.

126. Woo JH, Kim YH, Choi YJ, Kim DG, Lee KS, Bae JH, et al. Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. Carcinogenesis. 2003;24(7):1199-208.

127. Park S, Cho DH, Andera L, Suh N, Kim I. Curcumin enhances TRAIL-induced apoptosis of breast cancer cells by regulating apoptosis-related proteins. Molecular and cellular biochemistry. 2013;383(1-2):39-48.

128. Reuter S, Eifes S, Dicato M, Aggarwal BB, Diederich M. Modulation of anti-apoptotic and survival pathways by curcumin as a strategy to induce apoptosis in cancer cells. Biochemical pharmacology. 2008;76(11):1340-51.

129. Wang JB, Qi LL, Zheng SD, Wu TX. Curcumin induces apoptosis through the mitochondria-mediated apoptotic pathway in HT-29 cells. Journal of Zhejiang University Science B. 2009;10(2):93-102.

130. Shankar S, Chen Q, Sarva K, Siddiqui I, Srivastava RK. Curcumin enhances the apoptosis-inducing potential of TRAIL in prostate cancer cells: molecular mechanisms of apoptosis, migration and angiogenesis. Journal of molecular signaling. 2007;2:10.

131. Ravindran J, Prasad S, Aggarwal BB. Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? The AAPS journal. 2009;11(3):495-510.

132. Aggarwal S, Ichikawa H, Takada Y, Sandur SK, Shishodia S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IkappaBalpha kinase and Akt activation. Molecular pharmacology. 2006;69(1):195-206.

133. Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D'Alessandro N. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB activation levels and in IAP gene expression. Cancer letters. 2005;224(1):53-65.

134. Lev-Ari S, Vexler A, Starr A, Ashkenazy-Voghera M, Greif J, Aderka D, et al. Curcumin augments gemcitabine cytotoxic effect on pancreatic adenocarcinoma cell lines. Cancer investigation. 2007;25(6):411-8.

135. Ramachandran C, Resek AP, Escalon E, Aviram A, Melnick SJ. Potentiation of gemcitabine by Turmeric Force in pancreatic cancer cell lines. Oncology reports. 2010;23(6):1529-35.

136. Gupta SC, Patchva S, Aggarwal BB. Therapeutic roles of curcumin: lessons learned from clinical trials. The AAPS journal. 2013;15(1):195-218.

137. Shehzad A, Wahid F, Lee YS. Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. Archiv der Pharmazie. 2010;343(9):489-99.

138. Kunwar A, Barik A, Pandey R, Priyadarsini KI. Transport of liposomal and albumin loaded curcumin to living cells: an absorption and fluorescence spectroscopic study. Biochimica et biophysica acta. 2006;1760(10):1513-20.

139. Kunwar A, Barik A, Mishra B, Rathinasamy K, Pandey R, Priyadarsini KI. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. Biochimica et biophysica acta. 2008;1780(4):673-9.

140. Nardo L, Andreoni A, Masson M, Haukvik T, Tonnesen HH. Studies on curcumin and curcuminoids. XXXIX. Photophysical properties of bisdemethoxycurcumin. Journal of fluorescence. 2011;21(2):627-35.

141. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

142. Rebucci M, Michiels C. Molecular aspects of cancer cell resistance to chemotherapy. Biochemical pharmacology. 2013;85(9):1219-26.

143. Owens TW, Gilmore AP, Streuli CH, Foster FM. Inhibitor of Apoptosis Proteins: Promising Targets for Cancer Therapy. Journal of carcinogenesis & mutagenesis. 2013;Suppl 14.

144. Fulda S. Targeting apoptosis signaling in pancreatic cancer. Cancers. 2011;3(1):241-51.

145. Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, et al. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell proliferation. 2012;45(6):487-98.

146. Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO reports. 2006;7(10):988-94.

147. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer research. 1998;58(23):5315-20.

148. Marusawa H, Matsuzawa S, Welsh K, Zou H, Armstrong R, Tamm I, et al. HBXIP functions as a cofactor of survivin in apoptosis suppression. The EMBO journal. 2003;22(11):2729-40.

149. Choi YE, Butterworth M, Malladi S, Duckett CS, Cohen GM, Bratton SB. The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. The Journal of biological chemistry. 2009;284(19):12772-82.

150. Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes

in vitro monoubiquitination of caspases 3 and 7. The Journal of biological chemistry. 2000;275(35):26661-4.

151. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology. 2010;11(10):700-14.

152. Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 1999;5(1):119-27.

153. Liptay S, Weber CK, Ludwig L, Wagner M, Adler G, Schmid RM. Mitogenic and antiapoptotic role of constitutive NF-kappaB/Rel activity in pancreatic cancer. International journal of cancer Journal international du cancer. 2003;105(6):735-46.

