Molecular characterization of a type-II ribosome inactivating protein and BC1-2 specific small interfering RNA

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**MOLECULAR CHARACTERIZATION OF A TYPE-II
RIBOSOME INACTIVATING PROTEIN AND Bcl-2
SPECIFIC SMALL INTERFERING RNA**

YE WENHUI

**SCHOOL OF BIOLOGICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY**

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SUMMARY

Mistletoe (*Viscum album*) lectins exert unique biological effects inhibiting protein synthesis through the cleavage of ribosomal RNA and are classified as type-II ribosome-inactivating proteins and receive a rising attention due to their potential medical and therapeutic applications in cancer therapy. The heterodimeric glycoproteins contain the A-chain with RNA cleaving catalytic activity and the B-chain with sugar-binding properties. The mistletoe lectins induce apoptotic cell death and have been shown to be more cytotoxic to cancer cells than to normal cells. In recent years, studies on the lectins from the white berry European mistletoe (*Viscum album*) and the yellow berry Korean mistletoe (*Viscum album coloratum*) have been described. However, the mechanism of cytotoxic effect on cancer cells still remains unclear. For a better understanding of the biological function of the mistletoe lectins, we studied the molecular and structural characteristics of the type-II RIPs, by using recombinant Korean mistletoe lectin. To this end, the A-chain of the recombinant Korean Mistletoe isoform 1 (rKML-1A) was expressed, purified and characterized by biophysical methods. We demonstrated that the recombinant protein properly and effectively inactivated ribosome during protein synthesis, and induced apoptosis in cancer cells comparable to that of the natural Korean mistletoe lectin (KML). Information of three dimensional structure of the lectin may provide insights into molecular basis for its catalytic activity and sugar-binding properties. A high degree (90 %) of sequence identity between Korean and European mistletoe lectins prompted us to perform homology modeling studies. The results showed that the

overall structure was similar to a backbone RMSD between KML and the EML-1 of 0.218 Å and between KML and Ricin of 0.787 Å. However, minor differences were observed in the catalytic and galactose binding sites.

Modulation of apoptosis in cancer cells would offer potentially useful approaches to enhance the chemosensitivity of cancer cells to chemotherapeutic agents. Anti-apoptotic proteins Bcl-2 and Bcl-XL are central regulators of apoptotic pathway and implicated in various types of cancers. In this studies, small interfering RNAs (siRNAs) targeting the mRNA encoding two proteins were designed and evaluated in MCF-7/caspase 3 positive and MDA-MB-231 breast cancer cell lines. Our results demonstrated that expression of Bcl-2 or Bcl-XL was significantly suppressed by siRNAs and the down-regulation of Bcl-2 or Bcl-XL induced apoptosis in cancer cells. To examine the increase in chemosensitivity on cancer cells, the Bcl-2 specific siRNA was co-delivered with paclitaxel by using cationic core-shell polymer nanoparticles. The results showed that the nanoparticles were able to deliver both siRNA and the anti-cancer drug simultaneously, which resulted in a combined effect in killing cancer cells.

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LIST OF ABBREVIATION

AP	alkaline phosphate
Bcl-2	B-cell lymphoma/leukaemia 2
BSA	bovine serum albumin
CD	circular dichroism
DCF	2',7'-dichlorofluorescein
D-Gal	D-galactose
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	1, 4-Dithiothreitol
dsRNA	double-stranded RNA
EB	ethidium bromide
<i>E.coli</i>	Escherichia coli
EML	European mistletoe lectin
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
FMP	formycin monophosphate
FPLC	fast performance liquid chromatography
GalNAc	N-acetylgalactosamine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HML	Himalayan mistletoe lectin

HRP	horseradish peroxidase
IPTG	isopropyl-thio-B-D-galactopyranoside
KML	Korean Mistletoe lectin
LB	Luria-Bertani
MMP	mitochondrial membrane potential
NMR	nuclear magnetic resonance
PAP	pokeweed antiviral protein
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PKR	RNA-dependent protein kinase
PTGS	post-transcriptional gene silencing
PVDF	polyvinylidene difluoride
RISC	RNA-induced silencing complex
rKML	recombinant Korean Mistletoe lectin isoform 1
rKML-1A	A-chain of the recombinant Korean Mistletoe lectin isoform 1
rKML-1B	B-chain of the recombinant Korean Mistletoe lectin isoform 1
RIP	ribosome inactivating protein

RMSD	root mean square deviation
RNAi	RNA interference
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SRD	sarcin/ricin domain
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TMV	tobacco mosaic virus
TNF	tumor necrosis factor
VCA	<i>Viscum album</i> L. <i>coloratum</i> agglutinin

1 INTRODUCTION

1.1 Mistletoe Lectin

The term lectin was first used in 1954 to describe agglutinins from legumes that agglutinate erythrocytes through cross-linking reactions with exposed sugar residues (Boyd and Shapleigh, 1954). Studies on lectin began in 1888 when Stillmark discovered that extracts from *Ricinus communis* seeds caused the agglutination of erythrocytes. It was found that the active ingredient was a protein and the protein was named ricin. Early studies about lectins were largely focused on lectins' ability to stimulate the division of lymphocytes. Later, it was found that ricin and related toxins are more toxic to Ehrlich ascites than normal cells (Lin et al., 1970) and researchers began to study their structure, entry into cells and the mechanism of action.

Mistletoe is a semi-obligate parasite plant that lives on different kinds of trees. Extracts from white berry European mistletoe (*Viscum album. L*) and yellow berry Korean mistletoe have been used as alternative cancer therapy, and the extracts containing lectins have been shown to be more cytotoxic to cancer cells than to normal cells (Peumans et al., 2001). Several reports demonstrated that mistletoe lectins induce apoptosis (Bussing et al., 1996; Bussing et al., 1999; Lyu et al., 2002; Lyu et al., 2001; Yoon et al., 1999). The mistletoe lectin-induced apoptosis is associated with the release of cytochrome c from mitochondria and caspase activation.

Mistletoe lectins are classified into a group of type-II ribosome-inactivating protein (RIP) due to its ribosomal RNA cleavage activity. The lectins exist as dimers

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comprising an A-chain (catalytic subunit) and a B-chain (sugar-binding subunit) that are linked by a single disulfide bond (Eck et al., 1999a; Huguet Soler et al., 1996). The A-chain catalyses the cleavage of the N-glycosidic bond in the eukaryotic ribosomal RNA, whereas the B-chain is responsible for the specific binding to putative receptors on the surface of target cells. As observed with type-II RIPs, the B-chain-mediated binding of the holoprotein to the cellular surface results in a much higher cytotoxicity than that of the isolated A-chain.

Extracts from *Viscum album* leaves are widely used as immunomodulating agents and adjuvant cancer therapy (Beuth, 1997; Bocci, 1993; Hajto et al., 1990). The components of commercial preparations are different due to different isolation methods. Among various substances in the extracts, mistletoe lectin I (ML I) was identified as the main therapeutic component. However, ML I greatly varies and isoforms ML II and ML III are found in the extract. These isoforms are distinguished by different degrees of glycosylation. To clearly demonstrate the biological function and the relationship between the structure and mechanism, efforts have been made to obtain pure mistletoe lectin. Eck et al cloned mistletoe lectin gene successfully (Eck et al., 1999a). It was shown that from genomic DNA, an intron-free gene was obtained and genes coding for other type-II RIPs are also devoid of introns (Halling et al., 1985; Kaku et al., 1996; Wood et al., 1991). The studies showed that isoforms of ML exhibit sequence conservation, indicating the presence of isoforms due to post-translational modification, not due to genetic variation (Dietrich et al., 1992; Gabius et al., 1992). In contrast, multiple cDNAs of lectin genes of Korean mistletoe

have been cloned recently using several primers designed on the basis of amino acid sequences. The cDNA clones showed variations in their sequences at the level of nucleic acids. This suggests that isotypes of the Korean mistletoe lectin exist at genetic level, which is unlike the European mistletoe lectin (Eck et al., 1999a; Eck et al., 1999b).

1.1.1 Definition and classification of RIPs

The term ‘ribosome-inactivating protein’ refers to the proteins containing an RNA N-glycosidase domain that is structurally related to the classical RIPs. There are two major types of RIP: type-I single-chain RIPs and type-II double-chains RIPs.

Type-I RIPs consist of only one N-glycosidase domain. Most type-I RIPs are single chain proteins with about ~30 kD molecular weight, while a few type-I RIPs contain two chains which are non-covalently connected. The two chains were proteolytically processed from one ~30KD polypeptides.

Type-II RIPs consist of two-chains, A-chain and B-chain. The A-chain of type-II RIPs contains N-glycosidase domain and B-chain contains a structurally different carbohydrate-binding domain. These two chains are linked by one disulfide bond. The A-chain and B-chain are processed from one precursor. A link sequence between the two domains on the precursor is excised after translation to produce two chains (Peumans et al., 2001).

1.1.2 Distribution of RIPs in nature

The distribution of RIPs is wide in nature and may be more than it was originally thought. RIPs were initially found in plants, mostly in Angiospermae. Shiga toxin

produced by *Shigella dysenteriae* and shiga-like toxins produced by enterohaemorrhagic strains of *Escherichia coli* were also found to be RIPs (Obrig, 1997; Reisbig et al., 1981).

The level of RIPs in plant tissues varies, ranging from traces to hundreds of milligrams per 100 g. RIPs exist in different tissues (such as roots, leaves, stems and flower) of plant or sometimes only in one tissue. And in one tissue different isoforms are found or different RIPs are found in different tissues as well (Ferrerias et al., 1993; Girbes et al., 2003).

It was found that under some conditions the expression of RIPs increases. For example, when cells encounter viruses or stress, the level of RIPs is higher compared to the cells under normal condition (Chaudhry et al., 1994; Rippmann et al., 1997; Stirpe et al., 1996).

1.1.3 Enzyme activity

1.1.3.1 Classical enzyme activities on ribosomal RNA

Ricin was found to inhibit protein synthesis either in cells (Lin et al., 1971) or in cell-free system (Olsnes and Pihl, 1972). Endo and co-workers first discovered the mechanism of this inhibitory effect (Endo et al., 1987). They found that Ricin cleaved a specific glycosidic bond between an adenine and a sugar moiety of the nucleotide on the 28S rRNA. The adenine was removed and elongation step was then stopped. This specific site located in a highly conserved region of 28S rRNA called the sarcin/ricin loop, which is located in domain VII about 400 nucleotides from the 3' end of the rRNA. The cleavage site of the sarcin is near the ricin cleavage site. In the case of rat

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liver ribosome, this specific site is A4324 (fig 1.1). This site-specific RNA N-glycosidase activity is a common property of other type-I and type-II RIPs (Stirpe et al., 1988).

Copied from Stirpe, F. 2004. *Toxin* 44: 371-383

Fig 1.1 Schematic representation of the enzymatic action of ribosome-inactivating proteins on rRNA. RIPs cleave a single adenine base (A4324 in 28 S rat rRNA) at a site adjacent to the site of attack by a-sarcin, which cleaves the phosphodiester bond between G4325 and A4326 in rat 28 S rRNA.

The mammalian ribosomes are not the only substrates of RIPs. It was found that RIPs also depurinate ribosomes from insects (Zhou et al., 2000), plants (Iglesias et al., 1993), yeast (Roberts and Selitrennikoff, 1986), and bacteria (Girbes et al., 1993a).

The adenine removed by RIPs is in the middle of sequence 5'-AGUACGAGAGGA-3' on the rRNA where the underlined A is removed by RIPs. The nucleotide sequence is highly conserved and forms part of a stem-loop structure. Since the cleavage site of endoribonuclease α-sarcin is also in this sequence, it is called sarcin/ricin domain (SRD). This conserved loop is the recognition/binding site for both the eukaryotic/prokaryotic elongation factor 1 (eEF-1/EF-Tu) and the

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eukaryotic/prokaryotic elongation factor 2 (eEF-2/EF-G) complexes (Moazed et al., 1988).

Both depurination by RIP and cleavage by α -sarcin have severe effect on ribosome function. It was also reported that single point mutations introduced into *E. coli* SRD at several positions showed lethal results (Marchant and Hartley, 1994). NMR structure of a synthetic oligoribonucleotide (29-mer) that mimics the mammalian SRD revealed a compact, but irregular helix containing a bulged G across strand A stack and a GAGA containing tetraloop (Szewczak and Moore, 1995). Crystallographic structure of mammalian SRD and *E. coli* SRD confirmed this feature (Correll et al., 1998; Correll et al., 1999). In these models, the removed A4324 (A2660) (rat numbering; *E. coli* numbering in parentheses) is stacked with G4325 (G2661) and A4326 (A2662). The Watson–Crick faces in the minor groove are exposed and available for interaction with ligands.

Although all RIPs have RNA N-glycosidase activity toward ribosomes, their substrates are different. For example, ricin acts on mammalian and yeast ribosomes while poorly acts on plant and *Escherichia coli* ribosomes (Barbieri et al., 1993). Pokeweed antiviral protein (PAP) is active to plants, bacteria, yeasts, and lower and higher animal ribosomes. In general, type-II RIPs show preference for animal ribosomes. Type-I RIPs are active to substrates from rather broad species. Both RIPs and ribosomes could be reasons of different substrate specificity.

The structure of the target site on rRNA is conserved, but ribosomal proteins have different functions. Some ribosomal proteins allow the RIPs to go into the active site,

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but some proteins prevent this from happening. Studies about ricin showed that ribosomal proteins might not be necessary for its enzyme activity (Endo and Tsurugi, 1988). Naked 28S rRNA could also be the substrate of ricin. However the presence of ribosomal proteins can increase the reaction efficiency (Endo and Tsurugi, 1988). In some case, ribosomal proteins can also inhibit the RIPs activity. For example, ricin A-chain can remove the adenine from naked 23S rRNA in *E.coli* instead of native ribosome (Hartley et al., 1991). Yeast ribosomal protein L3 was found to be a binding factor of PAP (Hudak et al., 1999). L3 is highly conserved in different ribosomes. It's consistent with the diversity of substrates of PAP. Also the different structures of RIPs are related to substrate specificity.

RIPs depurinate not only ribosomal rRNAs, but also naked rRNAs. Moreover, they act on naked rRNAs from non-substrate ribosomes. Most RIPs deadenylate naked rRNA at multiple sites besides the site same as that in native ribosome. It can be concluded from these observations that deadenylation of naked rRNA does not have physiological meaning and ribosomes contribute to the substrate specificity of RIPs.

1.1.3.2 Activity on non-nucleic acid substrates

Other activities on non-nucleic acid substrates were also reported including superoxide dismutase (Li et al., 1996), chitinase, phosphatase and phospholipase (Helmy et al., 1999), though it is uncertain whether any of these is physiologically relevant to their toxic action.

Efforts were made to study the enzyme activity of RIPs and its molecular mechanism. It is clear that all RIPs have N-glycosidase and polynucleotide:adenosine

glycosidase activity. Other activities associated with RIPs need further confirmation with elimination of the contamination. Some studies already demonstrated the impurity of preparation of RIPs. For example, contaminated DNase and RNase have been detected in apparently pure ricin and momordin II, respectively (Day et al., 1998; Valbonesi et al., 1999).

1.1.4 Biological activities

1.1.4.1 Toxicity of type-II RIPs

Most type-II RIPs are potent toxins to higher and lower animals. The toxicity of type-II RIPs is explained by the carbohydrate binding activity of B-chain. B-chains of type-II RIPs are quite similar in the amino acid sequence and also 3-dimensional structure, which contain two or three binding sites (Frankel et al., 1996; Steeves et al., 1999). However there are still pronounced differences in sugar binding activity of B-chains of different RIPs.

Type-II RIPs have different cytotoxicity. For example, ricin inhibited protein synthesis by Hela cells with an IC_{50} (concentration causing 50% inhibition) below 1 ng/ml, whereas some elderberry type-II RIPs have no effect even at a higher concentration, i.e. 1 μ g/ml (Battelli et al., 1997). The reason could be due to a difference in sugar binding activity. Proteins with a low affinity to sugars show low toxicities.

1.1.4.2 Antiviral activity of type-I RIPs

Unlike some type-II RIPs, type-I RIPs are not cytotoxic. Type-I RIPs have only one chain that can not bind to certain residue on cell surface and subsequently enter

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into cells. The function of RIPs in nature is different between type-I and type-II RIPs. Type-II RIPs are thought to protect the plant from phytophagous invertebrates or herbivorous animals through their toxicity. In contrast, type-I RIPs are not cytotoxic and their antiviral activity is well documented.

Pokeweed antiviral protein (PAP) purified from pokeweed leaves is the first known type-I RIP. It was found to inhibit the transmission of tobacco mosaic virus (TMV) in plants by Duggar and Armstrong in 1925 (Peumans et al., 2001). Subsequently, RIPs from many sources were found to have antiviral activity against both plant viruses and animal viruses (Stirpe, 2004). The mechanism of this antiviral activity is not clear so far. Normally, RIPs are segregated from the cytoplasm. For example, ricin is located in protein bodies; PAP is in the cell wall matrix or in vacuoles and saporins stay in the intracellular space. It is possible RIPs are released to cytosol when cell is infected by virus. RIPs in cytosol then inactivate ribosome so that virus can not replicate or they act directly on virus RNA or DNA. It is also possible that RIPs activate the defense system of the plant to indirectly act on virus. Further investigation about antiviral mechanism was performed in transgenic plants. RIPs were transfected to improve the resistance of plants to viruses. As a matter of fact, RIPs protected plants from viral infection, but high-level expression of RIPs were toxic to plants themselves (Dai et al., 2003; Lodge et al., 1993; Moon et al., 1997).

Efforts were then made to identify antiviral RIPs with low toxicity. PAPc was a carboxyl-terminal deletion mutation of PAP, which had intact RNA N-glycosidase site. However when PAPc was expressed in transgenic tobacco plant, it had no

toxicity and did not depurinate the tobacco ribosome (Tumer et al., 1997). However, tobacco plants expressing PAPc were protected from potato virus X (Tumer et al., 1997) and from the fungal pathogen *Rhizoctonia solani* (Zoubenko et al., 1997). It was concluded that intact RNA N-glycosidase site but not ribosome inactivation was required for antiviral activity of PAP. Nevertheless it was not certain that toxicity and antiviral activity can be simply separated.

1.1.4.3 Other biological activities

Some RIPs were found to have antifungal and insecticidal activity. For example, barley RIPs, maize b-32 RIP and PAP (Tumer et al., 1999) could increase the resistance of plant against fungal. Maize RIP could increase the resistance of tobacco plants to insect *Helicoverpa zea* (Dowd et al., 2003).

RIPs are immunogenic, allergenic and also have immunosuppressive activity. Ricin was first found to induce formation of antibodies (Dowd et al., 2003). Other RIPs are also strongly immunogenic, which can induce formation of antibodies once administrated to animals.

It was observed that ricin induced an IgE response and it also increased the response against other antigens that were given at the same time (Dowd et al., 2003). Other RIPs also induce formation of IgE when introduced to mice or patients. When RIPs are given before other antigens, they show immunosuppressive effects preventing formation of antibodies. This might be related with their high toxicity for macrophages and their interference with an early step in immune response.

1.1.5 Structure

1.1.5.1 Primary structure

More than 50 type-I RIPs and about 15 type-II RIPs have been sequenced and/or cloned. High similarities exist between Type-I RIPs and A-chain of type-II RIPs by sequence comparison. B-chains of different type-II RIPs are also quite similar in primary structure.

1.1.5.2 Three-dimensional structure: an understanding of enzymatic activity and sugar-binding specificity

In general, the three-dimensional structures of the different RIPs are well conserved. It was demonstrated by the fact that the α -carbon traces of most RIPs are virtually superimposable. However, there are still some differences. The differences exist mainly in the surface loop and carboxyl-terminal region. These differences are thought to be related with activity difference and substrate specificity of the different RIPs (Peumans et al., 2001).

The structures of the type-I RIPs and the A chains of the type-II RIPs are very similar. Their tertiary structures of N-glycosidase domains of RIPs are highly conserved with the main differences in their surface loops. When each is compared with ricin A chain, the rms distance between corresponding C α positions is generally less than 2.0 Å. The only exceptions are saporin SO6 with an rms deviation of 2.2 Å and the A chain of Shiga toxin with an rms deviation of 2.8 Å (Robertus and Monzingo, 2004).

