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In Silico Modulation of Apoptotic Bcl-2 Proteins by Mistletoe Lectin-1: Functional Consequences of Protein Modifications

Tasneem A. Khwaja,[1,2] Tayyaba Wajahat,[1] Ishtiaq Ahmad,[1] Daniel C. Hoessli,[3] Evelyne Walker-Nasir,[1] Afshan Kaleem,[1] Wajahat M. Qazi,[1] Abdul R. Shakoori,[4] and Nasir-ud-Din[1,5]*

[1]Institute of Molecular Sciences and Bioinformatics, Lahore, Pakistan
[2]Medical School, University of Southern California, Los Angeles, California
[3]Department of Pathology & Immunology, CMU, University of Geneva, Geneva, Switzerland
[4]School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan
[5]HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan

Abstract The mistletoe lectin-1 (ML-1) modulates tumor cell apoptosis by triggering signaling cascades through the complex interplay of phosphorylation and *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification in pro- and anti-apoptotic proteins. In particular, ML-1 is predicted to induce dephosphorylation of Bcl-2-family proteins and their alternative *O*-GlcNAc modification at specific, conserved Ser/Thr residues. The sites for phosphorylation and glycosylation were predicted and analyzed using Netphos 2.0 and YinOYang 1.2. The involvement of modified Ser/Thr, and among them the potential Yin Yang sites that may undergo both types of posttranslational modification, is proposed to mediate apoptosis modulation by ML-1. J. Cell. Biochem. 103: 479–491, 2008. © 2007 Wiley-Liss, Inc.

Key words: posttranslational modifications; multifunctional protein; glycosylation; phosphorylation; Yin Yang sites; apoptosis

Lectins are sugar-recognizing proteins that function in a wide range of biological phenomena. Mistletoe (*Viscum album*) lectins are three (ML-1, ML-II, ML-III) toxic proteins among which ML-I, also known as type II ribosome inactivating protein, is often used in adjuvant cancer therapy [Franz et al., 1981]. ML-I is a galactose specific lectin exhibiting low affinity for *N*-acetylgalactosamine, ML-III is GalNAc specific with less affinity for Gal, and ML-II has the same affinity for both. ML-I has anti-tumor activities [Khwaja et al., 1986; Bussing et al., 1996]. Different phytotherapeutic preparations have extensively been used as chemotherapeutic agents in cancer treatment, though most of these produce undesirable side effects due to high toxicity [Dharap et al., 2005]. Mistletoe lectin preparations (ML-1/ML-II/ML-III or all) have been used in the treatment of cancer with low toxicity and no fatal side effects [Bruseth and Enge, 1990]. Mistletoe extracts show a positive effect on DNA repair in peripheral blood mononuclear cells [Kovacs, 2002]. Additionally, ML-I therapy has also been known to stimulate the immune system and to reduce tumor size [Heiny and Beuth, 1994]. ML-I extract has been helpful in reducing tumor pain [Heiny and Beuth, 1994]. All these mistletoe properties have made it a suitable candidate for cancer adjuvant therapy.

ML-I, a heterodimeric protein, is composed of a catalytically active A-chain and a B-chain with carbohydrate-binding specificities [Franz,

Abbreviations used: Apaf-1, apoptotic protease-activating factor 1; Bcl-2, B-cell lymphoma protein 2; BAD, Bcl-2-antagonist of cell death protein; BAX, Bcl-2-associated X protein; Gal, galactose; GalNAc, *N*-acetylgalactosamine; JNK, c-jun *N*-terminal kinase; ML-1, mistletoe lectin 1; *O*-GlcNAc, *O*-linked *N*-acetylglucosamine; PTM, posttranslational modification; TNF-α, tumor necrosis factor-alpha; Ser, serine; Thr, threonine; Tyr, tyrosine.

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*Correspondence to: Nasir-ud-Din, Institute of Molecular Sciences and Bioinformatics, 28 Nisbet Road, Lahore, Pakistan. E-mail: nasir@super.net.pk; prof_nasir@yahoo.com

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1986]. The two chains are disulfide linked, between Cys 247 in the A-chain and Cys 5 in the B-chain. ML-1 may induce apoptosis by activating different death pathways, that is, apoptosis by extrinsic, death receptor pathway, or by intrinsic, mitochondrial, pathway [Bantel et al., 1999]. The involvement of posttranslational modifications (PTMs) in regulating apoptosis has been investigated in the mitochondrial pathway in which proteins of the B-cell lymphoma protein 2, Bcl-2, family are the key regulators [Adams and Cory, 2001]. Few of these are anti-apoptotic proteins, Bcl-2 and Bcl-Xl, while others (the Bcl-2-associated X protein (BAX) and the Bcl-2-antagonist of cell death protein (BAD)) are pro-apoptotic proteins. Bcl-2 and Bcl-Xl are located in the outer mitochondrial membrane and promote cell survival, while BAX and BAD are in the cytosol, acting as sensors of cellular damage or stress [Cartron et al., 2004].