154. Aggarwal BB. Nuclear factor-kappaB: the enemy within. Cancer cell. 2004;6(3):203-8.

155. Fujioka S, Sclabas GM, Schmidt C, Frederick WA, Dong QG, Abbruzzese JL, et al. Function of nuclear factor kappaB in pancreatic cancer metastasis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9(1):346-54.

156. Xiong HQ, Abbruzzese JL, Lin E, Wang L, Zheng L, Xie K. NF-kappaB activity blockade impairs the angiogenic potential of human pancreatic cancer cells. International journal of cancer Journal international du cancer. 2004;108(2):181-8.

157. Greten FR, Weber CK, Greten TF, Schneider G, Wagner M, Adler G, et al. Stat3 and NF-kappaB activation prevents apoptosis in pancreatic carcinogenesis. Gastroenterology. 2002;123(6):2052-63.

158. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, et al. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene. 2003;22(21):3243-51.

159. 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):782-90.

160. Guo HC, Bu HQ, Luo J, Wei WT, Liu DL, Chen H, et al. Emodin potentiates the antitumor effects of gemcitabine in PANC-1 pancreatic cancer xenograft model in vivo via inhibition of inhibitors of apoptosis. International journal of oncology. 2012;40(6):1849-57.

161. Cao LP, Song JL, Yi XP, Li YX. Double inhibition of NF-kappaB and XIAP via RNAi enhances the sensitivity of pancreatic cancer cells to gemcitabine. Oncology reports. 2013;29(4):1659-65.

162. Bandala E, Espinosa M, Maldonado V, Melendez-Zajgla J. Inhibitor of apoptosis-1 (IAP-1) expression and apoptosis in non-small-cell lung cancer cells exposed to gemcitabine. Biochemical pharmacology. 2001;62(1):13-9.

163. Pan X, Arumugam T, Yamamoto T, Levin PA, Ramachandran V, Ji B, et al. Nuclear factor-kappaB p65/relA silencing induces apoptosis and increases gemcitabine effectiveness in a subset of pancreatic cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(24):8143-51.

164. Kong R, Sun B, Jiang H, Pan S, Chen H, Wang S, et al. Downregulation of nuclear factor-kappaB p65 subunit by small interfering RNA synergizes with gemcitabine to inhibit the growth of pancreatic cancer. Cancer letters. 2010;291(1):90-8.

165. Shrikhande SV, Kleeff J, Kayed H, Keleg S, Reiser C, Giese T, et al. Silencing of X-linked inhibitor of apoptosis (XIAP) decreases gemcitabine resistance of pancreatic cancer cells. Anticancer research. 2006;26(5a):3265-73.

166. Liu WS, Yan HJ, Qin RY, Tian R, Wang M, Jiang JX, et al. siRNA directed against survivin enhances pancreatic cancer cell gemcitabine chemosensitivity. Digestive diseases and sciences. 2009;54(1):89-96.

167. Dineen SP, Roland CL, Greer R, Carbon JG, Toombs JE, Gupta P, et al. Smac mimetic increases chemotherapy response and improves survival in mice with pancreatic cancer. Cancer research. 2010;70(7):2852-61.

168. Youns M, Fathy GM. Upregulation of extrinsic apoptotic pathway in curcumin-mediated antiproliferative effect on human pancreatic carcinogenesis. Journal of cellular biochemistry. 2013;114(12):2654-65.

169. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer. 2006;106(11):2503-13.

170. Lev-Ari S, Zinger H, Kazanov D, Yona D, Ben-Yosef R, Starr A, et al. Curcumin synergistically potentiates the growth inhibitory and pro-apoptotic effects of celecoxib in pancreatic adenocarcinoma cells. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2005;59 Suppl 2:S276-80.

171. Lev-Ari S, Starr A, Vexler A, Karaush V, Loew V, Greif J, et al. Inhibition of pancreatic and lung adenocarcinoma cell survival by curcumin is associated with increased apoptosis, down-regulation of COX-2 and EGFR and inhibition of Erk1/2 activity. Anticancer research. 2006;26(6b):4423-30.

172. Prakobwong S, Gupta SC, Kim JH, Sung B, Pinlaor P, Hiraku Y, et al. Curcumin suppresses proliferation and induces apoptosis in human biliary cancer cells through modulation of multiple cell signaling pathways. Carcinogenesis. 2011;32(9):1372-80.

173. Gupta SC, Prasad S, Kim JH, Patchva S, Webb LJ, Priyadarsini IK, et al. Multitargeting by curcumin as revealed by molecular interaction studies. Natural product reports. 2011;28(12):1937-55.

174. Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. Multiple biological activities of curcumin: a short review. Life sciences. 2006;78(18):2081-7.

175. Shehzad A, Lee YS. Molecular mechanisms of curcumin action: signal transduction. BioFactors (Oxford, England). 2013;39(1):27-36.