The N-glycosidase domain geometries are nearly identical. Sequence alignment shows about eight invariant residues, mostly clustered in active site, including Tyr 80,

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Tyr 123, Glu 177, Arg 180 and Trp 211 (ricin A-chain numbering). The complex structure with substrate analog gave insight about enzymatic activity. Crystal structures of ricin with adenosine analog formycin monophosphate (FMP) and ApG showed that the substrate purine ring was stacked between the invariant tyrosines. Arg 180 bound to N3 of the purine ring and Glu 177 interacted with the ribose (Monzingo and Robertus, 1992). Further more, mutation of these key residues affected the catalytic activity (Lord et al., 1994). Experiments with other RIPs complexed with both FMP and formycin were done subsequently. Results from these studies have shown essentially the same mode of binding (Huang et al., 1995; Monzingo et al., 1993; Ren et al., 1994; Weston et al., 1994).

The position and orientation of most residues in the active site are fixed in various RIPs complexes. However some variability was observed. The orientation of an active site residue (tyrosine 80 in ricin A) may be different among RIPs, and its conformational movement may play a role in catalytic activity of RIPs (Gu and Xia, 2000; Hosur et al., 1995; Husain et al., 1994; Ren et al., 1994; Tahirov et al., 1995). In the case of native ricin, the side chain of Tyr 80 is oriented so that it can form hydrogen bond with oxygen of Gly 121, across the active site (Katzin et al., 1991). When the substrate binds to ricin, this hydrogen bond is broken due to a slight movement and rotation of Tyr 80 about the C α -C β bond. The adenine ring of the substrate can then insert into the active site (Monzingo and Robertus, 1992). In other RIPs, the hydrogen bond has not been observed at corresponding site. The movement of the tyrosine residue only involves rotation about the C α -C β bond.

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However, there is no consistent pattern of conformational change of the tyrosine among RIPs. The orientation of this tyrosine residue is different among native RIPs; on the other hand, once bound to a substrate analog, rotation of the tyrosine occurs for a given RIP. The flexibility of this residue may facilitate substrate binding.

The structures of the B chains of the type-II RIPs from plants are also quite similar. When compared with the ricin B chain, rms distance between corresponding C α positions is 1.3 Å or less (Robertus and Monzingo, 2004). B-chains of type-II RIPs have a bilobal structure. Two domains of B-chains are homologous and each contains three homologous subdomains, called α , β , γ (Rutenber and Robertus, 1991). In the case of ricin B-chain, only 1 α and 2 γ subdomains have sugar-binding activity. 1 α subdomain shows low affinity and 2 γ subdomain shows high affinity (Hatakeyama et al., 1986). The sugar binding pockets are created by a sharp bend in the backbone. It is formed by the sequence Asp, Val and Arg, and a variable aromatic residue which provides the binding platform for the sugar. This residue is Trp37 in the 1 α /low affinity subdomain and Tyr248 in the 2 γ /high affinity site (Schlossman et al., 1989).

The difference of sugar binding residues in RIPs accounts for their different toxicity and specificity. Several type-II RIPs including ebulin, nigrin b and nigrin f, are nontoxic to cells, which is due to the reduced affinity (Citores et al., 1997; Girbes et al., 1993b; Pascal et al., 2001). In the case of ebulin, A-chain has the same enzymatic activity (Girbes et al., 1993c; Svinth et al., 1998). Compared to B-chain of ricin, Tyr 248 is replaced by Phe in ebulin B chain together with other subtle geometric rearrangements, which reduce the affinity of 2 γ subdomain (Pascal et al.,

2001). RCA, a ricin homologue with subunit composition A₂B₂ is weakly toxic. Sugar binding Site of 1 α subdomain is not changed, but Tyr 248 is replaced to histidine in 2 γ subdomain (Rutenber and Robertus, 1991).

1.1.5.3 Structure of Mistletoe Lectin I

The 3-D structure of Mistletoe Lectin I (MLI) is similar to that of ricin and abrin-a, two other type-II RIPs. The matched C α was 91.8% with rmsd 0.92 Å and 93.7% with rmsd 1.05 Å when the structure of MLI was superimposed to ricin and abrin-a respectively.

A-chain is a globular enzyme with active site located in a prominent central cleft. The conserved residues of active sites among all RIPs are Tyr76, Tyr115, Glu165, Arg168, Trp199 and Ser203 in MLI. Asn 112 is a glycosylation site located at the edge of active site cleft, which is unique among RIPs. From known structures of RIPs, Tyr76 in this region exhibits various conformations (Husain et al., 1994); conformational change of Tyr 76 is the main difference in active-site geometry when structure of sugar-free MLI was compared with that of ricin (Krauspenhaar et al., 1999).

B-chain of MLI consists of two homologous globular domains. Each domain consists of three subdomains located around a hydrophobic core. There are three conserved disulfide bonds in MLI B-chain. Mutation from Cys to Ser39 in 1 α subdomain makes B-chain lose a disulfide bond. Three glycosylation sites were found in MLI B-chain. Asn96 and Asn136 are conserved in ricin and abrin-a B-chains. There is still one additional glycosylation site at Asn61.

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Two sugar-binding sites exist in 1 α and 2 γ subdomains. Each binding site lies in a shallow cleft formed by contiguous stretches. Some common interactions between galactose and protein exist in both N-terminal and C-terminal. These interactions include hydrogen bond between aspartate (Asp23 in the N-terminal site, Asp235 in the C-terminal site) and galactose, hydrogen bond between asparagines (Asn47 in the N-terminal site, Asn256 in the C-terminal site) and galactose, and hydrophobic contact between an aromatic ring (Trp38 in the N-terminal site, Tyr249 in the C-terminal site) and galactose formed by parallel arrangement of the ring and C3-C4-C5 plane of galactose. Residues involved in these interactions are conserved in B-chains. The common feature of two binding sites sometimes was observed in proteins other than RIP B-chains. Results from sugar affinity study showed 3-OH and 4-OH of galactose are critical for sugar binding for MLI (Lee et al., 1994) and ricin (Solis et al., 1993). There are also some specific interactions in either N-terminal or C-terminal besides common ones. Comparing two binding sites, the electron density of galactose is more pronounced in the C-terminal site than in N-terminal site. This may suggest that N-terminal sugar binding site has a relative lower affinity to galactose, which is also found in ricin.

Formation of MLI dimer is consistent with previous electron microscopic study. The existence of dimer was thought to be a real dimer form instead of artefact due to crystallization. Two A-B monomers are symmetric and face each other at the N-terminal domain of B-chains. The distances between sugar-binding sites are calculated as straight distances between corresponding O4 atoms of galactose, which

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are 15Å for sites between B1-B1 domains, 87Å between B2-B2' domains, 47Å between either B1-B2 or B1'-B2'. A lot of polar and nonpolar interactions are observed at the dimer interface.

Sugar specificity, quaternary structure and differences in the arrangement of the sugar-binding sites of B-chain probably affect the binding ability of type-II RIPs to cell surface and subsequent intracellular biological processes. Thus the difference between B-chains contributes to different cytotoxicity of type-II RIPs. For example, the difference of cytotoxicity between MLI and ricin may due to B-chains since there was no significant difference in catalytic activity between plant-derived ML A-chain and plant-derived ricin A-chain (Eck et al., 1999a).

1.1.6 Apoptosis

Researchers are interested in Ribosome inactivating proteins because of their selective toxicity to tumor cells compared to normal cells. It was thought that RIPs caused cell death simply by inhibition of protein synthesis and consequent necrosis. However, from the morphology of cells, the cells seemed to undergo apoptosis after being treated with RIPs. Ricin and abrin were administered into rats intramuscularly (Griffiths et al., 1987). It was observed that cells in para-aortic lymph nodes and Peyer's patches underwent apoptosis, characterized by the formation of apoptotic bodies. Since then many studies reported that RIPs brought about cell death by inducing apoptosis in mammalian cells (Allam et al., 1997; Brinkmann et al., 1997; Kochi and Collier, 1993; Morimoto and Bonavida, 1992; Narayanan et al., 2004). Most of these studies revealed that RIPs could induce apoptosis in a variety of cell

lines, but the mechanism and pathway of RIP-induced apoptosis was not clear. Some possible pathways were proposed regarding different RIPs in certain cell lines.

1.1.6.1 Apoptosis induction through ribotoxic stress response pathway

One possible pathway of apoptosis induced by RIPs could be ribotoxic stress response. In 1997, Iordanov et al. found that ricin, α -sarcin and anisomycin were able to activate the SAP kinases or JNK1 in Rat-1 cells (Iordanov et al., 1997). This novel pathway was called ribotoxic stress response. It was caused by damage to 28S rRNA by RIPs and resulted in the activation of SAPK/JNK and its activator, SEK1/MKK4 and thus apoptosis (Laskin et al., 2002). RNA N-glycosidase activity is required for inducing apoptosis. Mistletoe lectin is able to induce apoptosis in many cell lines (Bussing et al., 1999; Langer et al., 1999; Vervecken et al., 2000). Site-specific mutation of E166Q and R169Q in recombinant ML resulted in the loss of protein synthesis inhibition as well as apoptosis inducing activity (Langer et al., 1999). The activation of SAPK/JNK1 pathway needs signaling from 28S rRNA. Nevertheless how signals are transmitted from damaged 28S rRNA to the MAPK pathway needs to be studied further.

1.1.6.2 Apoptosis induction through mitochondrial pathway

Studies about RIP-induced apoptosis revealed that mitochondria were involved. Normally, loss of mitochondrial membrane potential, MMP ($\Delta\psi_m$), rapid release of cytochrome c from mitochondrial and activation of caspase-9 were observed. Abrin was found to induce apoptosis through loss of MMP followed by caspase-3 activation and DNA fragmentation, but independent of death receptor pathway (Narayanan et al.,

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2005). Shih et al. demonstrated that abrin induced apoptosis through its direct interaction with a mitochondrial protein called anti-oxidant protein-1 (AOP-1) (Shih et al., 2001). It was found that abrin affected the anti-oxidant function of AOP-1 and consequently increased ROS production and released cytochrome c into the cytosol. Shih et al. also proposed that abrin could induce apoptosis independent of its RNA N-glycosidase activity. Another mode of mitochondria involved apoptosis is supposed by ROS production and increase of intracellular calcium levels. Ricin and Trichosanthin, a type-I RIP, were reported to increase cellular calcium level in hepatoma cells and human choriocarcinoma cells respectively (Hu et al., 2001; Zhang et al., 2001a). Recently, many studies have established a role for cellular calcium signaling in the mitochondrial pathway of apoptosis (Hajnoczky et al., 2003). It could be summarized that the RIP-induced apoptosis through mitochondrial pathway is a complex phenomenon, which could be regulated at multiple levels.

Other mechanisms were also reported. For example, it was reported that RIPs induced apoptosis through down-regulation of anti-apoptotic proteins or up-regulation of pro-apoptotic proteins (Erwert et al., 2003; Jones et al., 2000). Proteins involved in regulation varied in different cells since the basal levels are different. NAD^+ depletion and decrease of ATP level were observed in some researches (Komatsu et al., 2000).

The relationship between protein synthesis inhibition and apoptosis induction activities is not clear. Though all RIPs can inhibit protein synthesis, the mechanism and pathway of apoptosis induced by RIPs depend on the cell lines as well as the RIP

used. These pathways need not to be mutually exclusive. Further studies are needed to elucidate the signaling leading to apoptosis.

1.1.6.3 Mistletoe lectins induce cell death via apoptosis

Decreased cell death was observed in a large number of cancer cell lines treated with mistletoe lectin. It was reported by many studies that cell death induced by mistletoe lectin was related with apoptosis. When Molt 4 cells were treated with mistletoe lectin, the first observed event is membrane perforation (Ribereau-Gayon et al., 1997). Incubating leukemic T-cells and B-cells with ML-I induced apoptosis (Bantel et al., 1999). Korean mistletoe lectin was purified by Yoon et al. (Yoon et al., 1999). Typical apoptotic event was observed, such as morphological changes and DNA fragmentation, when tumor cells were treated with Korean mistletoe lectin. The mechanism underlying apoptosis induced by mistletoe lectin was also investigated by many research groups. In HL-60 cells, mistletoe lectin induced cell death and apoptosis in time-dependent and dose-dependent manner (Lyu et al., 2001). The activation of caspase-3 was investigated. Native and processed PARP form could be detected and expression of caspase-3 zymogen decreased. Apoptosis induced by mistletoe lectin was inhibited by z-DEVD-FMK, a caspase-3 specific inhibitor. When studied in hepatoma cells SK-Hep-1(p53-positive) and Hep 3B (p53 negative), it was found that mistletoe lectin could induce apoptosis in p53 and p21 independent manner and through down-regulation of Bcl-2 and up-regulation of Bax. Moreover, telomerase activity decreased in these two cell lines after treatment with mistletoe (Lyu et al., 2002). Pae et al. showed that treatment of Hela and MCF-7 cells with

mistletoe lectin either from European mistletoe or Korean mistletoe potentiated apoptosis induced by tumor necrosis factor, TNF (Pae et al., 2000a).

It was evident that both European mistletoe lectins and Korean mistletoe lectins could induce apoptosis in a large number of cancer cell lines.

1.1.7 Application of mistletoe lectin in vitro and in vivo

It has been clearly established that mistletoe lectin possess cytotoxicity and immuno-modulating activity. It could promote proliferation of peripheral blood mononuclear cells with increased TNF and IL-6 production (Stein et al., 1998), stimulate T-lymphocytes and enhance the activity of peripheral blood natural killer cells (NK) (Heiny et al., 1998).

Mistletoe lectins are immunogenic and can be used as adjuvant. Lavelle and coworkers (Lavelle et al., 2000) showed that Mistletoe lectin I owned potent immunogenic activity. In their experiments, ML-I and other lectins were introduced orally or by intranasal route in mice. ML-I induced high level of serum IgG, IgA and specific IgA in mucosal secretions, which is comparable to cholera toxin, a potent mucosal immunogen. ML-I, ML-II and ML-III were also demonstrated to enhance immunogenic activity of other proteins when co-administered (Lavelle et al., 2002; Lavelle et al., 2001).

In vitro study showed that the mistletoe lectin could induce apoptosis and increase the cytokine release. Yoon et al. isolated lectins (KML) from Korean mistletoe *Viscum album C. (coloratum)* (Yoon et al., 1999). KML possess strong cytotoxicity and induce apoptotic cell death in a variety of cell lines. Both EML and

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KML could promote apoptosis induced by TNF when studied in MCF-7 and HeLa cells (Pae et al., 2000a). Studies in human hepatoma cells SK-Hep-1 (p53-positive) and Hep 3B (p53-negative) revealed down-regulation of telomerase activity by KML (Lyu et al., 2002). Studies with VCA in Hep3B cells revealed increased reactive oxygen species (ROS) and loss of mitochondrial membrane potential. Antioxidant NAC suppressed ROS production and loss of mitochondrial membrane potential, and protected cells from apoptosis (Kim et al., 2004). They also demonstrated that VCA induced JNK phosphorylation. Hep3B cells overexpressing JNK1 were more susceptible to cell death from ROS and loss of mitochondrial membrane potential induced by VCA.

The cytotoxicity of mistletoe lectins *in vivo* was also investigated. It's clear that mistletoe lectins induced apoptosis and inhibited metastasis *in vivo*. Decrease of tumor weight and prolonged live time of animals were observed in experiments performed by many researchers. It was also demonstrated that the introduction of mistletoe lectin by intravenous injection or by oral route reduced tumor metastasis, which was mediated by NK activation (Yoon et al., 1998). Inhibition of tumor metastasis was believed to be due to inducing apoptosis as well as anti-angiogenic effect. Yoon and his colleagues showed administration of Korean mistletoe lectin reduced blood vessels towards the tumor mass. Oral delivery of mistletoe lectins has the immunomodulating advantage. Mistletoe lectins bind to gut mucosa and are presented to lymphocytes of Peyer patches, thus inducing a major cytokine response. On the other hand, mistletoe lectins also induced hyperplastic growth of the small

intestine in rats and did not show detrimental side effects (Pryme et al., 2002). Thus oral administration of mistletoe lectins could be a good alternative to conventional cancer therapy, which usually causes severe side effects.

1.1.8 Objective

For decades, several type-II RIPs have been discussed for a potential medicinal application. Mistletoe lectin has long been utilized for cancer therapy in Asia and Europe. The lectins purified from the mistletoe exact have been shown to be responsible for the production of the cytokines and are more toxic to cancer cells than normal cells. However, the detailed molecular mechanism of the cytotoxic effect on cancer cells largely remains to be answered. The information about the mechanism of cell death and a potential receptor on cell surface for lectin will provide a clinical basis of the lectin as anti-cancer drug. Recently, the cDNA sequences for European and Korean Mistletoes have been reported, indicating they are quite homologous (Park et al., 2001). With the help of the cDNAs, functional, biochemical, and structural studies on the mechanism of the lectin targeting cancer cells can be expedited.

Many cancer types are associated with the impairment in apoptosis which is related to resistance of cancer cells to radiation and chemotherapy treatments. Therefore, approaches to overcoming the apoptotic deficiencies in cancer cells may provide windows of therapeutic opportunity for cancer treatment. The ability of Mistletoe lectin for inducing apoptosis in cancer cells makes it a potential drug for cancer therapy.

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Therefore, the objectives of this study are to understand and define the molecular basis and biological effects of mistletoe lectin, through the studies of the recombinant Korean mistletoe lectin. Towards this end, the recombinant Korean mistletoe lectin (rKML) was expressed and purified, and its molecular characteristics in vitro were investigated.

To study the effect of rKML on the remission of cancer, in particular, the cytotoxicity and ability of rKML to induce apoptotic cell death in cancer cells were evaluated.

To gain structural basis for its ribosome inactivating activity and sugar binding properties, homology modeling studies were performed based on the high degree of sequence identity between Korean and other mistletoe lectins including European and Himalayan mistletoe lectins, and Ricin.

1.2 RNA Interference and chemotherapy

1.2.1 RNA interference (RNAi)

1.2.1.1 Introduction to RNAi

RNA interference phenomena was found by Fire and his co-workers in 1998 (Fire et al., 1998). They observed that double-stranded RNA (dsRNA) induced a potent silencing on homologous genes in the nematode *Caenorhabditis elegans*. RNAi has been used as a good technique to study gene function in plants, *C. elegans* and *Drosophila*.

The model about RNAi involves five steps in the process (Hutvagner and Zamore,

2002), which can be outlined as follows:

In first step, dsRNAs (>26 bp) are cleaved by Dicer to generate 21–23 nt fragments (Bernstein et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Dicer belongs to RNase III-like enzyme. The generated short duplexed RNA was called small interference RNA (siRNA), which contains 2 nt 3'-overhangs and 5'-phosphate groups (Elbashir et al., 2001b). These two properties are very important for their function.

In the second step, short duplexes were separated into two single stranded RNAs probably by an ATP-dependent RNA helicase (Nykanen et al., 2001). One strand is then incorporated into a high-molecular-weight protein complex which is called RNA- induced silencing complex (RISC) in step 3 (Nykanen et al., 2001), and in step 4, the homologous RNA sequences were cleaved by an as yet unidentified endonucleolytic component of RISC under the direction of this guide RNA (Elbashir et al., 2001b; Hammond et al., 2000).

In the last step, RISC is released from the cleaved mRNA. Released RISC can be recycled multiple times for catalysis, which could explain the potent silencing effect of RNAi.

RNAi has been developed into a powerful tool to study gene function. For example, researchers have studied individual gene function in *C. elegans* using RNAi and the study has extended to all genes (~19,000) of *C. elegans*. Similar studies were also done in other organisms, including plants and mammalian systems (Hannon, 2002). However, when it was applied to mammalian somatic cells, cells exhibited

nonspecific responses to dsRNA of ≥ 30 nt (Hutvagner and Zamore, 2002; McManus and Sharp, 2002). The non-specific response was caused by dsRNA of ≥ 30 nt and brought global changes of cellular gene expression, from which specific gene silencing is difficult to detect.

It was reported that this non-specific response could be overcome by utilizing small interfering RNAs (siRNA). siRNAs were demonstrated to cause potent and specific gene silencing effect in mammalian cells (Elbashir et al., 2001a). These siRNAs are about 21–23 nt, which are chemically synthesized and mimics of the key intermediate in the RNAi pathway. They were thought to incorporate into RISC and cleave homologous RNA substrates. These siRNAs are too small to induce nonspecific responses to dsRNA such as PKR (Elbashir et al., 2001a).

This landmark discovery by Tuschl and co-workers has led to routine RNA interference in mammalian cell culture, and siRNA may be a useful genetic analysis tool in mammalian cells.

1.2.1.2 Stable suppression in mammalian cells by siRNAs expressed in vivo

In C. elegans and plants, the signal of RNAi could be transferred from cell to cell, and the silencing effect could be amplified (Fire et al., 1998; Palauqui et al., 1997). But mammals lack this mechanism. One problem about RNAi in mammalian system is that the effects induced by siRNAs are transient. Although it was reported that the stable gene silencing could be achieved through the expression of a 500-base-pair dsRNA (Paddison et al., 2002b), this method can not be applied to all mammalian cell lines. It could only be utilized in cells without generic responses to dsRNA such as the

PKR pathway.

The remarkable finding that natural or designed siRNAs can be expressed *in vivo* (Brummelkamp et al., 2002; Paddison et al., 2002a; Paul et al., 2002; Zeng et al., 2002) and they induce stable suppression in mammalian cells resolve this problem. Short hairpin and pre-miRNA-based precursor RNAs can be transcribed from either RNA polymerase III (H1 or U6) (Brummelkamp et al., 2002; Paul et al., 2002) or RNA polymerase II promoters (Zeng et al., 2002) *in vivo*, and processed by Dicer to release functional siRNAs. Silencing effect induced by siRNAs expressed from DNA templates *in vivo* is the same as that mediated by exogenously introduced synthetic siRNAs (Brummelkamp et al., 2002; Paddison et al., 2002a; Zeng et al., 2002).

1.2.1.3 Biological function and application of RNAi

Since RNAi was discovered, it has evolved rapidly to a powerful tool in genomic study and its potential as an anti-virus or anti-cancer reagent is being investigated.

Post-transcriptional gene silencing (PTGS) and RNAi are conserved in the plant and animal. In plants, protecting cells from inappropriate expression of repetitive sequences, transposable elements and viruses was thought as the important function of RNAi (Li and Ding, 2001). Many plant viruses could encode suppresser of RNAi. Virus lacking the suppresser expression would subject to RNAi passway. These provided strong evidence that RNAi can be an innate cellular antiviral mechanism. This kind of virus also exists in animals, such as flock house virus, a small, positive-strand RNA virus, belonging to the Novaviridae. It was found to encode an RNAi antagonist (the B2 protein) (Li et al., 2002).

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Based on this native anti-virus mechanism, some researches targeting viral protein or other relative genes by siRNAs have been done and promising results have been achieved. The viruses targeted included RSV (respiratory syncytial virus) (Bitko and Barik, 2001), poliovirus (Gitlin et al., 2002) and HIV-1 (Coburn and Cullen, 2002; Jacque et al., 2002; Novina et al., 2002). In the case of HIV-1, not only the viral proteins but also the host proteins have been successfully targeted by siRNA to inhibit virus infection. The target viral proteins included Gag, Pol, Vif and the small regulatory proteins Tat and Rev (Coburn and Cullen, 2002; Jacque et al., 2002; Novina et al., 2002). Host proteins that are important for virus infection are also good targets for degradation by siRNAs. Tsg101 is required for vacuolar sorting and efficient budding of HIV-1 progeny. Chemokine receptor CCR5 is a co-receptor during HIV-1 cell entry process. These two proteins were already successfully suppressed to inhibit HIV-1 replication (Coburn and Cullen, 2002; Jacque et al., 2002; Novina et al., 2002).

One important application of RNAi is to study the function of individual genes. In *C.elegans*, this study has extended to nearly ~19,000 genes of the whole genome. Similar study was performed in other organisms including plants. After siRNA was successfully applied in mammalian cells, study on genes of human genome also started using expressed siRNAs (Hannon, 2002).

siRNA has been expanded from the availability of stable suppression. Induced phenotypes can now be observed over long time span. Stably engineered cells can be assayed either *in vitro* or *in vivo*. For example, suppression of growth of tumor cells

could be monitored in xenograft models. Finally, RNAi-based therapeutics could be achieved by combining siRNAs with existing high-efficiency gene delivery vehicles. Therefore the specificity of the siRNA make it a good tool to be utilized in therapy by silence the disease-causing genes and this could be very promising in cancer therapy.

1.2.2 Apoptosis and Bcl-2 family proteins

1.2.2.1 Apoptosis and central executioners in apoptosis pathway

Apoptosis or programmed cell death is a complicated process, which starts from membrane blebbing, goes through cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation and finally fragmentation of cells into apoptotic bodies that are rapidly cleared by phagocytes.

Apoptosis is an important cellular mechanism in maintaining homeostasis of cells, and allows organisms to tightly control cell numbers and tissue size. Dysfunction of apoptosis may lead to diseases, including developmental defects, autoimmune disease, neurodegeneration or cancer (Hengartner, 2000). There are two major apoptotic pathways in mammalian cells: mitochondrial pathway and death-receptor pathway. The mitochondrial pathway is triggered by either extracellular or internal signals, while the death-receptor pathway is triggered by members of the death-receptor superfamily.

In apoptotic pathway, caspases are thought as central executioners since most morphological changes are caused by caspases. Caspases are cysteine proteases and cleave Asp-xxx bond of substrates. They are divided into several subfamilies according to their sequence identity, structural similarity and substrate preference

(Thornberry et al., 1997). Caspases cleave target proteins, which mostly results in inactivation of target proteins while sometimes results in activation of target proteins. Some characteristic features of apoptosis could be explained by the cleavage of specific proteins by caspases. Nuclease that cuts the DNA to generate DNA ladder is one example. It is activated by caspase-3 through the cleavage of inhibitory subunit (Enari et al., 1998; Liu et al., 1997; Sakahira et al., 1998). This nuclease is now known as caspase-activated DNAase, or CAD.

During the apoptosis pathway, downstream caspases could be activated by upstream, previously activated caspases. This 'caspase cascade' strategy is potent to amplify and integrate pro-apoptotic signals. Two pathways of apoptosis converge at the level of caspase-3 activation and caspase-3 then activates downstream apoptotic cell progress (Hengartner, 2000).

1.2.2.2 Bcl-2 family proteins: important apoptotic regulators

There are many regulators involved in apoptosis pathway beside caspases. Among these are Bcl-2 family proteins. Bcl-2 family is named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (Tsujimoto et al., 1985). It is composed of a dozen of proteins and divided into three groups. 1) Bcl-2, Bcl-XL and several close relatives with anti-apoptotic activity. Members of this group contain four Bcl-2 homology domains (BH1~BH4) and TM domain. 2) Members of group II such as Bax, Bak are structurally similar to group I members containing three of the four conserved BH domains (BH1~BH3), but they possess pro-apoptotic activity. 3) Members of group III such as Bim or Bik share little

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sequence and structure similarity with the other two groups containing only BH3 domain. They are pro-apoptotic proteins.

The Bcl-2 family proteins are important regulator in apoptosis pathway. It regulates the release of cytochrome c from mitochondria intermembrane compartment into cytosol (Adams and Cory, 1998; Antonsson and Martinou, 2000; Gross et al., 1999). Cytochrome c is required for activation of caspase-9, which is initiator caspase in mitochondria pathway. Once cytochrome c is released from mitochondria, it associates with procaspase-9 together with Apaf-1 to form apoptosome, which is considered as active form of caspase-9 (Rodriguez and Lazebnik, 1999).

Bcl-2 family members can homodimerize, but more importantly, pro- and anti-apoptotic member can form heterodimers (Adams and Cory, 1998; Antonsson and Martinou, 2000; Reed, 1997). The amphipathic BH3 α helix can bind to the hydrophobic groove formed by α helix in BH3, BH1 and BH2 domains (Sattler et al., 1997). Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate the release of cytochrome c.

For pro-apoptotic family members, they translocate to mitochondria when they receive cytotoxic signal where they bind to pro-survive members and lock them into inactive form. Bcl-2 family protein might form new pores in mitochondria or affect pre-exist channel to affect the mitochondria homeostasis and release of several apoptogenic proteins (Muchmore et al., 1996; Shimizu et al., 1999).

1.2.3 Chemotherapy and drug-resistance

Chemotherapy is one of the main methods (radio-, chemo-, immuno- and gene

therapies) applied in clinical cancer treatment. While this method is successful for certain cancers, the effectiveness has often been limited by side effects on normal cells and by both intrinsic and acquired drug resistance from tumors cells. Drug resistance is an important clinical problem to be resolved. Several mechanisms have been found to be complicated. Increased efflux of drug from cells was observed during chemotherapy, which is mediated by energy dependent transporter such as P-glycoprotein (P-gp) and MDR-associated protein (MRP) (Tsuruo et al., 2003). Over-expression of P-gp was found in both intrinsically drug-resistant cancers and cancer cells, which acquired drug-resistance after chemotherapy.

After entering into cells, chemotherapeutic drugs induce a series of cellular responses, which are regulated by various cellular signal pathways. Cell cycle and cell proliferation are influenced. Dysfunction of these signal pathways also contributes to drug resistance. Apoptosis is one of such mechanism underlying drug resistance. However, there is increasing evidence that diverse drugs kill tumor cells through apoptosis pathway (O'Gorman and Cotter, 2001; Solary et al., 2000). Expression change or mutation of important regulator in apoptosis pathway provided cancer cells with survival advantage (Evan and Littlewood, 1998; Evan and Vousden, 2001). This indicates that apoptosis in tumor cells plays a critical role in chemotherapy-induced cell killing, and also suggests that blockade of apoptosis-inducing pathway could be one of mechanisms for drug resistance.

In principle, methods that could induce apoptosis or restore apoptotic potential are promising in enhancing cancer cell sensitivity to chemotherapeutic reagents.

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Inducing apoptosis has the advantage that it does not cause inflammation or damage to surrounding normal tissue compared to necrosis. Such efforts are being made. For example, mutation and inactivation of p53 are observed in many human tumors. Reintroduction of wild-type p53 into tumors has been demonstrated to induce apoptosis *in vitro* and increase cell death in xenograft models of different tumor origins (Lebedeva et al., 2003).

Bcl-2 and Bcl-XL proteins are also good targets to enhance the chemosensitivity. Bcl-2 and Bcl-XL proteins are important regulators in apoptosis pathway. They are pro-apoptotic proteins preventing the release of cytochrome c from mitochondria and inhibiting the activation of the caspase cascade (Adams and Cory, 2001). These two proteins are overexpressed in most types of cancers. And overexpression of Bcl-2 and Bcl-XL is implicated in the resistance of cancer cells to a variety of anti-cancer agents (Berardo et al., 1998; Simoes-Wust et al., 2000; Tanabe et al., 2003; Webb et al., 1997). For these reasons, down-regulation of anti-apoptotic protein Bcl-2 or Bcl-XL may increase chemosensitivity of cancer cells.

1.2.4 Objective

Drug resistance is an important clinical problem to be resolved in cancer therapy. Over-expression of anti-apoptotic proteins in cancer cells could block cellular apoptosis pathway and has been shown to be associated with resistance to chemotherapy and radiation therapy. Design and evaluation of antagonists suppressing such anti-apoptotic proteins could provide potential treatment options for cancer. Bcl-2 and Bcl-XL are important apoptotic regulators and thus are good targets

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in enhancing sensitivity of cancer cells. Recently siRNA has been developed as a good tool to silence the expression of gene in mammalian cells in a sequence-specific manner. Therefore, another objective of this study was to investigate the possibility of siRNA as a valuable approach in combination with conventional chemotherapeutic agents in cancer therapy. To this end, siRNAs targeting Bcl-2 mRNA or Bcl-XL mRNA were designed and evaluated in breast cancer cells. The effects of Bcl-2 or Bcl-XL-specific siRNAs in enhancing the sensitivity of cancer cells to anti-cancer drugs were studied.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Escherichia coli BL21 (DE3) was purchased from Invitrogen (Carlsbad, CA, USA). pET plasmids were purchased from Novagen (Madison, WI, USA). Ampicillin and kanamycin sulfate were purchased from US Biological (Massachusetts, USA) or Invitrogen.

The dNTP (dTTP, dATP, dGTP and dCTP) mixtures were purchased from Invitrogen. Restriction enzymes and T4 ligase were purchased from Roche (Indianapolis, IN, USA). The DNA polymerase pfu was purchased from Novagen (Madison, WI, USA). pfu UltraTM High-Fidelity DNA Polymerase and Dpn I were from stratagene (La Jolla, CA, USA). Plasmid purification kit, PCR purification kit, gel extraction kit were purchased from Qiagen (Hilden, Germany).

Protein molecular weight markers, the protein assay dye and polyvinylidene difluoride (PVDF) membrane were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Pre-stained protein molecular weight marker was from Fermentas.

Antibody against His-tag was from Qiagen. Antibodies against Bcl-2 (100), Bcl-X_{S/L} (S-18), Bax (Δ 21), Bad (C-7) and PARP (H-250) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibody against GADPH was purchased from Ambion. Protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN, USA). Alkaline peroxidase (AP)-coupled goat anti-rabbit immunoglobulin, alkaline

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peroxidase (AP)-coupled goat anti-mouse immunoglobulin and Immstar™ AP substrate were purchased from Bio-Rad.

Ni²⁺-NTA resin was from Qiagen (Hilden, Germany). HiPrep 16/60 Sephacryl S-200 was purchased from Amersham Biosciences (Buckinghamshire, UK). Lysozyme was purchased from Sigma-Aldrich (St. Louis, Mo, USA). Isopropyl-thio-B-D-galactopyranoside (IPTG) and 1, 4-dithiothreitol (DTT) were purchased from United States biological (Swampscott, MA)

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). BioPORTER was from Gene Therapy Systems (San Diego, CA, USA).

CellTiter 96 aqueous non-radioactive cell proliferation assay, TNT quick coupled transcription/translation system, luciferase assay system and CaspACE™ assay system were from Promega (Promega, Madison, WI, USA).

Generic chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2 Bacterial strain

Three bacterial strains were used for different purposes. *E.coli* (Escherichia coli) DH5α was used for plasmid purification. BL21 (DE3) was used for the protein expression. XL1-blue was used for the site directed mutation.

2.1.3 Cell lines

The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA). MCF-7 (caspase-3 positive) cell line was a gift from Prof. Alan Porter (Institute of Molecular and Cell Biology,

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Singapore). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were maintained in a 5 % CO₂ humidified incubator at 37 °C.

2.1.4 Medium

(1) Luria-Bertani (LB) medium

10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g NaCl in 1 L deionized (DI) water.

(2) LB agar plates

2 % agar, 10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g NaCl in 1 L DI water.

(3) SOC medium

2 % bactotryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂,

10 mM MgSO₄, 20 mM glucose

2.1.5 DNA and protein marker

DNA molecular ladders were purchased from Bio-Rad and New England Biolad. Protein molecular weight marker was purchased from BioRad containing following fractions: myosin 200 kDa, galactosidase 116.25 kDa, phosphorylase b 97.4 kDa, serum albumin 66 kDa, ovalbumin 45.0 kDa, carbonicanhydrase 31.0 kDa, trypsin inhibitor 21.5 kDa, lysome 14.4 kDa, aprotinin 6.5 kDa.

The prestained protein molecular weight marker was purchased from Fermentas containing β -galatosidase 118.0 kDa, bovine serum albumin 85.0 kDa, ovalbumin 47.0 kDa, carbonic anhydrase 36.0 kDa, β -lactoglobulin 26.0 kDa, lysozyme 20.0 kDa.

2.1.6 Small interfering RNAs (siRNAs)

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Six different siRNAs were used: HY-SR1, HY-SR2, HY-SR3, HY-SR4, HY-SR9 and FITC-siRNA, which were purchased from Qiagen-Xeragon (Germantown, MD, USA). They were annealed 21-nt double stranded siRNAs. HY-SR1, HY-SR2, HY-SR3 were targeting Bcl-2 mRNA; siRNA HY-SR9 was targeting Bcl-XL mRNA, HY-SR4 was scramble siRNA, and FITC-siRNA was control siRNA labeled with fluorescein at the 5' of the sense strand and used to quantify the transfection efficiency through flow cytometer. The siRNA was provided as lyophilized powder and was dissolved in sterile buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) to obtain 20 μ M solution and stored at -80 °C. It was heated to 90 °C for 1 min, and incubated at 37 °C for 60 minutes when siRNA solution was first used in order to disrupt higher aggregates, which may form during lyophilization process. This is necessary to ensure siRNA silencing efficiency.

The sequences of siRNAs are as follows:

HY-SR1: sense strand 5'-CUGUACGGCCCCAGCAUGCdTdT-3' antisense strand 5'-GCAUGCUGGGGCCGUACAGdTdT-3'. Its target site on Bcl-2 mRNA is 599-617.

HY-SR2: sense strand 5'-GUACAUCCAUAUAAGCUGdTdT-3' antisense strand 5'-CAGCUUAUAAUGGAUGUACdTdT-3'. Its target site on Bcl-2 mRNA is 49-67.

HY-SR3: sense strand 5'-CAUCGCCCUGUGGAUGACUdTdT-3' antisense strand 5'-AGUCAUCCACAGGGCGAUGdTdT-3'. Its target site on Bcl-2 mRNA is 514-532.

HY-SR4(scramble siRNA): sense strand 5'-GUCUCCAAGCGGAUCUCGUdTdT-3'

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antisense strand 5'-ACGAGAUCCGCUUGGAGACdTdT-3'

FITC-siRNA: sense strand 5'-UUCUCCGAACGUGUCACGUdTdT-3' antisense strand 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

HY-SR9: sense strand 5'-GCGUAGACAAGGAGAUGCAdTdT-3' antisense strand 5'-UGCAUCUCCUUGUCUACGCdTdT-3'. Its target site on Bcl-XL mRNA is 595-613.

2.1.7 Buffers and solutions

2.1.7.1 Solutions and buffers for DNA Electrophoresis

(1) 50 × TAE (Tris-acetate-EDTA)

242 g Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA, pH 8.0, and water to make 1 liter solution

(2) 1 % Agarose gel

1 % (w/v) agarose in 1× TAE buffer

(3) 6 × DNA loading buffer

0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 40 % (w/v) sucrose in H₂O

(4) Ethidium bromide (EB)

10 mg/ml in H₂O

2.1.7.2 Buffers for SDS-PAGE

(1) 5 × Tris-glycine electrophoresis buffer

15.1 g Tris, 94 g glycine pH 8.3, 50 ml 10 % SDS stock, and H₂O to make 1 liter solution

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(2) 2 × SDS-PAGE gel-loading buffer

100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4 % SDS, 0.2 % bromophenol blue, 20 % (v/v) glycerol, stored at -20 °C.

(3) 30 % acrylamide mixture

Dissolve 29 g acrylamide and 1 g N, N'-methylene-bisacrylamide in water, then adjusted to 100 ml. Stored at 4°C.

(4) 10 % ammonium persulfate

1 g ammonium persulfate in 10 ml water, stored at 4 °C.

(5) Staining solution

0.25 % (w/v) coomassie brilliant blue R-250: 0.25g coomassie brilliant blue R-250 in 100 ml destaining solution.

(6) Destaining solution

Methanol: H₂O: glacial acetic acid is 4.5:4.5:1 in volume.

(7) Storage buffer

5 % methanol, 7.5 % glacial acetic acid.

2.1.7.3 Buffers for Western blot

(1) 1 × Membrane transfer buffer

3.03 g Tris, 14.4 g glycine, H₂O to 1 liter

(2) 1 × TBS (Tris-buffered saline)

20 mM Tris, pH 7.5, 500 mM NaCl,

(3) 1 × TTBS

20 mM Tris, 500 mM NaCl, 0.1 % Tween-20, pH 7.5

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(4) Blocking solution (1 %)

1 g non-fat dry milk in 100 ml 1 × TBS

(5) Antibody buffer (1 %)

1 g non-fat dry milk in 100 ml 1 × TTBS

2.1.7.4 Buffers for protein purification

(1) Resuspension buffer for Ni-NTA purification

20 mM phosphate buffer, pH 7.8, 0.5 M NaCl, 5 mM 2-mercaptoethanol.