176. Dohi T, Okada K, Xia F, Wilford CE, Samuel T, Welsh K, et al. An IAP-IAP complex inhibits apoptosis. The Journal of biological chemistry. 2004;279(33):34087-90.

177. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: a cancer journal for clinicians. 2014;64(1):9-29.

178. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012;18(16):4266-76.

179. Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor-kappaB and IkappaB kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. Cancer. 2004;101(10):2351-62.

180. Tomita M, Kawakami H, Uchihara JN, Okudaira T, Masuda M, Takasu N, et al. Curcumin (diferuloylmethane) inhibits constitutive active NF-kappaB, leading to suppression of cell growth of human T-cell leukemia virus type I-infected T-cell lines and primary adult T-cell leukemia cells. International journal of cancer Journal international du cancer. 2006;118(3):765-72.

181. Lin YG, Kunnumakkara AB, Nair A, Merritt WM, Han LY, Armaiz-Pena GN, et al. Curcumin inhibits tumor growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-kappaB pathway. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(11):3423-30.

182. Howells LM, Sale S, Sriramareddy SN, Irving GR, Jones DJ, Ottley CJ, et al. Curcumin ameliorates oxaliplatin-induced chemoresistance in HCT116 colorectal cancer cells in vitro and in vivo. International journal of cancer Journal international du cancer. 2011;129(2):476-86.

183. Yu LL, Wu JG, Dai N, Yu HG, Si JM. Curcumin reverses chemoresistance of human gastric cancer cells by downregulating the NF-kappaB transcription factor. Oncology reports. 2011;26(5):1197-203.

184. Yang CL, Liu YY, Ma YG, Xue YX, Liu DG, Ren Y, et al. Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through Janus kinase-STAT3 signalling pathway. PloS one. 2012;7(5):e37960.

185. Kunnumakkara AB, Diagaradjane P, Guha S, Deorukhkar A, Shentu S, Aggarwal BB, et al. Curcumin sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting nuclear factor-kappaB-regulated gene products. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(7):2128-36.

186. Milacic V, Banerjee S, Landis-Piwowar KR, Sarkar FH, Majumdar AP, Dou QP. Curcumin inhibits the proteasome activity in human colon cancer cells in vitro and in vivo. Cancer research. 2008;68(18):7283-92.

187. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutation research. 2001;480-481:243-68.

188. Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD, Conney AH. Inhibitory effects of curcumin on in vitro lipoxygenase and cyclooxygenase activities in mouse epidermis. Cancer research. 1991;51(3):813-9.

189. Cho JW, Lee KS, Kim CW. Curcumin attenuates the expression of IL-1beta, IL-6, and TNF-alpha as well as cyclin E in TNF-alpha-treated HaCaT cells; NF-kappaB and MAPKs as potential upstream targets. International journal of molecular medicine. 2007;19(3):469-74.

190. Liu JY, Lin SJ, Lin JK. Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. Carcinogenesis. 1993;14(5):857-61.

191. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Alternative medicine review : a journal of clinical therapeutic. 2009;14(2):141-53.

192. Liu A, Lou H, Zhao L, Fan P. Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. Journal of pharmaceutical and biomedical analysis. 2006;40(3):720-7.

193. Marczylo TH, Verschoyle RD, Cooke DN, Morazzoni P, Steward WP, Gescher AJ. Comparison of systemic availability of curcumin with that of curcumin formulated with phosphatidylcholine. Cancer chemotherapy and pharmacology. 2007;60(2):171-7.

194. Antony B, Merina B, Iyer VS, Judy N, Lennertz K, Joyal S. A Pilot Cross-Over Study to Evaluate Human Oral Bioavailability of BCM-95CG (Biocurcumax), A

Novel Bioenhanced Preparation of Curcumin. Indian journal of pharmaceutical sciences. 2008;70(4):445-9.

195. Sahu A, Bora U, Kasoju N, Goswami P. Synthesis of novel biodegradable and self-assembling methoxy poly(ethylene glycol)-palmitate nanocarrier for curcumin delivery to cancer cells. Acta biomaterialia. 2008;4(6):1752-61.

196. Sou K, Inenaga S, Takeoka S, Tsuchida E. Loading of curcumin into macrophages using lipid-based nanoparticles. International journal of pharmaceutics. 2008;352(1-2):287-93.

197. Gupta V, Aseh A, Rios CN, Aggarwal BB, Mathur AB. Fabrication and characterization of silk fibroin-derived curcumin nanoparticles for cancer therapy. International journal of nanomedicine. 2009;4:115-22.

198. Mukerjee A, Vishwanatha JK. Formulation, characterization and evaluation of curcumin-loaded PLGA nanospheres for cancer therapy. Anticancer research. 2009;29(10):3867-75.