(2) Washing buffer for Ni-NTA purification

20 mM phosphate buffer, pH 7.2, 1 M NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol.

(3) Elution buffer for Ni-NTA purification

20 mM phosphate buffer, pH 6.0, 0.5 M NaCl, 0.5 M imidazole, 5 mM 2-mercaptoethanol

(4) Buffers of gel filtration

50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.01% NaN₃.

2.1.8 Medium and reagent for tissue culture

(1) Mammalian cell culture medium

Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % penicillin (100 unit/ml)-streptomycin (100 µg/ml).

(2) Freezing medium

Culture medium supplemented with 10 %DMSO

(3) Trypsin-EDTA (0.5 % Trypsin with EDTA·4Na)

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(4) 1 × PBS

9 g NaCl, 0.795 g Na₂HPO₄·7H₂O, 0.144 g KH₂PO₄, and H₂O to 1 liter, autoclaved

(5) Lysis buffer

50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 % Triton X-100

(6) siRNA transfection reagent

Oligofectamine

2.2 Methods

2.2.1 DNA quantization

To determine DNA concentration, absorbance at 260 nm wavelength was measured. The concentration is calculated by $50 \mu\text{g/ml} \times \text{dilution factors} \times \text{Abs}_{260}$. DNA purity was determined by the ratio of Abs at 260 nm / Abs at 280 nm. The value between 1.8~2.0 was considered to be in the normal range. If the ratio is less than 1.8, the sample may contain proteins or/and other UV absorbers. If the ratio is higher than 2.0, the sample may be contaminated with chloroform or phenol.

2.2.2 DNA electrophoresis

To make 1 % agarose gel, 1 g agarose powder was added in 100 ml electrophoresis buffer TAE. The solution was heated in a microwave oven until the agarose dissolved. EB was added to a final concentration of 0.1-0.5 $\mu\text{g/ml}$ when the gel solution cooled down. The gel solution was mixed by a gentle swirling and poured into the electrophoresis tank. DNA samples were mixed with 6 × the gel loading buffer and loaded into the gel submerged in 1 × TAE buffer. The gel was run at 100 volts for 20-30 minutes and visualized by UV light.

2.2.3 Polymerase Chain Reaction (PCR)

PCR reaction was performed in 50 μ l reaction volume: 5 μ l 10 $\times$ reaction buffer, 0.5 μ l 10 mM dNTP mixture, template plasmid, 1 μ l 10 μ M forward primer, 1 μ l 10 μ M reverse primer, 1 μ (1 unit) of polymerase, and water were added to 50 μ l. The mixture was transferred to 0.2 ml thin-wall PCR tube and put into GneeAmp® PCR system 9700 from Applied Biosystems. Tag polymerase was used for a colony screen and pfu polymerase was used for a high fidelity DNA fragment synthesis, since pfu DNA polymerase has 3'-5' proofreading activity. Different elongation temperatures were used. Denaturation was done at 95 °C for 1-2 minutes. For tag polymerase, the elongation temperature was 72 °C and for pfu polymerase, the elongation temperature was 68 °C. Most Annealing reactions were done at 50 °C for 1-3 minutes. The reactions were run for 25 -30 cycles.

2.2.4 Restriction endonuclease digestion

Plasmids or PCR products were digested with restriction endonucleases. The reactions were performed in 20~50 μ l volume with 1~3U restriction enzymes. Digestions with more than one enzyme can be performed at the same time with appropriate buffer according to manufacturers' instruction or performed or sequentially with individual buffer for each enzyme. The reactions usually lasted 2 hours at 37 °C.

2.2.5 Extract DNA from the agarose gel

DNA fragments or digested plasmids were purified by a recovery from agarose gel. Briefly, after separation of DNA fragments on a 0.8 % agarose gel, the gel slice

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containing the DNA fragment was cut out and purified using gel extraction kit according to the manufacturer's manual.

2.2.6 DNA ligation

PCR products and vector plasmids digested with suitable restriction endonucleases were ligated with T4 DNA ligase for 2 hours at room temperature or overnight at 4 °C. Ligation was usually performed in 20 µl volume: 1U T4 DNA ligase, 2 µl 10 × reaction buffer, PCR fragment and vector with a ratio of 5 to 1.

2.2.7 Colony PCR

Colony PCR was used to pick out positive colonies. Ligation product was transformed into DH5α and incubated overnight on an agar plate. The next day, colonies were inoculated into 2ml LB medium with antibiotic and incubated at 37 °C. Colony PCR was performed by using 2 µl of the culture medium as template, *Taq* polymerase and appropriate primers. Only positive colonies were screened and subjected to DNA sequencing.

2.2.8 Transformation

Transforming DNA (up to 25 ng per 50 µl competent cells) or ligation product was added to competent cells. Tubes were swirled gently several times to mix the contents. Two control tubes were set up, one was competent cells with a known amount of standard prepared plasmid DNA and one was competent cells without plasmid DNA. The tubes were incubated on ice for 30 minutes and placed in a 42 °C water bath for 45 seconds. 800 µl SOC medium was added to each tube and the cultures were incubated at 37 °C for 1 hour to allow the bacteria to recover and to

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express the antibiotic resistance marker encoded by the plasmid. Appropriate volume of transformed competent cells was transferred onto agar LB plates containing appropriate antibiotic. The plates were incubated in a 37 °C incubator and inverted when the liquid has been absorbed. Transformed colonies should appear in 12~16 hours.

2.2.9 Isolation of plasmid DNA

Plasmid was transformed into *E. coli* DH5 α and incubated overnight at 37 °C. A single colony was inoculated into 2 ml LB containing required antibiotics and incubated overnight at 37 °C. Plasmids were isolated from the 2 ml culture by using QIAprep Spin Miniprep Kit (Qiagen).

2.2.10 Site-directed mutagenesis

The site mutations were performed by using the QuickChange[®] II Site-Directed Mutagenesis Kit (Stratagene). The reaction mixture contained 5~50 ng plasmid template, 125 ng each primer, 0.2mM dNTP mixture, 1 μ l pfu Ultra[™] High-Fidelity DNA Polymerase (2.5 U/ μ l) and distilled, deionized water. After PCR reaction, 1 μ l *Dpn I* restriction enzyme (10 U/ μ l) was added into the PCR product and incubated at 37 °C for 1hour to remove the template DNA. 1 μ l *Dpn I* treated PCR product was transformed into *E. coli* XL1-blue competent cells and cultured on the LB plate containing both *Tet* and other antibiotic for the plasmid vector.

2.2.11 SDS-Polyacrylamide gel electrophoresis

The SDS-PAGE was used to check protein expression and purification. Different concentrations of acrylamide could be used according to protein size. 12 % resolving

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gel was used in this study, which was suitable to separate proteins of about 12~60 KD. Protein samples were mixed with 2 × loading buffer and heated at 90-100 °C for about 2~3 minutes. Gel was run at 30 mA until the dye reached the bottom of the gel. Gel was then put in staining buffer rotating on Belly Dancer for about 10 minutes and destained for some time. Protein bands were visualized with white light box.

2.2.12 Small scale induction of target protein

One colony containing the recombinant vector was picked up from the plate and inoculated into 2 ml LB medium with appropriate antibiotic. The tubes were incubated at 37 °C and 250 rpm. When the OD achieved 0.8~1.0, 1 ml culture was taken out as samples without IPTG, and 1 µl 1 M IPTG was added to the remainder of each culture. The induction was performed at different temperature, and the induction time was varied to optimize the induction conditions. The cells were harvested by centrifuge at 13.4×1000 rpm for 1 minute in a microfuge and supernatants were removed. The pellets were dissolved in 0.5 ml PBS and subjected to sonication. Samples of minus IPTG, total, soluble and insoluble fractions after adding IPTG were prepared and loaded on SDS-PAGE to analyze the yield and solubility of the protein.

2.2.13 Purification of histidine-tagged protein by Ni-NTA resin

One colony containing the recombinant plasmid was picked up from the plate, inoculated into 25 ml LB with antibiotic and incubated at 37 °C with shaking overnight. The next day, 2~2.5 ml overnight *E.coli* culture was inoculated into 250 ml LB with antibiotic in 1 L flask. The target protein was induced based on the optimized condition from previous small scale expression. The cells were harvested by

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centrifugation at $8000 \times g$ for 5 minutes.

The pellets were then suspended in suspension buffer (20 mM phosphate buffer, pH 7.8, 0.5 M NaCl, 5 mM 2-mercaptoethanol, 0.1 mg/ml lysozyme). The suspension was incubated on ice for 30 minutes; cells were sonicated for 20 minutes. The insoluble debris was removed by centrifugation at $180,000 \times g$ for 25 minutes.

Ni-NTA resin slurry was mixed and transferred to a small polypropylene column. The resin was packed under gravity flow and equilibrated with 3 column volumes of suspension buffer. Cell lysate was immediately loaded onto the column when the suspension buffer drained to the top of the resin. When all lysate passed through the resin, the column was washed with 10 times resin volumes of washing buffer (20 mM phosphate, pH 7.2, 1 M NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol). The bound protein was eluted with 5 times resin volumes of elution buffer (20 mM phosphate, pH 6.0, 0.5 M NaCl, 5 mM 2-mercaptoethanol, 0.5 M imidazole) in 1-ml fractions. Samples from different stages were kept and analyzed by 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.14 Purification of protein by fast performance liquid chromatography (FPLC)

Purified protein from affinity column was further purified with FPLC. Eluted fractions from Ni^{2+} -NTA affinity chromatography was concentrated to about 5mg/ml and loaded on Sephacryl S-200 gel filtration column pre-equilibrated with FPLC buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01 % sodium azide). The flow rate was 1 ml/min and UV_{280} was monitored. Fractions with

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high UV absorbance were collected. Sample from each fraction was analyzed by 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of purified proteins was determined by Bio-Rad Laboratories (Hercules, CA, USA) protein assay kit.

2.2.15 Protein concentration assay

The protein concentration was measured by Bio-Rad protein assay kit (Hercules, CA, USA). This assay was based on the absorbance maximum of coomassie brilliant blue G-250 dye shift from 465 nm to 595 nm when binding to proteins. The dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts distilled, deionized water. Dye reagent was filtered through Whatman #1 filter to remove particulates. Five dilutions of a protein standard were prepared. The linear range of the assay for BSA is from 0.2 to 0.9 mg/ml and the linear range for IgG is between 0.2 and 1.5 mg/ml. 100 μ l of each standard or sample solution was mixed with 5 ml dye reagent by vortex and incubated at room temperature for at least 5 minutes. The incubation time is no more than 1 hour. The absorbance of the mixture was measured at 595 nm. Standard curve was plotted by the concentration of standard protein dilutions versus the absorbance. The concentration of the samples was calculated accordingly.

2.2.16 Circular Dichroism (CD) measurement

The protein in 1 $\times$ phosphate buffered saline (PBS) was concentrated to 1 mg/ml and CD was measured using Chirascan Spectropolarimeter (Applied Photophysics Limited, Surrey, UK). 1 $\times$ PBS was used as a baseline, and the sample was loaded

into the curvet and the spectrum with 4 scans was recorded from 180 to 280 nm.

2.2.17 NMR spectroscopy

The purified rKML-1A was concentrated to 0.5 mM in a buffer containing 20 mM Na-PO₄, pH 7.0, 20 mM NaCl, 1 mM DTT, 0.01 % NaN₃, and 10 % D₂O. The NMR measurement was performed with water presaturation at 298 K on an Avance 700 spectrometer (Bruker, Fallanden, Switzerland) equipped with four radio frequency channels and a triple resonance probe. The spectra were processed and analyzed by using Topspin (Bruker).

2.2.18 Tissue culture

2.2.18.1 Sub-culture of mammalian cells

Prepared culture medium (DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin) and PBS were warmed up in 37°C water bath. Cell morphology was examined by microscope. The DMEM/FBS was aspirated from the tissue culture flask or dish. The flask was rinsed with PBS to remove traces of serum, which inhibited the effectiveness of trypsin. Trypsin (1 ml for T25 flask, 3 ml for T75 flask) was added and incubated at 37 °C. When the cells were detached, DMEM/FBS was added and clumps were dispersed gently through pipetting. Cell suspension was transferred to a centrifuge tube and spun for 5 minutes at 500 × g. The supernatant was removed and DMEM/FBS was added to re-suspend the cell pellets. Cell suspension was transferred to tissue culture flask or dish according to split plan and incubated at 37 °C in humidified, 5 % CO₂ atmosphere.

2.2.18.2 Freezing of mammalian cells

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First, cell morphology was examined by microscope. Only healthy cells in the logarithmic proliferation phase were frozen successfully. The DMEM/FBS was aspirated from the tissue culture flask or dish, rinsed with PBS to remove traces of serum. Trypsin was added and incubated at 37 °C. When the cells were detached, DMEM/FBS was added. Cell suspension was transferred to a centrifuge tube and centrifuged for 5 minutes at $500 \times g$. The supernatant was removed and cell pellet was re-suspended in 1.5 ml prepared DMEM with 10 % DMSO per vial. Cell suspension was transferred to labeled vials and frozen at minus 80 °C freezer. After 24 hour vials were transferred to liquid nitrogen.

2.2.18.3 Thawing of mammalian cells

DMSO was removed quickly from cells. Frozen vials were put in 37 °C water bath. As soon as the cell suspension was completely defrosted, they were transferred to a centrifuge tube containing 10 ml DMEM/FEB. Cells were spun down at $1000 \times g$ for 5 minutes. The supernatant was removed and cell pellets were re-suspended into 10 ml DMEM/FEB. Cell suspension was transferred to a culture flask or dish. After incubation for 24 hours, culture medium was changed.

2.2.18.4 Cell extraction

Cells were washed with ice-cold PBS three times, lysed with ice-cold lysis buffer (50 mM Tris.HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100) containing protease inhibitor cocktail (Roche Applied Science) and collected by scraping. The Lysates were centrifuged at 15,000 rpm for 20 minutes at 4 °C and the supernatants were collected. The protein concentration was determined using the protein assay dye

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reagent and bovine gamma globulin standard (Bio-Rad Labs).

2.2.19 Western blot

Equal amounts of proteins ($\mu\text{g}/\text{lane}$) were run on 12 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blocking with $1 \times \text{TBS}$ (20 mM Tris, 500 mM NaCl, pH 7.5) containing 1 % non-fat milk powder for 1 hour at room temperature, the membrane was incubated with primary antibody for 1 hour at room temperature. The membrane was washed twice with TTBS (20 mM Tris, 500 mM NaCl, 0.1 % Tween-20, pH 7.5) and incubated with second antibody for 1 hour at room temperature. The primary antibodies against Bcl-2 (100) (mouse monoclonal; 1:1,000), Bcl-X_{S/L} (S-18) (rabbit polyclonal; 1:1,000), Bax ($\Delta 21$) (rabbit polyclonal; 1:1000) and PARP (H-250) (rabbit polyclonal; 1:1000) and GAPDH (mouse monoclonal 1:30,000, Ambion) were used. The second antibody used was alkaline phosphate (AP)-conjugated goat anti-rabbit immunoglobulin (1:3,000, Bio-Rad) or horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (1:2,000, DAKO A/S, Denmark). Specific proteins were visualized after exposure to instant film.

2.2.20 Cell proliferation assay

Cells were seeded into 96-well plates at $5\sim 8 \times 10^3$ cells per well. Cancer cells were treated with siRNA or rKM-A1 and incubated for appropriate time. When cell proliferation assay was performed, 20 μl combined MTS/PMS solution (Promega) was added to each well containing 100 μl culture medium. After incubating the plate for 2 hours at 37 °C in a humidified, 5 % CO₂ atmosphere, the absorbance of each

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well at 490 nm was recorded by using a 96-well plate reader (Bio-Rad Labs).

2.2.21 Annexin-V-FLUOS staining

Approximately 2×10^5 cells/well were seeded in a 6-well plate. In the following day, cells were treated with rKM-A1/BioPORTER. After 24 hours, cells were harvested by trypsinization, and then resuspended in 100 μ l of Annexin-V-FLUOS staining solution (containing annexin V–fluorescein and propidium iodide) and incubated for 15 minutes at 15-25 °C. The samples were analyzed with a fluorescence-activated cell sorter (FACS) (BD Biosciences, San Jose, CA, USA) using 488 nm excitation and a 515 nm band pass filter for fluorescein detection, and a filter >600 nm for propidium iodide detection.

2.2.22 Caspase-3 activity assay

Caspase-3 activity was determined using the CaspACE™ assay system (Promega). One day before transfection, cells were seeded in 6-well plate. Cells were treated with rKM-A1 at different concentration and incubated for 24 hours at 37 °C in a humidified, 5 % CO₂ atmosphere. Cells were lysed by freezing and thawing. The cell lysates were centrifuged at 15,000 g for 20 minutes at 4 °C and the supernatant was collected. 100 μ g of the total protein from each sample was used for the assay. Cell lysate, 32 μ l of caspase assay buffer, 2 μ l of dimethyl sulfoxide, 10 μ l of DTT (100 mM) and deionized water were added to each well to make up a final 98 μ l of volume. 2 μ l of the substrate (Ac-DEVD-pNA) was added to each well and the plate was incubated at 37 °C for 24 hours. The absorbance was measured at 405 nm using a 96-well plate reader (Bio-Rad Labs).

2.2.23 Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS generation was monitored by flow cytometry, using fluorescence produced from 2',7'-dichlorofluorescein (DCF). H₂DCFDA was chemically reduced and acetylated form of 2',7'-dichlorofluorescein (DCF), which is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. 10 μ M H₂DCFDA stock solution was made and kept tightly sealed to avoid light at -20° C.

Cancer cells were seeded into 6-well plate. The next day, rKML-1A was delivered into cells by BioPORTER. Generation of ROS was detected after 24 hours incubation. Cells were trypsinized and re-suspended in PBS containing the H₂DCFDA to a final 10 nM working solution. After incubation at 37 °C for 10 minutes, the loading buffer was removed by centrifuge and cells were washed with PBS to remove unbound dye. The cells were returned to culture medium and incubated for 10 minutes at 37 °C. This recovery time allowed the cellular esterases to hydrolyze the acetate groups and render the dye responsive to oxidation. Cells were re-suspended in PBS and fluorescence intensity was measured using a FACSCalibur flow cytometer (BD Biosciences).

2.2.24 Assay of ribosome-inactivation activity of RIP

A non-radioactive assay for RIP activity was performed by utilizing a coupled transcription/translation system. Briefly, in this highly sensitive assay the inhibition of protein synthesis by the rRNA-N-glycosidase activity of RIPs was determined quantitatively by measuring luciferase activity in a cell-free rabbit reticulocyte lysate

system (Promega, WI, USA).

Equal amounts of reaction mixture (40 μ l TNT quick master mix, 1 μ l of 1 mM methionine, 2 μ l of luciferase control DNA (0.5 μ g/ μ l) and 7 μ l of nuclease-free water) were incubated at 30 °C for 16 minutes. Then 1 μ l of buffer (PBS, pH7.2, with 0.05 % (w/v) BSA) or rKML-1A was added to the test tubes containing 10 μ l reaction mixture, and then the samples were incubated at 30 °C for another 4 minutes. At this time (t_1) a sample (5 μ l) was taken from each tube and frozen immediately in liquid nitrogen to stop luciferase translation. After incubating for 6 minutes (t_2) a second sample (5 μ l) was taken and treated as described above.

The relative luciferase activity of the samples was determined with a luciferase assay system (Promega). For each reaction tube, the value for the first sample (t_1) was subtracted from the second sample (t_2), yielding the relative luciferase amount generated within this interval. Data from control reaction (no RIP added) were normalized to 100 % translational activity and the data obtained for increasing rKML-1A concentrations were corrected accordingly. By plotting the luciferase activity versus rKML-1A concentration, the IC_{50} value (50 % reduction of residual translational activity as compared to the control) was calculated using a three-parameter-fit equation. This IC_{50} value represents a system-specific value, characterizing the relative rRNA-*N*-glycosidase activity of the investigated RIPs.

2.2.25 Luciferase activity assay

The lucifersase activity was measured with luciferase assay system. Light is produced by converting the chemical energy of luciferin oxidation through an electron

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transition, forming the product molecule oxyluciferin. Firefly luciferase, a monomeric 61 kDa protein, catalyzes luciferin oxidation using ATP · Mg²⁺ as a cosubstrate. To prepare the luciferase assay reagent, luciferase assay buffer was added to the vial containing the lyophilized luciferase assay substrate. This reconstituted reagent was prepared in working aliquots to avoid multiple freeze-thaw cycles and stored at -70 °C avoiding light.

Before beginning measurement, the luciferase assay reagent was fully equilibrated to room temperature. To ensure the temperature equilibration, the thawed aliquots were equilibrated for at least 30 minutes. Luciferase assay reagent (100 µl) was added into a luminometer tube, one tube per sample. The luminometer was programmed to perform a 3-second measurement delay followed by a 15-second measurement read for luciferase activity. Reaction mixture (5 µl) was added to the tube containing the luciferase assay reagent and mixed by pipetting 2 or 3 times. The tube was placed in luminometer and the reading was recorded.

2.2.26 Transfection of mammalian cells with siRNAs

Oligofectamine reagent is a formulation suitable for the transfection of oligonucleotides into eukaryotic cells. The day before transfection, the cells were trypsinized and seeded into 6-well plate or 96-well plate so that they are 30~50 % confluent the day of transfection. Cells were incubated at 37 °C in a humidified 5 % CO₂ incubator until they reach the desired confluency. It was important to maintain a standard seeding protocol and use cells within 20 passages.

For 96-well plate, 0.6 µl 20 µM stock siRNA was diluted with 20 µl Opti-MEN I;

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and 0.6 μ l oligofectamine was diluted with 5.4 μ l Opti-MEN I. After incubation of 5~10 minutes, the diluted oligofectamine reagent was added into diluted oligonucleotide, mixed gently and incubated at room temperature for 15~20 minutes. 23.4 μ l Opti-MEN I was added to the mixture to obtain a final volume of 50 μ l. Culture medium was removed and cells were washed once with Opti-MEN I. siRNA/oligofectamine complexes were then added to each well. The cells were incubated for 2 hours at 37 °C in a CO₂ incubator. Following the incubation, 150 μ l DMEM supplemented with 10 % FBS, without PS was added to each well. The final concentration of siRNA was 60 nM.

For 6-well plate, 6 μ l 20 μ M stock siRNA was diluted with 200 μ l Opti-MEN I; and 6 μ l oligofectamine was diluted with 54 μ l Opti-MEN I. After incubation of 5~10 minutes, the diluted oligofectamine reagent was added into diluted oligonucleotide, mixed gently and incubated at room temperature for 15~20 minutes. 234 μ l Opti-MEN I was added to the mixture to obtain a final volume of 500 μ l. Culture medium was removed and cells were washed once with Opti-MEN I. siRNA/oligofectamine complexes were then added to each well. The cells were incubated for 2 hours at 37 °C in a CO₂ incubator. Following the incubation, 1.5 ml DMEM supplemented with 10 % FBS, without PS was added to each well. The final concentration of siRNA was 60 nM.

2.2.27 Treatment of mammalian cells with rKML-1A

The BioPORTER reagent reacts quickly and interacts non-covalently with the protein creating a protective vehicle for immediate delivery into cells. The

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BioPORTER reagent can fuse directly with the plasma membrane and deliver the captured protein into the cells or the BioPORTER/protein complexes are endocytosed by the cells and then fuse with the endosome releasing the BioPORTER-captured protein into the cytoplasm.

Cells were seeded in 6-well plate and incubated overnight. The second day, protein was delivered into cells when the confluency was about 50 %. Appropriate amount of protein was diluted in 40 μ l PBS (1 μ g protein was used to achieve final 1 mg/ml during transfection). Quickeease tube containing the dry film of BioPORTER reagent was hydrated with the diluted protein solution by pipetting up and down 3 to 5 times. After incubating at room temperature for 3~5 minutes, Quickeease tube was vortexed briefly and gently at low to medium speed for a few seconds. OPTI-MEM I was added to the BioPORTER/protein complex to 1 ml. The growth medium was removed and cells were washed with OPTI-MEM I. The BioPORTER/protein complex diluted in OPTI-MEM I was added to wells containing cells. Cells were incubated in 5 % CO₂ incubator at 37 °C for 4 hours. After 4 hours incubation, same volume of DMEM supplemented with 20 %FBS and 1 %PS was added.

2.2.28 Deliver siRNA by P(MDS-co-CES)

2.2.28.1 Preparation of 0.02 M sodium acetate/acetic acid (NaOAc/HAc) buffer, pH 4.6

The buffer was prepared by mixing 490 ml 0.2 M NaOAc and 510 ml 0.2 M HAc together and subsequently diluting 10 times.

2.2.28.2 Preparation of siRNA and P(MDS-co-CES) complexes

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For preparation of the complexes, the polymeric micelle solution was sterilized by filtration using 0.22 μm filter. Proper volume of polymeric micelles and siRNA was mixed gently at different N/P ratio. The micelles/siRNA complexes were allowed to incubate for 45 minutes at room temperature and topped up to appropriate volume (50 μl for 96-well plate or 500 μl for 6-well plate) with OPTI-MEM I (Invitrogen) before adding into plate.

2.2.28.3 siRNA transfection by P(MDS-co-CES)

MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin and 100 $\mu\text{g/mL}$ of streptomycin at 37 °C under an atmosphere with 5 % CO_2 . Cells were seeded in a 6-well or 96-well plate and incubated overnight. The next day when the cells were 30-50 % confluent, siRNAs were transfected into cells by P(MDS-co-CES). The siRNA and polymer were mixed at different N/P ratio and incubated for 45 minutes at room temperature. Culture medium was removed from MDA-MB-231 cells and siRNA/polymer complexes were added into wells containing cells. After 4 hours of incubation at 37 °C in a 5 % CO_2 humidified incubator, 150 μl or 1.5 ml DMEM supplemented with 10 % FBS was added to each well for 96-well plate or 6-well plate respectively.

3 CHARACTERIZATION OF RECOMBINANT KOREAN MISTLETOE LECTIN

3.1 Expression of the recombinant Korean mistletoe lectin A and B chains

The Korean mistletoe lectins were shown to exist in several isoforms (Park et al., 2001). In the present work, for study we chose KML-1A and KML-1B, which is one of isoforms of Korea mistletoe lectin. Efforts were made to obtain the recombinant Korean mistletoe lectin (rKML). Both KML-1A and KML-1B were cloned into pET14 vector, which contained T7 promoter and N-terminal His-tag. The calculated molecular weights of KML-1A and KML-1B with His-tag are 30.7 kDa and 31.8 kDa, respectively. Both the A- and the B-chains of rKML (rKML-1A and rKML-1B) were expressed separately in the bacterial expression system. Our results showed that rKML-1A was expressed in a soluble form (Fig. 3.1A) while rKML-1B was exclusively expressed in an insoluble form (Fig. 3.1B). Several attempts, which included refolding and different expression conditions, were made to obtain the soluble form of the B-chain but our attempts failed to improve its solubility. Therefore, we focused on the purification and molecular characterization of rKML-1A.

3.2 Purification of A chain of recombinant Korean mistletoe lectin

To obtain a large amount of rKML-1A, cells (10 liter) were grown to exponential phase ($OD_{600\text{ nm}} = 1.0$) and protein was induced with 1 mM IPTG at 30 °C for 4 hours. The hexahistidine-tag fused rKML-1A was then purified by Ni-NTA affinity resin. Nonspecifically and weakly bound proteins were removed by extensive washing using a buffer containing low concentration of imidazole (20 mM). The rKML-1A was

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eluted from the resin using elution buffer containing a high concentration of imidazole (500 mM). Samples of every stage were collected and analyzed on a 12% SDS-PAGE. As shown in Fig. 3.2, the protein sample with little impurity was obtained after the metal affinity purification.

The fractions eluted from Ni^{2+} -NTA affinity column were loaded onto a Sephacryl S-200 gel filtration column for a further purification. Fractions containing rKML-1A were collected and analyzed on a 12% SDS-PAGE. As shown in Fig. 3.3, after S-200 gel filtration, rKML-1A was nearly homogenous. The protein was concentrated to about 1 mg/ml using a concentrator. The purified rKML-1A was stable for 1~2 months when stored at 4 °C. For a longer storage, the protein was frozen in liquid nitrogen and stored at -80 °C.

The purified rKML-1A was confirmed by Western blot. Since rKML-1A contained His-tag fusion protein at N-terminal, it was subjected to Western blotting analysis with the hexahistidine specific antibody. Western blotting result showed a clear single band at 30 kDa position (Fig. 3.3 C), suggesting the purified protein might be rKML-1A with his-tag.

3.3 Molecular characterization of the recombinant Korean mistletoe lectin A-chain (rKML-1A)

Natural mistletoe lectins exist in a heterogeneous form and results in a difficulty in studying their molecular and physical characteristics. The availability of the homogeneous sample would make the characterization more efficient and reliable. To this end, in this study we focused on the molecular characteristics of the purified

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Fig. 3.1 Expression of rKML-1A and rKML-1B. rKML-1A (A) and rKML-1B (B) were expressed in *E. coli* BL21 (DE3) cells. Protein samples were analyzed on 12% SDS-PAGE gel. Lane 1, MW marker, lane 2, before IPTG induction, lane 3-5, the total, soluble and insoluble fractions, respectively after IPTG induction.

Fig. 3.2 Purification of rKML-1A by Ni-NTA. rKML-1A was expressed in *E. coli* BL21 (DE3) cells, purified by Ni²⁺-NTA affinity resin. Protein samples at various stages were analyzed by 12% SDS-PAGE. Lane 1, MW marker; lane 2, loading sample; lane 3, flow-through; lane 4, wash sample; lane 5~9, elution fractions from Ni²⁺-NTA affinity resin.

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Fig. 3.3 Purification of rKML-1A by Sephacryl S-200 gel filtration chromatography. A, Ni-NTA purified rKML-1A was further separated on a gel filtration column Sephacryl S-200. B, fractions 6~12 were collected and analyzed on a 12% SDS-PAGE. C, rKML-1A purified from S-200 gel filtration chromatography was identified by Western blot with anti-His antibody.

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rKML-1A and first performed Circular Dichroism (CD) analysis (Fig. 3.4A), showing that rKML-1A contained both α -helix and β -sheet, and also suggests apparently folded pattern of a globular protein.

To further probe whether rKML-1A is correctly folded, we then monitored 1D ^1H NMR spectrum of rKML-1A (Fig. 3.4B). The 1D ^1H NMR spectrum of rKML-1A shows the characteristics of properly folded proteins featuring good dispersion of resonance lines in the three spectral regions where the methyl protons, α -protons, and amide protons are present and separated (Page et al., 2005). The identification of the purified rKML-1A was further characterized by Mass Spectrometry (MS). The protein was digested with trypsin and analyzed by MS. Blast search showed that the purified protein was KML-1A with 44% sequence coverage (Fig. 3.5). Taken together, the data indicated that the purified protein was rKML-1A and was correctly folded and ready for biochemical and structural studies.

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Fig. 3.4 CD and NMR analysis of the rKML-1A. (A) Circular Dichroism (CD) analysis of the rKML-1A. The purified rKML-1A in PBS buffer was concentrated to 1 mg/ml. The spectrum was recorded in the range of the wavelengths from 190 nm to 250 nm. (B) ¹H NMR analysis of the rKML-1A. The rKML-1A was purified and concentrated to 0.5 mM in the buffer containing 20 mM phosphate, pH 7.0, 20 mM NaCl, 1 mM DTT, 0.01 % NaN₃, and 10 % D₂O. The ¹H spectrum was recorded on Avance 700 MHz NMR spectrometer (Bruker) as described in “Materials and methods”.

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Fig. 3.5 MS analysis of rKML-1A. Pure rKML-1A in PBS buffer was used for MS spectrum analysis. A, rKML-1A was digested by trypsin and analyzed by mass spectrometry. Matched peptide was shown in bold red. The sequence coverage is 44%. B, the MS spectrum showed the digested fragment with observed molecular weight.

3.4 Ribosome-inactivating activity of rKML-1A

The ribosome-inactivating activity of rKML-1A was analyzed using a coupled transcription/translation assay (Langer et al., 1996). Luciferase DNA was used in this system and the inhibition effect was determined by measuring synthesis of luciferase protein, which was determined by luciferase activity assay. To establish optimized assay conditions, the formation of the luciferase protein was monitored over a period of 30 minutes. After an initial transcription phase of about 15~18 minutes, a linear increase of translated protein was observed (Fig. 3.6 A). For each protein, suitable concentration range was chosen to enable reliable IC_{50} value determination. After testing experiments (data not shown), a concentration range from 10 to 1000 $\mu\text{g/ml}$ of rKML-1A was used to determine IC_{50} . Our data showed that the in vitro synthesis of luciferase in a rabbit reticulocyte lysate system was inhibited by the addition of the purified rKML-1A. To determine IC_{50} of rKML-1A, we separately measured the luciferase activity in the absence of the rKML-1A and then normalized to 100 % translational activity. After plotting the luciferase activity versus protein concentration, IC_{50} of rKML-1A was calculated, showing that it was 3.05 nM (Fig. 3.6B). Interestingly, rKML-1A showed an increased IC_{50} in the ribosome-inactivating activity assays compared to the European mistletoe lectin-1, which showed the IC_{50} of 20.5 pM (Eck et al., 1999a).

The Ribosome-inactivating activity of natural KML, which was purified from plant, was also analyzed using the same method in this study, and the IC_{50} was 3.23 nM. Therefore the results suggest that both the rKML-1A and the native KML carry

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Fig. 3.6 Ribosome-inactivating activity of the rKM-1A and KML. A, Time course of luciferase activity, generated in the *in vitro* transcription/translation assay. B, Ribosome-inactivating activity of the rKML-1A (■) and the KML (▲) was determined by measuring the degree of inhibition of luciferase translation in a rabbit reticulocyte lysate system. The luciferase activity of control (without protein) was normalized to 100 % translational activity. And the data from samples with increasing concentration of rKML-1A or KML were corrected accordingly. IC_{50} was about 3.05 nM for rKML-1A and 3.23 nM for KML calculated by a three-parameter-fit equation.

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similar levels of the ribosome-inactivating activity under the assay condition used in this study.

3.5 rKML-1A induces apoptosis in cancer cells

3.5.1 rKML-1A induces cell death related to apoptosis

The natural Korean mistletoe lectin was shown to induce apoptosis in cancer cells (Lyu et al., 2002; Lyu et al., 2001; Yoon et al., 1999). In this study, to confirm and demonstrate the biological effects of rKML-1A in cancer cells, we first performed cell proliferation assays in MCF7/caspases-3 positive cells. The cytotoxic effect of the mistletoe lectins requires both the A- and B-chains: the A-chain for catalytic activity and the B-chain for internalization (Peumans et al., 2001). Since the recombinant B-chain was not available, in this study, a protein delivery reagent BioPORTER was employed for the delivery of rKML-1A into cells, and cytotoxicity of rKML-1A was then evaluated and also compared with that of natural KML. MCF-7/caspases-3 positive cells were treated with rKML-1A or natural KML and after 24 hours, the cell viabilities were measured by cell proliferation assay. We demonstrated that under our condition used in this study the growth of MCF7/caspases-3 positive cells was inhibited by the treatment of rKML-1A in a dose-dependent manner (Fig. 3.7 A). The cells apparently showed reduced cell viability at 1 µg/ml of the recombinant KML-1A, while in the case of the natural KML, it showed cytotoxic effect at 10 ng/ml level (Fig. 3.7 B). These data suggest that the efficiency by using BioPORTER as an artificial delivery vehicle for transporting rKML-1A into cells was considerably lower compared to the B-chain of the natural KML.

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To further define whether the growth inhibition of cancer cells is related to the induction of apoptosis, the cells treated with the rKML-1A/BioPORTER were analyzed by Annexin-V-FLUOS staining (Fig. 3.8). The results showed that the degree of apoptotic cells increased with the concentration of rKML-1A: the degree of apoptotic cells was 10.74% at 1 μ g/ml of rKML-1A and increased to 21.16 % at 10 μ g/ml of rKML-1A, indicating a significant increase in apoptotic cell population over control cells which showed marginal apoptosis by 3.48 %. These data suggest that the cytotoxic effect of rKML-1A in the cancer cells was due to the induction of apoptosis.

3.5.2 Effect of rKML-1A on activation of caspase-3 in cancer cells

Caspase-3 activation is an important event during apoptosis. Thus the effect of rKML-1A on activation of caspase-3 was investigated in MCF-7/caspases-3 positive cells. After the cells were treated with rKML-1A/BioPORTER for 24 hours, the cell lysates were collected to measure caspase-3 activity. As shown in Fig. 3.9 A, the caspase-3 activity significantly increased compared to control cells without treatment with rKML-1A; at 1 μ g/ml of the rKML-1A, the caspase-3 activity increased 1.93 fold and at 10 μ g/ml of the rKML-1A, the activity increased 3.03 fold. The cleavage of PARP, which is one of substrates of caspase-3, was also examined by Western blot analysis. The cleaved ~85KD band could be clearly observed (Fig. 3.9B), which was consistent with the caspase-3 activity assay.

3.5.3 Expression of Bcl-2 family proteins in cancer cells treated with rKML-1A

Bcl-2 family proteins play important role in apoptosis. The balance of pro-apoptotic and anti-apoptotic members of Bcl-2 family is essential for apoptotic

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regulation. Thus proteins with opposite functions compete to regulate the release of cytochrome c from mitochondria. Recent studies showed that the treatment of the mistletoe lectin could alter the expression of Bcl-2 family proteins (Bussing et al., 1999; Kim et al., 2004). Thus, in this study, the expression levels of pro-apoptotic protein Bax, and anti-apoptotic proteins Bcl-2 and Bcl-XL were examined. MCF-7/caspases-3 positive cells were treated with 10 µg/ml rKML-1A and the cell lysates were collected after 24 and 48 hours incubation. As shown in Fig. 3.10, the expression of Bcl-XL and Bcl-2 was reduced while the expression of Bax increased, indicating the rKML-1A induced apoptosis by changing the ratio between anti- and pro-apoptotic proteins.

3.5.4 Effect of rKML-1A on generation of ROS in cancer cells

Reactive oxygen species (ROS) including superoxide anion, H₂O₂ (intracellular hydrogen peroxide), and hydroxyl radicals are constantly formed in all aerobic cells, which is the result of mitochondrial electron transport and several enzymatic reactions. Oxidative stress occurs in cells when there is an imbalance between the rate of generation and removal of ROS. Oxidative stress induces a variety of cellular responses, including apoptosis, activation of MAP kinases and caspase family proteases (Guyton et al., 1996; Tournier et al., 1997; Wilmer et al., 1997). In this study the generation of ROS was examined to determine whether ROS is involved in the apoptosis induced by rKML-1A. MCF-7/caspase-3 positive cells were treated with 1 µg/ml of rKML-1A for 24 hours. The generation of ROS was measured by detecting the fluorescent product from H₂DCFDA by flow cytometry. As shown in Fig. 3.11,

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rKML-1A induced apparent production of ROS compared to control cells (M1, 53.83 % in control cells versus 73.69 % in rKML-1A-treated cells) (ROS peak shifted to the right).

Fig. 3.7 Cytotoxicity of the rKM-1A and the KML on MCF-7/caspase-3 positive Cells. Cells were treated with the rKML-1A (A) and the KML (B) for 24 hours. Cell viability was measured by MTS assay at 490 nm. Cell viability was expressed as a percent relative to untreated control. Experiment was done in triplicate. Data were presented as mean $\pm$ SD (n=3).

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Fig. 3.8 rKM-1A induces apoptosis. MCF-7/caspase-3 positive cells were treated with different concentrations of the rKML-A1 with BioPORTER reagent. 1, control cells without the rKML-1A; 2, cells treated with 1 μ g/ml rKML-1A; 3, cells treated with 10 μ g/ml rKML-1A. After 24 hours, cells were stained with Annexin-V-Fluorescein and Pridium iodide, and analyzed by FACS. The apoptotic cells appeared in the lower right area.