199. Shaikh J, Ankola DD, Beniwal V, Singh D, Kumar MN. Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9-fold when compared to curcumin administered with piperine as absorption enhancer. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2009;37(3-4):223-30.

200. Takahashi M, Uechi S, Takara K, Asikin Y, Wada K. Evaluation of an oral carrier system in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. Journal of agricultural and food chemistry. 2009;57(19):9141-6.

201. Anand P, Nair HB, Sung B, Kunnumakkara AB, Yadav VR, Tekmal RR, et al. Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. Biochemical pharmacology. 2010;79(3):330-8.

202. Das RK, Kasoju N, Bora U. Encapsulation of curcumin in alginate-chitosan-pluronic composite nanoparticles for delivery to cancer cells. Nanomedicine : nanotechnology, biology, and medicine. 2010;6(1):153-60.

203. Koppolu B, Rahimi M, Nattama S, Wadajkar A, Nguyen KT. Development of multiple-layer polymeric particles for targeted and controlled drug delivery. Nanomedicine : nanotechnology, biology, and medicine. 2010;6(2):355-61.

204. Mosley CA, Liotta DC, Snyder JP. Highly active anticancer curcumin analogues. Advances in experimental medicine and biology. 2007;595:77-103.

205. Sato A, Kudo C, Yamakoshi H, Uehara Y, Ohori H, Ishioka C, et al. Curcumin analog GO-Y030 is a novel inhibitor of IKKbeta that suppresses NF-kappaB signaling and induces apoptosis. Cancer science. 2011;102(5):1045-51.

206. Ghalandarlaki N, Alizadeh AM. Nanotechnology-applied curcumin for different diseases therapy. 2014;2014:394264.

207. Shi HS, Gao X, Li D, Zhang QW, Wang YS, Zheng Y, et al. A systemic administration of liposomal curcumin inhibits radiation pneumonitis and sensitizes lung carcinoma to radiation. International journal of nanomedicine. 2012;7:2601-11.

208. Matabudul D, Pucaj K, Bolger G, Vcelar B, Majeed M, Helson L. Tissue distribution of (Lipocurc) liposomal curcumin and tetrahydrocurcumin following two- and eight-hour infusions in Beagle dogs. Anticancer research. 2012;32(10):4359-64.

209. Ranjan AP, Mukerjee A, Helson L, Gupta R, Vishwanatha JK. Efficacy of liposomal curcumin in a human pancreatic tumor xenograft model: inhibition of tumor growth and angiogenesis. Anticancer research. 2013;33(9):3603-9.

210. Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. Molecular therapy : the journal of the American Society of Gene Therapy. 2010;18(9):1606-14.

211. Savina A, Furlan M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. The Journal of biological chemistry. 2003;278(22):20083-90.

212. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current protocols in cell biology / editorial board, Juan S Bonifacino [et al]. 2006;Chapter 3:Unit 3.22.

213. Lancaster GI, Febbraio MA. Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. The Journal of biological chemistry. 2005;280(24):23349-55.

214. Franzen CA, Simms PE, Van Huis AF, Foreman KE, Kuo PC, Gupta GN. Characterization of uptake and internalization of exosomes by bladder cancer cells. BioMed research international. 2014;2014:619829.

215. Christianson HC, Svensson KJ, van Kuppevelt TH, Li JP, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(43):17380-5.

216. Atai NA, Balaj L, van Veen H, Breakefield XO, Jarzyna PA, Van Noorden CJ, et al. Heparin blocks transfer of extracellular vesicles between donor and recipient cells. Journal of neuro-oncology. 2013;115(3):343-51.

217. Rieu S, Geminard C, Rabesandratana H, Sainte-Marie J, Vidal M. Exosomes released during reticulocyte maturation bind to fibronectin via integrin alpha4beta1. European journal of biochemistry / FEBS. 2000;267(2):583-90.

218. van der Meel R, Fens MH, Vader P, van Solinge WW, Eniola-Adefeso O, Schiffelers RM. Extracellular vesicles as drug delivery systems: lessons from the liposome field. Journal of controlled release : official journal of the Controlled Release Society. 2014;195:72-85.

219. Neoptolemos JP. Adjuvant treatment of pancreatic cancer. European Journal of Cancer. 2011;47:S378-S80.

220. Demory Beckler M, Higginbotham JN, Franklin JL, Ham AJ, Halvey PJ, Imasuen IE, et al. Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies intercellular transfer of mutant KRAS. Molecular & cellular proteomics : MCP. 2013;12(2):343-55.

221. Martinez-Lorenzo MJ, Anel A, Alava MA, Pineiro A, Naval J, Lasierra P, et al. The human melanoma cell line MelJuSo secretes bioactive FasL and APO2L/TRAIL on the surface of microvesicles. Possible contribution to tumor counterattack. Experimental cell research. 2004;295(2):315-29.

222. Rana S, Malinowska K, Zoller M. Exosomal tumor microRNA modulates premetastatic organ cells. Neoplasia (New York, NY). 2013;15(3):281-95.

223. Jung T, Castellana D, Klingbeil P, Cuesta Hernandez I, Vitacolonna M, Orlicky DJ, et al. CD44v6 dependence of premetastatic niche preparation by exosomes. Neoplasia (New York, NY). 2009;11(10):1093-105.

224. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Molecular pharmaceutics. 2007;4(6):807-18.

APPENDIX A

FUTURE DIRECTIONS

Part 1: To determine the mechanism of action by which curcumin induces caspase-independent apoptosis

Currently, therapy for pancreatic cancer consists of anti-metabolite drugs (Gemcitabine), topoisomerase inhibitors (Irinotecan) and mitotic spindle stabilizers (Paclitaxel). While these agents target different intracellular processes, all converge upon induction of cell death by apoptosis, leading to the activation of caspases (cysteine-asparic proteases) that cause endonuclease-mediated fragmentation of DNA and cellular demise. Pre-clinical and clinical studies have extensively demonstrated that pancreatic cancer cells acquire resistance to these therapies by up-regulating anti-apoptotic proteins, such as the inhibitor of apoptosis (IAP) protein family, that prevent the activation of caspases. Based on this thesis, curcumin effectively reduces the protein and mRNA expression of the IAPs. However, IAP protein function is closely related to the inhibition of caspase-dependent apoptosis; it is still unclear how curcumin may affect caspase-independent apoptotic cell death to prevent cancer propagation. Hence, it is imperative to conduct experiments to investigate curcumin's capabilities to induce caspase-independent cell death in the event that caspase-dependent cell death is circumvented by pancreatic cancer cells. Since circumvention of caspase-dependent cell death is a common event in the development of chemotherapy resistance, these studies will address one of the crucial obstacles to the eradication of pancreatic cancer, which is the persistent resistance to therapy. The objective of this study is to determine curcumin's effects on intracellular proteins crucial for activation of caspase-independent apoptosis *in vitro* and *in vivo*.

Using an *in vitro* cell-based model, the expression levels of proteins essential for the activation of caspase-independent apoposis, including apoptosis inducing factor (AIF), endonuclease G (Endo G) and calpains, will be determined in the cytoplasm, mitochondria, lysosomes and nucleus of pancreatic cancer cells (PANC-1, MIA PaCa-2, Capan-1 and Capan-2) following curcumin treatment. Furthermore, studies using siRNAs and Isothermal Titration Calorimetry (ITC) will be performed to confirm that the modulation of these proteins is directly related to curcumin treatment. Using an *in vivo* orthotopic PANC-1-derived xenograft model, tumor growth and expression of AIF, Endo G and calpains will be determined following curcumin treatment.

Part 2: To investigate the effects of pancreatic adenocarcinoma cell-derived exosomal curcumin on T regulatory cells (Tregs) in vitro

In the context of pancreatic cancer, components of the tumor microenvironment including cancer cells, stromal cells and immune cells, have been linked to the aggressive phenotype and chemoresistant nature of the disease. In particular, investigation of the function of the immunosuppressive T regulatory cell, Treg, has gained increased attention due to the ability of these cells to repress immune surveillance and immune targeting of tumors. Associated with this, tumor-derived exosomes, which are present in the tumor microenvironment, have been shown to have the ability to transport material between components of the tumor microenvironment, including immune cells. Furthermore, tumor-derived exosomes have been shown to suppress the anti-cancer functions of recipient immune cells. For these reasons, exosomes have been considered significant mediators of the tumor microenvironment. Interestingly, studies have shown that

therapeutic compounds conserve their properties after being packaged into exosomes and are capable of affecting recipient cells. Therefore, the release of exosomes can be used as a therapeutic advantage, particularly in situations where low bioavailability of drugs hinders their success. This thesis demonstrates that curcumin is able to be packaged within exosomes and retain its anti-cancer function upon delivery to recipient pancreatic cancer cells. Additionally, other studies have confirmed that curcumin retains its immunomodulatory properties following exosomal trafficking, suggesting that exosomal curcumin may aid in the reprogramming of immune system toward a cancer-targeting profile. In this context, studies involving exosomal curcumin's effects on Tregs may provide significant insight into the role of curcumin on the immune component of the tumor microenvironment. The objective of this study is to determine the effects of exosomal curcumin on the immunosuppressive function of Tregs *in vitro*. To accomplish this objective, exosomes isolated from PANC-1 cells cultured in the presence or absence of curcumin will be used to treat Tregs from pancreatic cancer patients or healthy donors. Subsequently, activation or suppression of Treg cells will be assayed using real-time PCR (RT-PCR), enzyme-linked immunosorbent assays (ELISA) and flow cytometry profiling.