Fig. 3.9 Effect of rKML-1A on activation of caspase-3 and cleavage of PARP. Cells were incubated in the absence or presence of rKML-1A. At 24 hours after the treatment, cell extracts were prepared and subjected to caspase-3 activity assay (A: Data was presented as mean $\pm$ SD (n=2)) and Western blot using anti-PARP antibody (B). 1, control cells without treatment; 2, cells treated with 1 μ g/ml rKML-1A; 3, cells treated with 10 μ g/ml rKML-1A.

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Fig. 3.10 Expression of pro-apoptotic and anti-apoptotic proteins in MCF-7/caspase-3 positive cells. After cells were treated with rKML-1A, the whole cell lysates were collected and total proteins were separated on a 12 % SDS-PAGE, followed by immunoblotting with anti-Bax, Bcl-2, Bcl-XL, GAPDH antibody. 1, cells without any treatment; 2, cells treated with BioPORTER only; 3, cells treated with rKML-1A for 24 hours; 4, cells treated with rKML-1A for 48 hours.

Fig. 3.11 Effect of rKML-1A on generation of ROS in cancer cells. MCF-7/caspase-3 positive cells were incubated with rKML-1A for 24 hours and then harvested by trypsinization. After incubation with H₂DCFDA for 10 minutes, the unbound dye was washed out and intracellular fluorescence intensity was measured by flow cytometry. The green line represents control cell without treatment; the pink line represents cells treated with 1 μg/ml rKML-1A.

3.6 Homology modeling of Korean mistletoe lectin

The sequence of KML-1 was compared with that of European mistletoe lectin-1, Himalayan mistletoe lectin and Ricin (Fig. 3.12 and Fig. 3.13). KML-1 is highly homologous to European mistletoe lectin-1, showing 92.9 % sequence identity of A-chain and 83.2 % sequence identity of B-chain. This high degree sequence homology led us to study the 3-dimensional structure of KML-1 by building a homology model. The model of KML-1 was obtained by using Modeller 8v1, and European mistletoe lectin-1 complexed with galactose (PDB code 1OQL) was used as template. The model obtained was checked by the program PROCHECK, yielding 93 % of the backbone conformations within the most favored regions of the Ramachandran plot. For the X-ray structure of 1OQL (Niwa et al., 2003), the percent of the backbone conformations within the most favored regions of the Ramachandran plot are 87.6 %.

X-ray structures of Himalayan mistletoe lectin (PDB ID:1YF8) and Ricin (PDB ID:2AAI) were also used to analyze RMSD of the C α -trace. When analyzing the structural alignment of the C α -traces between KML-1 and EML-1, HML and Ricin, the RMSD between KML-1 and EML-1, HML and Ricin was 0.218, 0.533 and 0.787 Å, respectively. The structure generated from homology modeling was highly homologous to that of European ML-1, indicating that the overall three-dimensional topologies between two proteins were nearly identical. (Fig. 3.14A)

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Chain A

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KML-1A  -YERLRLRVTHQTG--DEYFRFITLLRDYVSSGS-F SNEIPLLROSTIPVSDAQRFLV 55
EML-1A  -YERLRLRVTHQTG--DEYFRFITLLRDYVSSGS-F SNEIPLLROSTIPVSDAQRFLV 55
HML-A    -YERLDLDVTSQTG--BEYFRFITLLRDYVSSGS-F SNEIPLLROSGGGV-BAARFVL 54
Ricin-A  IFPKQYPIINFTTAGATVQSYTNFIRAVRGRITGADVREHIPVLPN-RVGLPINQRFIL 59
          : :   :.  ***  :.*.**  :*. :***: . :****: * : :.  ***:

KML-1A  VE LTNQGGDSITAAIDVTNIYVVAQAGDQSYFLRDAPD---GAERHLFTGTT-RSSLPF 111
EML-1A  VE LTNQGGDSITAAIDVTNIYVVAQAGDQSYFLRDAPR---GAETHLFTGTT-RSSLPF 111
HML-A    VE LTNQGGDSITAAIDVTNIYVVAQAGSQSYFLS-----GPGTHLFTGTT-RSSLPF 106
Ricin-A  VELSNHAELSVTLALDVTFYVVGIRAGNSAYFFHPDNQEDAEAITHLFTDVQNRFTFAF 119
          ***:*. .  *: * :***** :*.*:***: .  *****  * :*.*

KML-1A  TGSYTDLERFAG-HRDQIP LGREELIQSVSALRFPGS--NTRAQARSFIILLIQMI:EAA 167
EML-1A  NGSYPDLEFYAG-HRDQIP LGIDQLIQSVTALRFPGG--STRTQARSILILLIQMI:EAA 167
HML-A    NGSYPDLEQYAG-HRQIP LGIDQLIQSVTALRFPG---NTRTQARSILILLIQMI:EAA 161
Ricin-A  GGNVDRLEQLAGNLRENIELGNPLEBAISALYYYSTGGIQLPTLARSFIICIQMI:EAA 179
          *. *  ** : *  * :.* *  *  * :***: * : .  .  : ****: * *****

KML-1A  RFNPIILWRARQYISGGSEFLPDVTYLQLETSGGQSTQVQHSTDGVEFNNPIRLTISTGVF 227
EML-1A  RFNPIILWRARQYINSGASEFLPDVYMLELETSGGQSTQVQHSTDGVEFNNPIRLAIPPGNF 227
HML-A    RFNPIILWRARQYINSGASEFLPDVYMLELETSGGQSTQVQSTEGVEFNNPIRLAIP-GNF 220
Ricin-A  RFQYIEGEMRTRIIRYNRRSAPDPSPVITLENSWGRLLSTAIQESNQGAFAPIQLQRFNGEK 239
          **: *  .  *  *  .  **  : : ** :***: ** :*.*:*. * . ** : *

KML-1A  VTLENVROVIASLAIMLFVCEDRPSSS- 254
EML-1A  VTLENVROVIASLAIMLFVCG----- 248
HML-A    VTLENVROVIASLAIMLFVC----- 240
Ricin-A  FSVYDVSIILIPILALMVYRCAPPPSSQF 267
          . : : *  :*.* :***: *  . :*.*

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Fig. 3.12 Multiple amino acid sequence alignment of A chains of rKML-1, EML-1, HML and Ricin. Key residues in the catalytic site are shadowed by grey color, and residues involved in the glycosylation are shadowed by turquoise color. KML-1A: the amino acid sequence of the recombinant Korean mistletoe lectin I A chain; EML-1A: the amino acid sequence of the European mistletoe lectin I A chain; HML-A: the amino acid sequence of the Himalayan mistletoe lectin A chain; Ricin-A: the amino acid sequence of the Ricin A chain.

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[illegible]

Fig. 3.13 Multiple amino acid sequence alignment of B chains of rKML-1, EML-1, HML and Ricin. Residues that form hydrogen bonds with galactose are shadowed by red color, residues that provide the hydrophobic contact are shadowed by green color and residues involved in the glycosylation are shadowed by turquoise color. KML-1B: the amino acid sequence of the recombinant Korean mistletoe lectin I B chain; EML-1B: the amino acid sequence of the European mistletoe lectin I B chain. HML-B: the amino acid sequence of the Himalayan mistletoe lectin B chain; Ricin-B: the amino acid sequence of the Ricin B chain.

3.7 Conservation of catalytic site of KML

As with the A-chain of EML-1, the A-chain of KML-1 was predicted to excise a specific adenine base from a highly conserved loop region of 28S rRNA (Endo et al., 1988). In the structure obtained from homology modeling, the active site region of KML-1 was located in a prominent cleft (Fig. 3.14B). The alignment of the amino acid sequences indicated that all four invariant residues in the active site, which were conserved throughout the wide range of RIPs (Katzin et al., 1991), were also conserved in KML-1A. The overall architecture of the active site cleft region remained almost constant as well.

The key catalytic residues Tyr76, Tyr115, Glu165, and Arg168 in the A-chain of EML-1 (Niwa et al., 2003) were superimposed with KML-1A, showing a high degree of structural similarity with only a minor variation (Fig. 3.15A). There was one N-linked glycosylation site in EML-1, and the residue involved was Asn112. On the other hand, in the KML-1A, Asn112 was replaced by Thr112. Whether this has any influence on the overall anti-tumor activity of KML-1A needs to be further explored.

3.8 Molecular basis of glycosylation and sugar binding characteristics of KML

B-chain of rKML-1 was supposed to bind to putative receptors on the cell surface and subsequently KML was transported into cells through endocytosis (Narayanan et al., 2005). So the B-chain is necessary for cytotoxicity of type-II RIPs.

There were three N-glycosylation sites in the EML-1B, but only one remained in KML-1B, as shown in the sequence alignment of B-chains (Fig. 3.13). The N-glycosylation motif is N-X-S/T, where X is any amino acid but proline. The three

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Fig. 3.14 3D structure of rKML by homology modeling. (A) 3D structural model of the rKML. (B) Rectangle indicates the nucleotide binding site ("NUC") for the KML-1A. This figure was generated using PyMol version 0.97 (Delano).

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N-glycosylation sites of EML-1B were NGS (61-63), NGT (96-98), NDT (136-139). The corresponding residues at the same sites in the rKML-1B were NGR (61-63), NGI (96-98) and NDT (139-141). The replacement of R63 and I98 at the 1st and 2nd N-glycosylation sites in KML-1B led to the loss of glycosylation.

Sugar binding characteristics of rKML was also studied by molecular modeling. The hydrogen bonding between galactose and EML-1B was determined after adding hydrogens to the protein at pH 7. The residues of the EML-1B involved in the hydrogen bonding with galactose at the N-terminal site were Asp23, Asp26, Lys41 and Asn47 with Trp38 providing the hydrophobic contact, while at the C-terminal site the residues involved were Asp235, Gln238 and Asn256 with Tyr249 providing the hydrophobic contact; In EML-1B, both the Asp23 and Asp235 satisfied the donor-acceptor distance criteria of 3.9Å for the hydrogen bonding. But when we added the hydrogens to the protein at pH 7, the hydrogen-acceptor distance criteria of 2.5 Å was not met, due to which we were unable to see the hydrogen bonding in our model. However the corresponding residues in KML-1B were Asp23 and Asp238, which were very well conserved. We then superimposed the KML structure with that of EML and studied the differences at both the C and N-terminal sites. At the N-terminal site, while Asp23, Lys41, Asn47 and Trp38 were conserved, the only residue mutated in KML-1B was Glu26 instead of Asp26 (Fig. 3.15 B). At the C-terminal site while Asp238 and Asn259 were conserved, the residue mutated was Glu241 instead of Gln238, and the Tyr249 which provided the hydrophobic contact was mutated to Phe252 in KML-1B (Fig. 3.15 D). Since only the main chain atoms of Asp26 and Gln

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238 in EML-1B were involved in the hydrogen bonding, the corresponding residues mutated into Glu26 and Glu241 in KML-1B were speculated to make little influence on the galactose binding. Also the Tyr249 in EML-1B mutated to Phe252 in KML-1B can still provide the hydrophobic contact for galactose. From the above studies it was clear that the catalytic and galactose binding sites of KML were similar and conserved as that of EML-1. Therefore, KML might have a bit different degree of biological effect or activity compared to EML-1.

Recently, a third sugar binding site was reported for Himalayan mistletoe lectin (HML) (Mishra et al., 2005). We then performed multiple sequence alignment of KML, EML-1, HML and Ricin by using clustal W program (Thompson et al., 1994) which was shown in Fig. 3.12 and Fig. 3.13. From the sequence alignment, interestingly all the residues at the third sugar binding site of HML were absolutely conserved in EML; while in KML the residues mutated were Gly85, Tyr79 and in Ricin the residues mutated were Tyr78, Ala84. We then superimposed KML structure with X-ray structure of HML after adding the hydrogens at pH 7 and then studied the differences at the third sugar binding site. As shown in Fig. 3.15C, the residues of HML-B involved in providing hydrophobicity to galactose and glucose moiety of lactose were Tyr66, Phe75 and Tyr64 and the corresponding residues in KML-1B were Tyr70, Tyr79 and Tyr68. The residues of HML-B involved in hydrogen bonding with galactose and glucose moiety of lactose were Thr63, Val81 and Tyr71 and the corresponding residues in KML-1B were Thr67, Gly85 and Tyr75. Since only main chain amide nitrogen of Val81 in HML-B was involved in the hydrogen bonding, the

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corresponding residue mutated into Gly85 was speculated to make little influence on the binding of lactose. Also the Phe75 in HML-B mutated to Tyr79 in KML-1B can still provide the hydrophobic contact for galactose moiety of lactose; in HML-B, both the Thr63 and Tyr71 satisfy the donor-acceptor distance criteria of 3.9 Å for the hydrogen bonding. But when we added the hydrogens to the protein at pH 7, the hydrogen-acceptor distance criteria of 2.5 Å was not met, due to which we were unable to see the hydrogen bonding in our model.

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Fig. 3.15 Analysis of the active site of rKML-1A and sugar-binding sites of rKML-1B. (A) Active site residues of the KML-1A (green) superimposed with the active site residues of the A-chain of EML-1 (yellow). Annotations for the EML-1A and KML-1A are indicated by red and blue colors, respectively. (B) Superimposition of N-terminal galactose binding sites of KML-1B. Superimposition of N-terminal galactose binding site residues of KML-1B (green) with EML-1B (yellow). Galactose (GAL) is shown in yellow color and the corresponding hydrogen bonding

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between galactose and EML-1B is indicated in dashed lines. Annotations for the EML-1B and KML-1B are indicated by red and blue colors, respectively. (C) Superimposition of lactose binding residues of KML-1B (green) with HML-B (yellow). Lactose (LAC) is shown in yellow color and the corresponding hydrogen bonding between galactose moiety of lactose and HML-B is indicated in dashed lines. Annotations for the HML-B and KML-1B are indicated by red and blue colors, respectively. (D) Superimposition of C-terminal galactose binding site residues of KML-1B (green) and EML-1B (yellow). Galactose (GAL) is given in yellow color and the corresponding hydrogen bonding between galactose and EML-1B is indicated in dashed lines. Annotations for the EML-1B and KML-1B are indicated by red and blue colors, respectively.

3.9 Mutagenesis study of active site of KML-1A

3.9.1 Construction, expression, and purification of active mutants

The modeling study indicated that the active site region of the KML was located in a prominent cleft. The four invariant residues in the active site, which are conserved throughout the wide range of RIPs, are conserved in KML-1A. The overall architecture of the active site cleft also remains conserved.

The key catalytic residues Tyr76, Tyr115, Glu165 and Arg168 of the A-chain of KML-1A are superimposed with EML-1 (Niwa et al., 2003), showing a high degree of structural similarity with only a minor variation. The catalytic importance of these key residues was investigated by site-directed mutagenesis. Four mutations Y76F, Y115F, E165Q and E168Q were made in the present study. As shown in Fig. 3.16, the four active mutations were expressed in our expression system, but only rKML-1A Y76F and rKML-1A Y115F were expressed in soluble forms while the other two mutants (E165Q and E168Q) were expressed insoluble. Thus the two soluble proteins were characterized.

3.9.2 Enzyme activity analysis of active site mutants

To retermine the critical role of these two amino acids in the catalytic site, the ribosome-inactivating activity assays of rKML-1A Y76F and rKML-1A Y115F were performed in a coupled transcription/translation system by using the same protocol as used for rKML-1A. Preliminary experiments were performed to choose suitable concentration for IC₅₀ determination (data were not shown). As shown in Fig. 3.19, rKML-1A Y76F and rKML-1A Y115F were still able to inhibit the synthesis of

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luciferase, but the concentration required to exert the similar catalytic effect was significant higher compared to that of the wild-type. The IC₅₀ was 28.95 nM and 27.47 nM respectively, which were about ten times higher than that of rKML-1A. The result obtained in the present study was consistent with the previously published data about ricin, which showed that conversions of tyrosines 80 and 123 to phenylalanines caused decrease of catalytic activity roughly 10-fold (Lord et al., 1994).

Table 3.1 Sequence of the primers used to construct four mutants rKML-1A Y76F, Y115F, E165Q and E168Q

Forward primer for rKML-1A Y76F in pet14	CCATCGACGTTACTAACCTGTTCGTGGTGGC TACCAAGCAGG
Reverse primer for rKML-1A Y76F in pet14	CCTGCTTGGTAAGCCACCACGAACAGGTTA GTAACGTCGATGG
Forward primer for rKML-1A Y115F in pet14	CCTCCCATTCACCGGAAGCTTCACAGATCTG GAGCGATTC
Reverse primer for rKML-1A Y115F in pet14	GAATCGCTCCAGATCTGTGAAGCTTCCGGTG AATGGGAGG
Forward primer for rKML-1A E165Q in pet14	CTCATT CAGATGATCTCCCAGGCCGCCAGAT TCAATCCC
Reverse primer for rKML-1A E165Q in pet14	GGGATTGAATCTGGCGGCCTGGGAGATCAT CTGAATGAG
Forward primer for rKML-1A R168Q in pet14	GATGATCTCCGAGGCCGCCCAATTCAATCCC ATCTTATGG
Reverse primer for rKML-1A R168Q in pet14	CCATAAGATGGGATTGAATTGGGCGGCCTCG GAGATCATC

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Fig. 3.16 Expression of rKML-1A Y76F, Y115F, E165Q and E168Q. Four mutants were expressed in *E. coli* BL21 (DE3) cells. Protein samples were analyzed on a 12 % SDS-PAGE gel. Lane 1, MW marker, lane 2, -IPTG, lane 3 and 4, total and soluble fractions of rKML-1A Y76F after IPTG induction, lane 5 and 6, total and soluble fractions of rKML-1A Y115F after IPTG induction, lane 7 and 8, total and soluble fractions of rKML-1A E165Q after IPTG induction, lane 9 and 10, total and soluble fractions of rKML-1A E168Q after IPTG induction.

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Fig. 3.17 Purification of rKML-1A Y76F by Ni-NTA and Sephacryl S-200 gel filtration chromatography. A, rKML-1A Y76F was expressed in *E. coli* BL21 (DE3) cells, purified by Ni^{2+} -NTA affinity resin. Lane 1, MW marker; lane 2, total cell lysate after IPTG induction; lane 3, loading sample; lane 4, flow-through; lane 5, washed sample; lane 6~10, elution fractions from Ni^{2+} -NTA affinity resin. B, Purification of rKML-1A Y76F by Sephacryl S-200 gel filtration column chromatography. Fractions were collected and analyzed on a 12% SDS-PAGE. C, profile of Sephacryl S-200 gel filtration column chromatography is shown.

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Fig. 3.18 Purification of rKML-1A Y115F by Ni-NTA and Sephacryl S-200 gel filtration chromatography. A, rKML-1A Y115F was expressed in *E. coli* BL21 (DE3) cells, purified by Ni^{2+} -NTA affinity resin. Lane 1, MW marker; lane 2, total sample after IPTG induction; lane 3, loading sample; lane 4, flowthrough; lane 5, wash sample; lane 6–10, elution fractions from Ni^{2+} -NTA affinity resin. B, Purification of rKML-1A Y115F by Sephacryl S-200 gel filtration chromatography. Fractions with high UV absorbance were collected and subjected to 12% SDS-PAGE. C, profile of Sephacryl S-200 gel filtration column chromatography is shown.

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Fig. 3.19 Ribosome-inactivating activity of the rKM-1A Y76F and Y115F. Ribosome-inactivating activity of the rKML-1A Y76F (▲) and the Y115F (■) were determined by measuring the degree of inhibition of luciferase translation in a rabbit reticulocyte lysate system. The luciferase activity of control (without protein) was normalized to 100 % translational activity. And the data from samples with increasing concentration of proteins were corrected accordingly. IC₅₀ was calculated by a three-parameter-fit equation.

3.10 Discussion

3.10.1 Characterization of rKML-1A

In recent years, the potential of mistletoe lectins and their biological effects in cancer therapy have been studied. The A-chain of the heterodimeric mistletoe lectins induces apoptosis in cancer cells upon its entry to cells; B-chain binds a potential receptor on the cell surface through its sugar binding properties. The physiological function of the type-II RIPs has been shown to inhibit protein synthesis, modulate telomerase, and regulate the phosphorylation of protein kinases in mammalian cells (Choi et al., 2004; Narayanan et al., 2005). Yet, the detailed molecular basis for the effects of the mistletoe lectins is unclear. In this work, to better understand and define the function of the mistletoe lectins, KML was chosen and characterized. The recombinant KML-1A and KML-1B (rKML-1A and rKML-1B) were expressed in our bacterial expression system, showing that rKML-1A was expressed in a soluble form, while rKML-1B was insoluble. Thus, in the current study focus was made on the characterization of KML-1A. Our bacterial expression system was efficient to produce rKML-1A and allowed us to obtain homogeneous rKML-1A, after purification by Ni-NTA and gel filtration chromatography. The CD and NMR results suggest that the purified A-chain showed characteristics of a well-folded protein.