APPENDIX B

BIOGRAPHICAL SKETCH

Carlos J. Díaz Osterman

1548 Alta Street
Redlands, CA 92374
(909) 583-5935
cdiazosterman@gmail.com

Academic Background

Loma Linda University, Loma Linda, CA	Ph.D.	2010 - current	Biochemistry
Universidad Metropolitana, San Juan, PR	B.S.	2008	Chemistry, *magna cum laude*

Research Experience

Graduate Dissertation

Dissertation title: Curcumin: a multi-dimensional approach to pancreatic cancer, targeting cell death and exosomes

Loma Linda University School of Medicine, Loma Linda, CA

Advisor: Dr. Nathan R. Wall, Department of Biochemistry

Computational chemistry technician

Project title: Ab initio study of the effect of radical attack on the folding of Ala-Ala-Ala tripeptide

Purdue University, Indiana, May 2007 - August 2007, Committee on Institutional Cooperation (CIC) Summer Research Opportunity Program (SROP) and the National Science Foundation Model Institutions for Excellence (NSF-MIE) Program

Advisors: Joseph Francisco, Ph.D. and Alex Davis, Ph.D.

Biophysics technician

Project title: Analyzing the biochemical and functional similarities between azurin and plantacyanin organometal proteins to assess whether plantacyanin is an anti-cancer agent

Universidad Metropolitana, Puerto Rico, August 2006 - May 2007, National Science Foundation Model Institutions for Excellence (NSF-MIE) Program

Advisor: Lymari Fuentes, Ph.D.

Organic chemistry and biochemistry technician

Project title: Chemical ligation with N-alkylaminooxypeptides

Santa Clara University, California, May 2005 - August 2005, National Science Foundation Research Experience for Undergraduates (NSF-REU) Program

Advisor: Michael R. Carrasco, Ph.D.

Biotechnology technician

Project title: The role of barrier to autointegration factor (BAF) in vertebrate nuclear structure and function

Universidad Metropolitana, Puerto Rico, August 2004 - May 2005, National Science Foundation Model Institutions for Excellence (NSF-MIE) Program

Advisor: Miriam Segura-Totten, Ph.D.

Biology technician

Project title: Diseases transmitted by Anopheles gambiae and Plasmodium falciparum

University of Utah, Utah, May 2004 - August 2004, Committee on Institutional Cooperation (CIC) Summer Research Opportunity Program (SROP) and the National Science Foundation Model Institutions for Excellence (NSF-MIE) Program

Advisor: Rosemary Gray, Ph.D.

Analytical and organic chemistry technician

Project title: High performance liquid chromatographic and gas chromatographic analysis of organic compounds that exhibit anti-tumor properties

Universidad Metropolitana, Puerto Rico, August 2003 - May 2004, National Science Foundation Model Institutions for Excellence (NSF-MIE) Program

Advisor: Osvaldo Cox, Ph.D.

Biology technician	Project title: Impact of Cycadophyta on the natural behavior of insects Universidad Metropolitana, Puerto Rico, August 2003 - May 2004, National Science Foundation Model Institutions for Excellence (NSF-MIE) Program Advisor: Eva Davila, M.S.

Teaching Experience

Graduate school lecturer	Loma Linda University, CA, Winter 2015 Course: Integrated Biomedical Graduate Studies (IBGS) 512 - Cellular Mechanisms and Integrated Systems II Lecture: Intracellular Compartments and Protein Sorting, Intracellular Membrane Trafficking and Extracellular Communication
Junior high and high school science teacher	Colegio Laico de Levittown, Puerto Rico, August 2009 - May 2010 Courses taught: Biology, chemistry, geology, physical and environmental sciences
Math and science tutor	Learning Alliances, LLC, Puerto Rico, August 2009 – May 2010 Courses tutored: Biology, chemistry, algebra, geometry and physics
Chemistry tutor	Universidad Metropolitana, School of Science and Technology, Department of Chemistry, Puerto Rico, 2005 and 2006 Courses tutored: General chemistry and organic chemistry

Publications

Peer-reviewed	**Díaz Osterman CJ**, Wall NR. Curcumin and pancreatic cancer: a research and clinical update. Journal of Nature and Science, 1(6):e124, 2015

Díaz Osterman CJ, Lynch JC, Leaf P, Wall NR. Curcumin modulates pancreatic adenocarcinoma cell-derived exosomal function. (Accepted with revisions in PLoS One, 2015.)

Díaz Osterman CJ, Gonda A, Stiff T, Sigaran U, Asuncion Valenzuela MM, Ferguson Bennit HR, Moyron R, Khan S, Wall NR. Curcumin induces pancreatic adenocarcinoma cell death via reduction of the Inhibitors of Apoptosis. (Accepted for publication in Pancreas, 2015.)

Khan S, Ferguson Bennit H, Asuncion Valenzuela MM, Turay D, **Díaz Osterman CJ**, Moyron RB, Esebanmen GE, Ashok A, Wall NR. Localization and upregulation of Survivin in cancer health disparities: a clinical perspective. (Accepted for publication in Biologics: Targets and Therapy, 2015.)

Asuncion Valenzuela MM, Ferguson Bennit HR, Gonda A, **Díaz Osterman CJ** , Hibma A, Khan S, Wall NR. Exosomes secreted from human cancer cell lines contain inhibitors of apoptosis (IAP). Cancer Microenviron. 2015.

Asuncion Valenzuela MM, Castro IV, Gonda A, **Díaz Osterman CJ**, Jutzy JMS, Aspe JR, Neidigh JW, Wall NR. Cell death in response to antimetabolites directed at ribonucleotide reductase and thymidylate synthase. Onco Targets Ther. 2015;(8):495-507

Aspe JR, **Díaz Osterman CJ**, Jutzy JMS, Deshields S, Wall NR. Enhancement of Gemcitabine sensitivity in pancreatic adenocarcinoma by novel exosome-mediated delivery of the Survivin-T34A mutant. J Extracell Vesicles. 2014;(3):1-9.

Galloway NR, **Díaz Osterman CJ**, Reiber K, Jutzy JMS, Li F, Sui G, Soto U, Wall NR. Ying Yang 1 regulates the transcriptional repression of Survivin. Biochem Biophys Res Commun. 2014; (1):208-213.

Pending completion

Khan S, Jutzy JMS, **Díaz Osterman CJ**, Daniels T, Neidigh JW, Peniche M, Wall NR. Cancer Cell Import of the Inhibitor of Apoptosis (IAP) Protein Survivin Requires Transferrin and Tumor Necrosis Factor Alpha (TNF-α) Receptors

Turay D, Khan S, **Díaz Osterman CJ**, Aspe JR, Curtis MP, Neidigh JW, Mirshahidi S, Lilly MB, Casiano CA, Wall NR. Proteomic profiling of serum-derived exosomes from prostate cancer patients

Presentations

Posters

Díaz Osterman CJ, Wall NR. Curcumin induces cellular death in pancreatic cancer through a caspase-independent, apoptosis-inducing factor (AIF)-dependent manner. 81st Annual Postgraduate Convention, Loma Linda University School of Medicine, California, 2015.

Díaz Osterman CJ, Ferguson HR, Asuncion Valenzuela MM, Khan S, Wall NR. Curcumin suppresses pancreatic cancer growth by targeting IAPs. 105th Annual American Association for Cancer Research Conference, California, 2014

Díaz Osterman CJ, Ferguson HR, Asuncion Valenzuela MM, Khan S, Wall NR. Curcumin suppresses pancreatic cancer growth by targeting survivin. Gordon Research Conference on Pancreatic Diseases: From Molecules to Patients, Massachusetts, 2013

Díaz Osterman CJ, Sigaran U, Stiff TR, Ferguson HR, Asuncion Valenzuela MM, Khan S, Wall NR. Curcumin induces pancreatic cancer cell death by targeting the inhibitor of apoptosis proteins. 13th Annual Loma Linda University Health Disparities Research Symposium, Loma Linda University, California, 2013

Díaz Osterman CJ, Ferguson H, Asuncion Valenzuela MM, Khan S, Wall NR. 6-gingerol and curcumin suppress pancreatic cancer growth by targeting the inhibitors of apoptosis. American Association for Cancer Research Conference on Pancreatic Cancer: Progress and Challenges, California, 2012

Díaz Osterman CJ, Ferguson HR, Asuncion Valenzuela MM, Khan S, Wall NR. 6-gingerol and curcumin suppress pancreatic cancer growth by targeting the inhibitors of apoptosis. 15th Annual Loma Linda University Basic Science Research Symposium, California, 2012

Díaz Osterman CJ, Khan S, Turay D, Wall NR. Differences in exosomal protein composition between ethnic groups. 14th Annual Loma Linda University Basic Science Research Symposium, Loma Linda University, California, 2011

Díaz Osterman CJ, Khan S, Turay D, Wall NR. Differences in exosomal protein composition between ethnic groups. 11th Annual Loma Linda University Heath Disparities Research Symposium, Loma Linda University, California, 2011

Díaz Osterman CJ, Davis A, Francisco J. Ab initio study of the

effect of radical attack on the folding of Ala-Ala-Ala tripeptide. Purdue University, Indiana, 2007

Díaz Osterman CJ, Davis A, Francisco J. Ab initio study of the effect of radical attack on the folding of Ala-Ala-Ala tripeptide. 18th Annual Undergraduate Research Symposium, Universidad Metropolitana, Puerto Rico, 2007