We first examined the ribosome-inactivating activity of the rKML-1A. The IC_{50} value of rKML-1A was comparable to that of the native KML, indicating that the recombinant A-chain maintains the similar level of the catalytic activity in inhibiting

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translation as compared to the natural KML.

The cytotoxicity of native KML was investigated in various human and murine tumor cells. It was shown that cytotoxic activity of KML was higher than that of EML (Yoon et al., 1999). Cell proliferation assay done in this study indicates that the rKLM-1A was active in exerting cytotoxicity in cancer cells. However, the cytotoxicity was lower than that of the native KML; the native KML induced apoptosis in human cancer cell at concentration range of ng/ml (Lyu et al., 2002; Yoon et al., 1999). The cytotoxicity of rEML was similar when compared with that of EML (Eck et al., 1999b). Thus we speculate that the difference between the native KML and rKML-1A is due to the delivery efficiency of the artificial delivery system used in the study, which might be lower than that of the native KML B-chain.

Study with *Viscum album* L. *coloratum* agglutinin (VCA) isolated from the Korean mistletoe (*Viscum album* L. *coloratum*), showed that VCA induced apoptosis when various cells were treated with VCA (Choi et al., 2004; Pae et al., 2000a; Pae et al., 2000b; Yoon et al., 1999). To confirm that rKML-1A induces cell death through apoptosis, Annexin-V-FLUOS staining was performed. Results from this study indicate that rKML-1A induced apoptotic cell death in MCF-7/caspase-3 positive cells, and the expression of Bcl-2 family proteins changed after the rKML-1A treatment.

Isolation of mistletoe lectins from plant produces inhomogeneous material probably due to posttranslational modifications. The impurity of the preparation of native lectin sample caused the studies on the mechanisms of the lectins inducing

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apoptosis in cancer cells or action on immune system to be difficult. Compared to the recombinant European mistletoe lectin, which was expressed in inclusion body and refolded to an active protein, homogeneous rKML A-chain could be purified from soluble fraction with a high yield in the present study and led us to perform the structural and mechanistic studies, allowed us to gain insights into the mode of action, and also explore the application of rKML A-chain in cancer therapy.

3.10.2 Purification of rKML and rKML-1B

Type-II RIPs exert their cytotoxicity more to cancer cells than to normal cells through sugar binding activity of B-chain. Therefore the expression and purification of the recombinant B-chain is very important to study the specificity and undefined receptors on cancer cell surface. The sugar-binding specificity is different between three isoforms of EML. EML-1 is mainly specific to D-galactose, EM III is mainly specific to N-acetyl-D-galactosamine and EM II is specific for both (Franz et al., 1981; Olsnes et al., 1982). In the case of KML-C purified from plant extract, it showed affinity to both GalNAc and D-Gal (Yoon et al., 1999). When expressed in *E. coli* system, the rKML-1B was insoluble (Fig. 3.1). We speculate that the insolubility of rKML-1B might be attributable to the requirement of disulfide bond formation in the course of folding. To obtain rKML-1B, various attempts including refolding, co-expression with rKML-1A and co-expression with bacterial chaperones were made, but we failed to obtain the B-chain in soluble form.

In plants, Mistletoe lectin is initially synthesized as a single precursor protein, in which the A subunit and the B subunit are connected by a link sequence. During the

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biosynthetic process, the link peptide is removed and inter-chain disulfide bond is formed to generate the heterodimeric native protein (Hartley and Lord, 2004). On the basis of these, it would be reasonable to design a single chain on which A-subunit and B-subunit were connected by a link peptide. To this end, several link sequences were designed, with TEV protease recognition site included. The genes encoding KML-1A and KML-1B were cloned into several vectors (pET16, pRSET, pETM30, pETSUMO) to produce single chain lectins with variable link sequences. But the expressions of single chain proteins were very low and obtained little proteins.

The cytoplasm of *E. coli* is in reduced environment, which makes the formation of disulfide bonds difficult (Rudolph and Lilie, 1996). Refolding of the denatured proteins in a buffer containing a defined reduced/oxidized system is an alternative strategy. In this study, co-refolding method was tried according to the published protocol (Eck et al., 1999b). Association of two chains was achieved (after two proteins were rebuffed to the same buffer containing only 2 mM dithiothreitol) by dropwise addition of the two proteins to an association buffer in the presence of the cysteine/cystine reduced/oxidized system. A 90-fold dilution was described as adequate (Clark, 1998; Eck et al., 1999a; Rudolph and Lilie, 1996) by excessive dilution of the protein dissolved in chaotropic salts. Two subunits were covalently associated to form heterodimer through the refolding process. Although the recombinant European mistletoe lectin was successfully obtained by this method, the refolding efficiency of rKML-1A and rKML-1B was extremely low and dimer formation was barely detected on a non-reducing SDS-PAGE gel.

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Small Ubiquitin-like Modifier (SUMO) system is frequently used to increase the expression of recombinant proteins and enhance the solubility. And the tertiary structure of the SUMO protein is specifically recognized and cleaved by an ubiquitin-like protein-processing enzyme, SUMO C-terminal peptidase. Thus SUMO fusion can be easily removed to generate native protein. The gene of rKML-1B was also cloned into pET SUMO vector. The expression and purification conditions were optimized. For expression, the induction was performed at 20 °C overnight in presence of 1 % glucose and 0.5 mM IPTG. The imidazole concentration in wash buffer was increased to 150 mM to remove nonspecific bands. SUMO system increased the solubility of rKML-1B and led us to obtain a small amount of the rKML-1B, but the isolated rKML-1B was unstable. Taken together, our results suggest that rKML-1B shows different molecular characteristics as compared to European mistletoe lectin.

3.10.3 Modeling study of rKML

Type-II RIPs are highly homologous among the family members (Park et al., 2001) and encouraged us to perform homology modeling on KML. Based on the stereochemical quality and residue-by-residue geometry checked by PROCHECK, the overall structural fold of KML is similar to the X-ray structure of EML-1 (Niwa et al., 2003). The alignment of the amino acid sequences indicates that the key catalytic residues of the A-chain of rKML-1 were well conserved in the active site of EML-1 as well as other RIPs (Katzin et al., 1991). Our structural model confirmed the conservation of the catalytic sites in KML (Fig. 3.15A). Mutational study

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demonstrated that these conserved key catalytic residues were critical for the ribosomal-inactivating activity of rKML-1A. The rKML-1A mutants (Y76F and Y115F) significantly affected catalytic activity of KML-1A, which was consistent with similar study about ricin (Lord et al., 1994).

The Asn112 of the A-chain of EML-1 was shown to be important in the N-linked glycosylation (Niwa et al., 2003). Interestingly, the Asn112 is replaced by Thr112 in KML-A1 instead (Fig. 3.12). Previous studies showed that in European mistletoe lectin no significant difference in N-glycosidase activity between the native A-chain and recombinant A-chain was observed (Eck et al., 1999a). Also, the plant-derived ricin A-chain, recombinant unglycosylated ricin A-chain and chemically deglycosylated plant-derived ricin A-chain showed similar cytotoxicity when examined in a human cancer cell line (Shire et al., 1990). In this study rKML-1A and native KML had similar ribosome-inactivating activity. However, whether the mutation in the glycosylation site on the A-chain of the lectins has any influence on the overall activity of KML-A remains to be answered.

The B-chains of the type-II RIPs contain a few sugar binding sites. They shared a high degree of sequence similarity, and so were their 3-dimensional structures. However, their sugar binding specificities are predicted to vary. These expected differences in the specificities might be important (Frankel et al., 1996; Steeves et al., 1999), because the biological effects of the type-II RIPs were determined by the binding of the B-chains to the potential sugar-containing receptors on the cell surface. The structural superimposition of the B-chain of EML-1 with rKML-1B revealed that

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some differences at both the C- and N-terminal sites existed. At the N-terminal site, while Lys41, Asn47 and Trp38 were conserved, Asp26 was changed to Glu26 in rKML-1B instead. At the C-terminal site, while Asn256 was conserved, Gln238 was changed to Glu241 in rKML-1B (Fig. 3.15B, D). Since only the main chain atoms of Asp26 and Gln238 in the B-chain of EML-1 were involved in the hydrogen bonding, the above mentioned mutations were expected to affect little on the molecular interaction of the B-chains with the sugar. Also from our studies at the third sugar binding pocket recently identified in HML-B, all the residues at the third sugar binding site were absolutely conserved in EML-1B while the residues mutated in rKML-1B were Gly85, Tyr79. Since only main chain amide nitrogen of Val81 in HML-B was involved in the hydrogen bonding, the corresponding residue mutated into Gly85 in rKML-1B was speculated to make little influence on the binding of lactose. Also the Phe75 in HML-B mutated to Tyr79 in rKML-1B can still provide the hydrophobic contact for galactose moiety of lactose. Taken together, our studies suggested that the galactose binding sites of rKML-1B are similar but not the same compared to that of EML-1. Apart from sugar-binding specificities, we have noticed that the B-chain of EML-1 or HML contained three N-linked glycosylation sites; B chain of ricin had two N-linked glycosylation sites; while rKML-1B contained only one N-glycosylation site (Fig. 3.13) and whether the absence of the two N-glycosylation sites in the rKML-1B has any influence on its biological function might be worthy of further investigation.

In summary, in the current study, we successfully purified and characterized

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rKML-1A by biophysical and biological methods. rKML-1A was demonstrated to induce cell death through apoptosis involving activation of caspase-3, regulation of pro- and anti-apoptotic proteins and generation of ROS. Modeling study indicated that the catalytic and galactose binding sites of KML showed a similar topology, but difference were observed in the sugar binding site. Future study involving the sugar binding characteristics of B-chain of KML might provide a clue for better comprehension of the biological effects of mistletoe lectins and its molecular basis in inducing apoptotic cell death in cancer cells.

4 siRNA-MEDIATED CHEMOSENSITIVITY ENHANCEMENT OF CANCER CELLS

4.1 Design of siRNAs

In the current experiment, three siRNAs targeting Bcl-2, one siRNA targeting Bcl-XL, one FITC-labeled siRNA and one scramble siRNA were designed, which were all 21-mers with two dT overhanging at 3' end. The strategy for choosing siRNA target sites was based on the observation by Elbashir et al. (Elbashir et al., 2001a) in which siRNA with 3' overhanging UU dinucleotides was the most effective. Since some regions of mRNA may be either highly structured or bound by regulatory proteins, normally several target sites of the mRNA were selected. Target sequences were aligned in a BLAST search to eliminate those with significant homology to other genes. For Bcl-2, three siRNAs corresponding to target sequences located in 5', middle and 3' regions of the mRNA sequence were designed. The nucleotide sequences, corresponding positions at target mRNA and GC content of the siRNAs were shown in Table 4.1. A scramble siRNA, which lacked significant sequence homology to the genome, was used as a negative control. FITC-labeled siRNA was also used to monitor the transfection efficiency of siRNA. All of the siRNAs were chemically synthesized and resuspended in the RNase-free buffer and stored at -80 °C.

4.2 Protein expression level in breast cancer cells

Before siRNA transfection, the expression levels of anti-apoptotic proteins Bcl-2 and Bcl-XL were examined in breast cancer cell lines MCF-7 / caspase-3 positive and MDA-MB-231. These two cell lines expressed different levels of Bcl-2 and Bcl-XL.

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Table 4.1 Sequences of Bcl-2, Bcl-XL siRNAs, scramble siRNA and FITC-siRNA. The GC content of siRNAs is also indicated.

siRNA	Sequence	Position	GC%
HY-SR1	Sense strand 5'-CUGUACGGCCCCAGCAUGCdTdT-3'	Corresponding to 599-619 nt of Bcl-2 mRNA	62 %
	Antisense strand 5'-GCAUGCUGGGGCCGUACAGdTdT-3'		
HY-SR2	Sense strand 5'-GUACAUCCAUAUAAGCUGdTdT-3'	Corresponding to 49-69 nt of Bcl-2 mRNA	33 %
	Antisense strand 5'-CAGCUUAUAAUGGAUGUACdTdT-3'		
HY-SR3	Sense strand 5'-CAUCGCCCUGUGGAUGACUdTdT-3'	Corresponding to 514-534 nt of Bcl-2 mRNA	52 %
	Antisense strand 5'-AGUCAUCCACAGGGCGAUGdTdT-3'		
HY-SR4	Sense strand 5'-GUCUCCAAGCGGAUCUCGUdTdT-3'	Scramble siRNA	52 %
	Antisense strand 5'-ACGAGAUCCGCUUGGAGACdTdT-3'		
HY-SR9	Sense strand 5'-GCGUAGACAAGGAGAUGCAdTdT-3'	Corresponding to 595-613 nt of Bcl-XL mRNA	48 %
	Antisense strand 5'-UGCAUCUCCUUGUCUACGCdTdT-3'		
FITC-siRNA	Sense strand 5'-UUCUCCGAACGUGUCACGUdTdT-3'		48 %
	Antisense strand 5'-ACGUGACACGUUCGGAGAAdTdT-3'		

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As shown in Fig. 4.1, the expression level of Bcl-2 protein was higher in MCF-7/caspase-3 positive cells compared to that in MDA-MB-231 cells. In contrast, Bcl-XL protein level was higher in MDA-MB-231 cells.

4.3 Transfection efficiency of siRNA in two cancer cell lines

Transfection efficiency of siRNAs was first evaluated in MCF-7/caspase-3 positive cells and MDA-MB-231 cells. After 24 hours of incubation, the cells were washed with PBS twice and observed under fluorescent microscope to monitor FITC-siRNAs. The cells were then harvested through trypsinization and transfection efficiency was analyzed by FACS. As shown in Fig. 4.2, the majority of siRNA were internalized into MCF-7/caspase-3 positive cells and MDA-MB-231 cells. Transfection efficiency varied with the concentration of siRNA and cell lines. In MCF-7/caspase-3 positive cells, the efficiency was 59.4 % (M1) at 120 nM; 61.4 % (M1) at 60 nM; 60.3 % (M1) at 40 nM; 55.5 % (M1) at 20 nM (Fig. 4.3). In MDA-MB-231 cells, the efficiency was 73.6 % (M1) at 120 nM; 76.9 % (M1) at 60 nM; 75.7 % (M1) at 40 nM; 60.3 % (M1) at 20 nM (Fig. 4.4). The results indicated that the transfection efficiency was independent of siRNA concentration in the range between 40 nM and 120 nM, while at 20 nM, the efficiency was relatively low. At concentration of 60 nM, siRNA showed high transfection efficiency and low toxicity. This concentration was chosen for further experiments.

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Fig. 4.1 Bcl-2 and Bcl-XL protein levels in MCF-7/caspase-3 positive and MDA-MB-231 cells. Extracts from two cancer cell lines were prepared and analyzed on a 12% SDS-PAGE followed by Western blot analysis. A: Bcl-2, B: Bcl-XL. 1: MDA-MB-231, 2: MCF-7/caspase3 positive.

Fig. 4.2 FITC-siRNAs were transfected into MCF-7/Caspase-3 positive cells (A, B) and MDA-MB-231 cells (C, D) and observed with fluorescent microscope. A and C were control cells treated with oligofectamine; B and D were cells treated with 60 nM of FITC-siRNAs.

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Fig. 4.3 Trasnfecion efficiency of siRNA by oligofectamine in MCF-7/caspase-3 positive cells using flow cytometer. Cells were transfected with FITC-labeled siRNA by oligofectamine at different concentrations. The efficiency was analyzed by flow cytometer. A, 120 nM; B, 60 nM; C, 40 nM and D, 20n M.

Fig. 4.4 Trasnfecion efficiency of siRNA by oligofectamine in MDA-MB-231 cells using flow cytometer. Cells were transfected with FITC-labeled siRNA by oligofectamine at different concentrations. The efficiency was analyzed by flow cytometer. A, 120 nM; B, 60 nM; C, 40 nM; and D, 20 nM.

4.4 Comparison of three siRNAs in cancer cells

Three siRNAs targeting Bcl-2 were transfected into MCF-7/caspase-3 positive cells twice in two days. Cells transfected with scramble siRNA or treated with oligofectamine were used as controls. After 24 hours, cells were harvested and total proteins were separated by SDS-PAGE. Bcl-2 protein level was analyzed by Western blot with antibody against Bcl-2. As shown in Fig. 4.5, siRNA HY-SR2 exhibited the best result. And scramble siRNA had no effect on Bcl-2 protein. The same result was also observed in MDA-MB-231 cells (Fig. 4.6). GC content could be one of the reasons that affect the potency of siRNA. GC content of siRNA HY-SR2 is 33 % while those of HY-SR1 and HY-SR3 is 62 % and 52 % respectively. This is consistent with the general observation that siRNAs with 30 %~50 % GC content are more active than those with a higher GC content.

4.5 Downregulation of Bcl-XL by siRNA HY-SR9 in MCF-7/caspase-3 positive and MDA-MB-231 cells

Only one siRNA was designed to target Bcl-XL mRNA. MCF-7/caspase-3 positive cells and MDA-MB-231 cells were transfected with siRNA HY-SR9 twice in two days. After 24 hours, cells were collected and subjected to Western blot analysis. Results showed that siRNA HY-SR9 was effective in down-regulating anti-apoptotic protein Bcl-XL in both cell lines. So siRNA HY-SR9 was used in the following experiments. As shown in Fig. 4.7, the treatment with siRNA HY-SR9 significantly reduced Bcl-XL protein expression compared to cells without treatment, whereas the scramble siRNA was ineffective.

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Fig. 4.5 Down-regulation of Bcl-2 protein level in MCF-7/caspase-3 positive cells. Three siRNAs targeting Bcl-2 or scramble siRNA were transfected into MCF-7 cells; the Bcl-2 protein level was detected by Western blot after 24hours. 1, DMEM 2, oligofectamine 3, siRNA HY-SR1, 4, siRNA HY-SR2, 5, siRNA HY-SR3, 6, scramble siRNA. GAPDH protein level was shown as an internal control.

Fig. 4.6 Down-regulation of Bcl-2 protein level in MDA-MB-231 cells. Three siRNAs targeting Bcl-2 were tranfected into MDA-MB-231 cells for two times; the Bcl-2 protein level was detected by Western blot after 24hours. 1, oligofectamine 2, siRNA HY-SR2 3, siRNA HY-SR3 4, siRNA HY-SR1 5, scramble siRNA. GAPDH protein level was shown as an internal control.

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Fig. 4.7 Down-regulation of Bcl-XL protein level by siRNA HY-SR9 in cancer cells. MCF-7 /caspase-3 positive cells (A) and MDA-MB-231 cells (B) were treated with oligofectamine (lane 1), siRNA HS-SR9 (lane 2), or scramble siRNA (lane 3). Bcl-XL protein level was detected by Western blot after 24 hours. GADPH protein level was shown as an internal control.

4.6 Enhancement of the sensitivity to anti-cancer drug after siRNA treatment in breast cancer cells

Apoptosis in tumor cells plays a critical role in chemotherapy-induced cell death. The blockade of apoptosis-inducing pathway could be one of the mechanisms for drug resistance. Bcl-2 and Bcl-XL are important regulators in apoptosis pathway. Overexpression of Bcl-2 and Bcl-XL is implicated in the resistance of cancer cells to a variety of anti-cancer agents. Thus down-regulation of anti-apoptotic protein Bcl-2 or Bcl-XL may enhance sensitivity of cancer cells to anti-cancer drugs.

siRNA HY-SR2 targeting Bcl-2 and siRNA HY-SR9 targeting Bcl-XL have successfully suppressed the expression of Bcl-2 and Bcl-XL in the present study. Therefore they were used to evaluate whether the pre-treatment of cancer cells with siRNAs would enhance the sensitivity to chemotherapeutic drugs.

Breast cancer cells MCF-7/caspase-3 positive and MDA-MB-231 were treated with siRNA targeting Bcl-2 or Bcl-XL twice in two days and then incubated with anti-cancer drugs at different concentrations for 24 hours. Cell proliferation assay was performed and IC_{50} was calculated. IC_{50} value represents the drug concentration required to inhibit 50 % cell growth compared with untreated control cells. As shown in Table 4.2, the treatment of MDA-MB-231 cells with siRNA HY-SR2 resulted in 1.8 and 1.6 fold increase in sensitivity to cisplatin and betucilline acid, respectively. In MCF-7/caspase-3 positive cells, the treatment with siRNA HY-SR2 resulted in 1.2 and 1.3 fold increase in sensitivity to cisplatin and betucilline acid, respectively.

This effect was also investigated with siRNA HY-SR9 in MDA-MB-231 cells.

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Reduction of cell viability by cisplatin was higher in MDA-MB-231 cells when pretreated with siRNA HY-SR9 compared to oligofectamine pre-treated cells; the combined treatment resulted in 1.7 fold increase in sensitivity to cisplatin (Table 4.3).

Table 4.2 Modulation of sensitivity to anticancer drugs in MDA-MD-231 and MCF-7/caspase-3 positive cells treated with siRNA HY-SR2 targeting Bcl-2. Cells were treated with 60 nM of siRNA HY-SR2 and anti-cancer drug. IC₅₀ was calculated using KaleidaGraph software. a, cells treated with oligofectamine and anti-cancer drug. b, cells treated with siRNA and anti-cancer drug. Experiment was carried out in triplicate. Data are presented as mean ± S.D. (n=3).

Cell line	Drug	IC 50(μM)		Fold increase
		Control ^a	siRNA ^b	
MDA	Cisplatin	246.2 ± 15.8	139.7 ± 18.2	1.7
MCF-7 (+)	Cisplatin	46.0 ± 4.5	37.3 ± 1.0	1.2
MDA	Betucilline acid	41.4 ± 5.4	25.8 ± 2.5	1.6
MCF-7 (+)	Betucilline acid	34.4 ± 3.1	26.1 ± 0.9	1.3

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Fig. 4.8 Cell viability of MDA-MB-231 cells treated with siRNA HY-SR2 and cisplatin. Left: cells were treated with oligofectamine and cisplatin, and cell viability was measured after 24 hours by MTS assay. Right: cells were treated with siRNA HY-SR2 and cisplatin, and cell viability was measured after 24 hours by MTS assay.

Fig. 4.9 Cell viability of MCF-7/Caspase-3 positive cells treated with siRNA HY-SR2 and cisplatin. Left: cells were treated with oligofectamine and cisplatin, and cell viability was measured after 24 hours by MTS assay. Right: cells were treated with siRNA HY-SR2 and cisplatin, and cell viability was measured after 24 hours by MTS assay.

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Fig. 4.10 Cell viability of MDA-MB-231 cells treated with siRNA HY-SR2 and betucilline acid. Left: cells were treated with oligofectamine and betucilline acid, and cell viability was measured after 24hours by MTS assay. Right: cells were treated with siRNA HY-SR2 and betucilline acid, and cell viability was measured after 24 hours by MTS assay.

Fig. 4.11 Cell viability of MCF-7/Caspase-3 positive cells treated with siRNA HY-SR2 and betucilline acid. Left: cells were treated with oligofectamine and betucilline acid, and cell viability was measured after 24 hours by MTS assay. Right: cells were treated with siRNA HY-SR2 and betucilline acid, and cell viability was measured after 24 hours by MTS assay.

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Table 4.3 Modulation of sensitivity to anticancer drugs in MDA-MD-231 cells treated with siRNA HY-SR9 targeting Bcl-XL. Cells were treated with siRNA HY-SR9 and anti-cancer drugs. IC50 was calculated using KaleidaGraph software. a: cells treated with oligofectamine and cisplatin. b: cells treated with siRNA and cisplatin. Experiment was carried out in triplicate. Data are presented as mean $\pm$ S.D. (n=3).

	IC ₅₀ (μ M)		
Drug	Control ^a	siRNA ^b	Fold increase
Cisplatin	192.6 $\pm$ 12.6	116.3 $\pm$ 1.4	1.7

Fig. 4.12 Cell viability of MDA-MB-231 cells treated with siRNA HY-SR9 and cisplatin. Left: cells were treated with oligofectamine and cisplatin, and cell viability was measured after 24 hours by MTS assay. Right: cells were treated with siRNA HY-SR9 and cisplatin, and cell viability was measured after 24 hours by MTS assay.

4.7 Discussion

Anti-apoptotic protein Bcl-2 and Bcl-XL are important in apoptosis. It is the ratio of anti-apoptotic to pro-apoptotic proteins that decides the fate of cells instead of the absolute protein amount (Adams and Cory, 1998; Korsmeyer et al., 1993). Over-expression of anti-apoptotic proteins Bcl-2 or Bcl-XL has been linked to cancer and chemo resistance in various cancer cells (Coultas and Strasser, 2003; Kaufmann et al., 2003; Reed, 1995; Wiessner et al., 1999). This suggests that the down-regulation of Bcl-2 or Bcl-XL may change the balance between pro- and anti-apoptotic proteins, inducing apoptosis in cancer cells and rendering cancer cells more susceptible to anti-cancer agents.

Small molecules, peptidemimetics and antisense deoxyoligonucleotides targeting antiapoptotic proteins have been used to suppress antiapoptotic proteins. Design and functional evaluation of these antagonists could provide options for cancer therapy. Recently, siRNA has been developed to be an effective and specific tool to suppress gene expression. The possibility of siRNA as one of the valuable approaches in combination with conventional chemotherapeutic drugs in treating cancer was investigated in this study.

The ability of siRNA targeting Bcl-2 was first examined. The siRNA HY-SR2 showed the best silencing effect on Bcl-2 protein in MCF-7/caspase-3 positive cells and MDA-MB-231 cells. Second, the ability of siRNA targeting Bcl-XL was investigated. The siRNA HY-SR9 showed effective silencing effect on Bcl-XL in MCF-7/caspase-3 positive and MDA-MB-231 cells while scramble siRNA was

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ineffective. The siRNA HY-SR2 and HY-SR9 were used in the rest of the study. The levels of Bcl-2 and Bcl-XL were different in the cancer cells studied. Between MCF-7/caspase-3 and MDA-MB-231 cells, Bcl-2 protein was expressed higher in MCF-7/caspase-3 while Bcl-XL protein was expressed higher in MDA-MB-231. The chemosensitization was first investigated in MDA-MB-231 cells by using siRNA HY-SR9. IC_{50} decreased by 1.7 fold. MDA-MB-231 cells demonstrated moderate increase in chemosensitivity by using siRNA HY-SR2. Pretreatment with siRNA HY-SR2 rendered MDA-MB-231 cells more sensitive to betulinic acid and cisplatin. MCF-7/caspase-3 positive cells showed lower increase in sensitivity, 1.3 folds to betulinic acid and 1.2 folds to cisplatin. Both cell lines did not show much sensitivity increase to paclitaxel and camptothecin. Similar study was also performed in HeLa cells (Feng Lin, et al., manuscript in preparation). Among the cell lines tested, HeLa cells showed the highest effect: to paclitaxel, the sensitivity increased by 3.5 fold; to camptothecin by 2.7 fold; to betulinic acid by 2.1 fold; and to cisplatin by 1.6 fold.

These data suggested that down-regulation of anti-apoptotic protein Bcl-2 would increase the chemosensitivity of cancer cells, but this effect appeared to be cell line dependent. In conclusion, the siRNA molecules designed in this study were shown to be effective tools to suppress gene expression, and it provided potential to overcome drug resistance in clinical cancer therapy.

5 CO-DELIVERY OF siRNA AND DRUG BY P(MDS-co-CES)

A biodegradable cationic amphiphilic copolymer, which consists of cholesterol side chains and a cationic main chain, was obtained from Dr Yang's group (Institute of Bioengineering and Nanotechnology, Singapore). This polymer can easily form core-shell nanoparticles having a hydrophobic cholesterol core and a cationic shell in an aqueous solution by a self-assembly process. Hydrophobic drugs can be incorporated into the core during the self-assembly process. The cationic shell of the resulting drug-loaded nanoparticles can be employed to bind DNA. These nanoparticles are easier to produce when compared with liposomes, and their size can be more readily varied by changing the composition of the polymer.

The toxicity of this new polymer and delivery efficiency were tested in human embryonic kidney (HEK293) cell line, HepG2 cell line and 4T1 mouse breast cancer cell line using GFP and luciferase report genes. Data from Dr Yang's group showed this polymer was less toxic and exhibited comparable or higher gene delivery efficiency compared to the golden standard vector polyethylenimine (PEI). It was also shown that co-delivery of hydrophobic drug paclitaxel and DNA can increase the expression of the gene. In vivo study showed significantly decreased tumor growth rate by co-delivery of DNA and drugs (Wang et al., 2006).

To further demonstrate the combined effect of co-delivery of drug and gene by this polymer, the co-delivery of siRNA targeting anti-apoptotic protein Bcl-2 and paclitaxel was investigated in the present study.

5.1 Cytotoxicity of P(MDS-co-CES) in MDA-MB-231 cells

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Cytotoxicity of P(MDS-*co*-CES) was first investigated in breast cancer cell line MDA-MB-231. The cells were incubated with different concentrations of these nanoparticles. After 24 hours incubation, cell viability was measured by MTS assay. As shown in Fig. 5.1, the toxicity increased markedly with the increase of the concentration of P(MDS-*co*-CES). Cytotoxicity of the cationic polymers or nanoparticles is caused by electrostatic interactions with negatively charged glycocalyx of the cell surface (Fischer et al., 2003). The nanoparticle/DNA complexes displayed lower cytotoxicity than the nanoparticles due to the reduced surface charge. In MDA-MB-231 cells, at 2.8 mg/L, the nanoparticles showed low toxicity and this concentration was chosen for further experiments.

5.2 Deliver siRNA to MDA-MB-231 cells by P(MDS-*co*-CES)

siRNA HY-SR2 targeting anti-apoptotic protein Bcl-2 has demonstrated that it could successfully down-regulate Bcl-2 protein level and cause cell apoptosis in section 4. In the current experiment, siRNA HY-SR2 was transfected into MDA-MB-231 cells by P(MDS-*co*-CES). The concentration of polymer used was 2.8 mg/L according to the cytotoxicity experiment. siRNA used was 60 nM, thus the N/P ratio was 4. To investigate whether P(MDS-*co*-CES) could successfully deliver siRNA, Bcl-2 protein expression was evaluated. siRNA HY-SR2 was delivered to MDA-MB-231 cells by P(MDS-*co*-CES) twice at N/P=4. After 24 hour, the extracts were harvested and total proteins were separated by SDS-PAGE and subjected to Western blot analysis by using Bcl-2 and GAPDH antibodies. As shown in Fig. 5.2, the expression of Bcl-2 dramatically decreased (Fig 5.2, lane 3) compared to cells

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without transfection (Fig 5.2, lane 1) and cells incubated with P(MDS-*co*-CES) only (Fig. 5.2, lane 2). Western blot results demonstrated that at N/P=4 (siRNA used was 60 nM and P(MDS-*co*-CES) was 2.8 mg/L), the nanoparticles can successfully deliver siRNA to mammalian cells and the toxicity of the nanoparticles was relatively low.

Fig. 5.1 Cytotoxicity of P(MDS-*co*-CES). MDA-MB-231 cells were incubated with different concentrations of P(MDS-*co*-CES) for 24 hours. Cell viability was measured by MTS assay. Experiment was done in triplicate. Data were presented as mean $\pm$ SD (n=3).

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Fig. 5.2 Down-regulation of Bcl-2 protein level in MDA-MB-231 cells. siRNA targeting Bcl-2 was delivered into MDA-MB-231 cells by P(MDS-co-CES) twice. After 24 hours, cell lysates were collected and analyzed by Western blot with Bcl-2 and GAPDH antibody. Lane 1, cells without any treatment, lane 2, cells treated with P(MDS-co-CES) only. Lane 3, cells treated with 60 nM siRNA delivered by P(MDS-co-CES), N/P=4.

5.3 Deliver siRNA by P(MDS-co-CES) at different N/P ratios

The effect of down-regulation of Bcl-2 protein level by siRNA HY-SR2 delivered by P(MDS-co-CES) was then investigated at different N/P ratios. The concentration of P(MDS-co-CES) was fixed, and different N/P ratios were achieved by varying the concentration of siRNA. Since the P(MDS-co-CES) used was 2.8 mg/L, when N/P=12, 8 and 4, the siRNA used was 20 nM, 30 nM and 60 nM respectively. MDA-MB-231 cells were transfected with siRNA HY-SR2 by P(MDS-co-CES) twice at different N/P ratios. After 24 hours, cell lysates were collected and subjected to Western blot analysis. As shown in Fig. 5.3, the Bcl-2 protein expression was significantly suppressed at all these N/P ratios especially at N/P=12 (Fig 5.3, lane 3) compared to control cells without treatment (Fig 5.3, lane 1)

and cells treated with nanoparticles only (Fig 5.3, lane 2). Immuno-blotting with antibody specific for GAPDH was done as a loading control.

5.4 Co-Delivery of paclitaxel and siRNA

To illustrate the advantage of co-delivery of drug and gene, paclitaxel-loaded nanoparticles were used to deliver siRNA to investigate the synergy. MDA-MB-231 cells were first transfected with siRNA HY-SR2 by blank nanoparticles. After 24 hours, the cells were again transfected with siRNA HY-SR2 delivered by paclitaxel-loaded nanoparticles, and 48 hours later, cell viability was measured. As shown in Fig. 5.4, when the concentration of paclitaxel was 100 nM, cell viability decreased to 59 % when siRNA and paclitaxel were co-delivered by P(MDS-*co*-CES), while cell viability only decreased to 78 % when cells were treated with paclitaxel only. When concentration of paclitaxel was 400 nM, cell viability decreased to 39% when siRNA and paclitaxel were co-delivered by P(MDS-*co*-CES), while cell viability only decreased to 58 % when cells were treated with paclitaxel only.

Bcl-2 is an anti-apoptotic protein. The blockade of apoptosis-inducing pathway could be one reason for drug resistance. Thus down-regulation of Bcl-2 could enhance the sensitivity of cancer cells to anti-cancer drugs. Results obtained here suggested that the combined effect of co-delivery of anti-cancer drug and siRNA that silences the anti-apoptotic protein Bcl-2 could enhance the sensitivity of cancer cells to anti-cancer drugs.

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Fig. 5.3 Down-regulation of Bcl-2 protein level in MDA-MB-231 cells at different N/P ratios. siRNA targeting Bcl-2 was delivered into MDA-MB-231 cells by P(MDS-co-CES) at different N/P ratios twice. After 24 hours, cell lysates were prepared and subjected to Western blot with Bcl-2 and GAPDH antibody. Lane 1, cells without any treatment, lane 2, cells treated with P(MDS-co-CES) only. Lane 3-5, cells treated with siRNA delivered by P(MDS-co-CES) at different N/P ratios: 12, 8 and 4. Polymer used was 2.8 mg/L. siRNA used was 20, 30 and 60 nM respectively.

Fig. 5.4 Co-delivery of Paclitaxol and siRNA by P(MDS-co-CES). MDA-MB-231 cells were first transfected with siRNA targeting Bcl-2 by P(MDS-co-CES). After 24 hours, cells were transfected with siRNA by PCT-loaded P(MDS-co-CES). Cell viability was measured after 48 hours. 1, control cells without treatment; 2, cells were treated with siRNA by P(MDS-co-CES); 3 and 4, cells were treated with 100 nM PCT-loaded P(MDS-co-CES) without and with siRNA respectively; 5 and 6, cells were treated with 400 nM PCT-loaded P(MDS-co-CES) without and with siRNA respectively.

5.5 Discussion

The cationic core-shell nanoparticles which were used in the present study were self-assembled from a biodegradable amphiphilic copolymer P(MDS-co-CES). Compared to viral gene-delivery systems, it is safer and easier to produce; compared to liposomes, these nanoparticles are easier to manufacture and modulate their size and degree of positive charge.

The copolymer consists of cholesterol side chains, which form a hydrophobic core and a cationic main chain, which forms a cationic shell in an aqueous solution by a self-assembly process. The main chain of the copolymer is polyester and the pendant chain contains potentially hydrolytically labile urethano groups, which make this copolymer degradable. Hydrophobic drugs can be incorporated into the core during the self-assembly process and DNA or RNA can bind to cationic shell of the resulting drug-loaded nanoparticles. Since most anticancer drugs are hydrophobic, these nanoparticles provided a great potential for co-delivering anticancer drugs and genes for improved cancer therapy.

The transfection efficiency of these nanoparticles was investigated in HEK 293, Hep G2, and 4T1 cells. It was demonstrated that the transfection efficiency increased with N/P ratio and achieved the highest value at N/P ratio of 15. When compared to polyethylenimine (PEI), the transfection efficiency was either comparable or much higher than that of PEI. More important, when luciferase or GFP genes were delivered by paclitaxel-loaded nanoparticles, the gene expression level did increase by about 11-fold and 8-fold respectively. It was expected that the presence of paclitaxel would

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enhance gene expression possibly because of its anti-mitotic function (Nair et al., 2002; Son and Huang, 1996). In vivo experiments further demonstrated that co-delivery of paclitaxel and IL-12 gene inhibited growth of tumor much more efficiently than treatments with either paclitaxel or IL-12 gene alone. The effect of co-delivery of siRNA HY-SR2 and paclitaxel was investigated in the present study. The siRNA HY-SR2 was designed to target anti-apoptotic protein Bcl-2. It has been shown to successfully suppress Bcl-2 expression when delivered by lipofectamine. Delivery of siRNA HY-SR2 by the nanoparticles was studied in MDA-MB-231 cancer cells. The Bcl-2 protein level decreased when cells were treated with siRNA by the nanoparticles and the suppression effect also increased with increasing N/P ratio. When siRNA HY-SR2 and paclitaxel were co-delivered into MDA-MB-231 cells, the cell viability was much lower than that of cells treated only with paclitaxel. The possible reason is that siRNA HY-SR2 decreased the Bcl-2 protein level, changing the balance between pro- and anti-apoptotic proteins and thus making cells easier to undergo apoptosis. This effect made the cells more sensitive to the drugs.

Co-delivery of drugs and DNA has been proposed to enhance gene expression or achieve combined effect of drug and gene therapy (Cotten et al., 1992; Janat-Amsbury et al., 2004; Kishida et al., 2003; Malone et al., 1994; Zhang et al., 2001b). Nanoparticles used in this study were demonstrated to achieve high delivery efficiency and significant combined effect in vitro and in vivo. Moreover, by using these nanoparticles, drugs and DNA or RNA could be delivered to the same cells since they are bound to the same carrier. The unique property of nanoparticles makes the

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combined use of anti-cancer drugs and siRNAs more feasible especially when in vivo or clinical study is performed.

Further study about combinational effect of siRNA targeting Bcl-2 and paclitaxel should be studied in animal model in the near future.

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Articles

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2. Wang, Y., Gao, S., Ye, W.H, Yoon, H.S., and Yang, Y.Y. (2006) Co-delivery of drugs and DNA from cationic core-shell nanoparticles self-assembled from a biodegradable copolymer. **Nature Materials** 5, 791-796.
3. Lee, AL, Wang, Y, Ye WH, Yoon, HS, Chan, SY, Yang, YY (2008) Efficient intracellular delivery of functional proteins using cationic polymer core/shell nanoparticles. **Biomaterials** 29, 1224-1232.

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1. Ye W.H., Nanga R., Kang C.B., Song J.H., Song S.K., Yoon H.S. Catalytic Activity And Sugar-Binding Property Of Type II Ribosome-inactivating Protein From *Viscum Album Coloratum*. The 20th Symposium of the Protein Society, San Diego, USA (August, 2006).
2. Feng L., Ye W. H., Yu C.W. and Yoon H.S. RNA interference of Bcl-2 enhances sensitivities of cancer cells to therapeutic drugs, but their effects appear to be cancer cell-dependent. The 4th Annual Congress of International Drug Discovery Science and Technology, China (2006).