Díaz Osterman CJ, Carrasco MR. Chemical ligation with N-Alkylaminooxypeptides. Annual More Graduate Education at Mountain States Alliance Arizona Student Research Conference, Arizona State University, Arizona, 2006

Díaz Osterman CJ, Carrasco MR. Chemical ligation with N-Alkylaminooxypeptides. Society for Advancement of Chicanos and Native Americans in Science National Conference, Colorado, 2005

Díaz Osterman CJ. Diseases transmitted by Anopheles gambiae and Plasmodium falciparum. 15th Annual Undergraduate Research Symposium, Universidad Metropolitana, Puerto Rico, 2004

Díaz Osterman CJ. Diseases transmitted by Anopheles gambiae and Plasmodium falciparum. Society for Advancement of Chicanos and Native Americans in Science National Conference, Texas, 2004

Díaz Osterman CJ. Diseases transmitted by Anopheles gambiae and Plasmodium falciparum. Summer Research Opportunity Program Symposium, University of Utah, Utah, 2004

Díaz Osterman CJ. High performance liquid chromatographic and gas chromatographic analysis of organic compounds that exhibit anti-tumor properties. Undergraduate Research Symposium, Universidad Metropolitana, Puerto Rico, 2004

Oral presentations

Keynote Speaker, “White Coat, Now What?” 2nd Annual Family Day Awards Banquet, Loma Linda University School of Medicine, California, 13 February 2015

“Curcumin modulates pancreatic adenocarcinoma cell derived exosome function.” Initiative for Maximizing Student Development (IMSD) Meeting, Loma Linda University, California, 2015

“Curcumin: a multi-dimensional approach to pancreatic cancer

targeting cell death and exosomes." Loma Linda University, California, 2014

"Survivin's role in pancreatic cancer autophagy." Initiative for Maximizing Student Development (IMSD) Meeting, Loma Linda University, California, 2012

"The effects of turmeric compounds on pancreatic cancer cell lines." Loma Linda University, California, 2012

"Ab initio study of the effect of radical attack on the folding of Ala-Ala-Ala tripeptide." Purdue University, Indiana, 2007

"Chemical ligation with N-Alkylaminooxy peptides." Santa Clara University, California, 2005

"The interaction between Anopheles Gambiae and Plasmodium Falciparum: the diseases they transmit and their effects on the human body." University of Utah, Utah, 2004

Academic Distinctions

Scholarships

National Institutes of Health (NIH) Initiative for Maximizing Student Development (IMSD), Loma Linda University, California, 2010 - present

National Science Foundation Model Institutions for Excellence (NSF-MIE) Program, Universidad Metropolitana, Puerto Rico, 2003 - 2008

Science, Mathematics and Research Transformation (SMART) Program, Universidad Metropolitana, Puerto Rico, 2005 - 2006

Awards

First Place Poster Award, Basic Sciences, 81st Annual Postgraduate Convention, Loma Linda University School of Medicine, California, 2015.

Outstanding Teaching, Colegio Laico de Levittown, Puerto Rico, 2009 - 2010

Dean's List, Universidad Metropolitana, Puerto Rico, 2003 - 2008

Academic Excellence Award in Chemistry, Puerto Rico Chemistry Academy, Puerto Rico, 2007

Excellent High School Poster Judge Award, Universidad Metropolitana, Puerto Rico, 2007

Best Poster Award, Organic Chemistry, Society for Advancement of Chicanos and Native Americans in Science National Conference (SACNAS), Colorado, 2005

Memberships

Scientific societies

American Chemical Society, 2014 - present
American Society of Clinical Oncology, 2014 - present
American Association for Cancer Research, 2012 - present
Society for Advancement of Chicanos and Native Americans in Science, 2004 - 2006

Scholarly societies

American Association of Chemistry Teachers, 2014 - present
National Science Teachers Association, 2014 - present
California Science Teachers Association, 2014 - present

Student societies

Association of Latin American Students, Loma Linda University, 2010 and 2013

Service

Leadership

Social Affairs Vice President, Loma Linda University Basic Science Student Council, 2013 - 2014

Community

Poster Judge, Ana G. Mendez University System (AGMUS) Research Symposium, Puerto Rico, September, 2013

Volunteer, Project C.H.A.N.G.E. Black Men's Health Fair, Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine and Behavioral Health, Riverside, California, August 18, 2013

Interviewer, High School Apprenticeship Bridge to College (ABC) Program Invitational, Loma Linda University, California, 2012 and 2013

Hirshberg Foundation for Pancreatic Cancer Research Los Angeles Cancer Challenge 5 K race, California, 2013 and 2012

Contributing to a cleaner environment by collecting and recycling garbage from the beaches of Puerto Rico, sponsored by Colegio

Laico de Levittown, Puerto Rico, 2010

Poster Judge, Regional Science Fair, Department of Education and Universidad Metropolitana, Puerto Rico, 2007