Phosphorylation of Bcl-2 proteins regulates their ability to inhibit apoptosis [Adams and Cory, 2001]. Phosphorylated Bcl-2, BAD, and BAX have an anti-apoptotic function and their dephosphorylation is required for pro-apoptotic activity [Verma et al., 2001]. ML-1 binding to a sugar-containing receptor often results in Bcl-2, BAD, and BAX dephosphorylation, followed by heterodimerization of BAD and BAX with Bcl-2. The heterodimer produces pores in the mitochondrial membrane, thereby releasing cytochrome *c* from mitochondria to cytosol. The released cytochrome *c* then combines with the heterodimer of apoptotic protease-activating factor 1 (Apaf-1) and caspase 9 to form the apoptosome [Van Cruchten and Van Den Broeck, 2002] that binds procaspase 3 and completes the apoptosis reaction.

Hence, the activity of ML-1 in cancer treatment lies in its ability to induce apoptosis of cancerous cells [Niwa et al., 2003], by promoting PTMs in pro- and anti-apoptotic proteins.

Glycosylation, *O*-GlcNAc modification, is a dynamic and a regulatory process similar to phosphorylation and which can inhibit phosphorylation on the same Ser and/or Thr residue [Kearse and Hart, 1991]. Such alternative modifications by phosphorylation and glycosylation underlie the Yin Yang hypothesis [Zachara and Hart, 2002], according to which these modifications compete for the same site or on the same region of a polypeptide [Zachara and Hart, 2002]. These PTMs induce temporary conformational and functional changes that are difficult to assess in vivo. Computer-assisted protein modification studies can help exploring structural and functional changes, and suggest experiments to verify functional variations.

This article describes the potential phosphorylation and glycosylation sites on evolutionarily conserved residues of BAD, BAX, and Bcl-2 proteins, identifying false negative prediction sites in these proteins. We propose that ML-I may induce downstream signaling events for tumor cells apoptosis through binding to the cell surface receptors. These signaling events include alternative phosphorylation and *O*-GlcNAc modification of Bcl-2, BAX, and BAD.

MATERIALS AND METHODS

Sequence Data of Proteins

The sequence data used for predicting phosphorylation and glycosylation sites of human Bcl-2, BAD, and BAX, were retrieved from the Swiss-Prot sequence database [Boeckmann et al., 2003] (http://www.expasy.ch/sprot/sprot-top.html). The sequences of retrieved Bcl-2, BAD and BAX had entry names Bcl-2_Human, BAD_Human and BAXA_Human respectively. A BLAST search was made using BLAST from NCBI database, selecting nonredundant sequences with all other default parameters [Altschul et al., 1997] to find sequences most similar to human Bcl-2, BAD, and BAX. The search was made for all organisms' sequences. A total of 101 hits for Bcl-2, 23 for BAD, and 100 for BAX were obtained. All the sequences with higher bit score and zero or negative expect value from the respective blast searches for BAD, BAX, and Bcl-2 were selected. The selection included the sequence only belonging to the respective category from each of the BLAST searches. For the purpose of reducing redundancy in selection, synthetic construct, isoforms of selected sequences, hypothetical, predicted, similar, and unnamed proteins were neglected.

Five sequences of mammals were selected from the BLAST results of BAD. These sequences were of *Homo sapiens* (NP_004313.1), *Ovis aries* (AAS78591.1), *Mus musculus* (NP_031548.1), *Rattus norvegicus* (NP_073189.1), and *Canis familiaris* (XP_854947.1). Seven sequences selected from the BLAST search of BAX belonging to the mammals were *Homo sapiens*

(NP_620116.1), *Ovis aries* (AAF98242.1), *Mus musculus* (NP_031553.1), *Canis familiaris* (NP_001003011.1), *Rattus norvegicus* (NP_058755.1), *Bos taurus* (NP_ 776319.1), and *Felis cattus* (NP_001009282.1). Similarly, ten sequences were selected from the BLAST results of Bcl-2 from the following mammals: *Homo sapiens* (NP_000624.2), *Rattus norvegicus* (AAP47159.1), *Cricetulus longicaudatus* (CAB92245.1), *Canis familiaris* (AAR92491.1), *Felis cattus* (BAC 24136.1), *Bos taurus* (O02718), *Mus musculus* (AAH68988.1), *Cervus elaphus* (AF512029_1), *Ovis aries* (ABB02264.1), and *Oryctolagus cuniculus* (ABF71071.1).

All sequences were multiple aligned using CLUSTALW [Thompson et al., 1994], to find the conserved residues. CLUSTALW generates biologically significant multiple sequence alignments by matching all possible biological elucidations of the sequences, suggesting that the similarity of the two sequences is not fortuitous. This method calculates the best match for the selected sequences, aligning them in such a way that the individualities, similarities, and dissimilarities of sequences can be seen. First, selected sequences from different mammals for each BAD, BAX, and Bcl-2 were aligned one by one. During alignment the sequence of *Cervis elevatis* and *Ovis aries* from Bcl-2, BAD, and BAX were neglected in the final alignment list, because they were actually C-terminal fragments. In a second phase, BAD, BAX, and Bcl-2 were aligned to find similarity among pro- and anti-apoptotic proteins. Finally, alignment was made between BAD and BAX to find the similarity among the two pro-apoptotic proteins.

Posttranslational Modification Prediction Methods

The prediction method used for predicting phosphorylation, in Bcl-2, BAX, and BAD sequence of human, was NetPhos 2.0 (http://www.cbs.dtu.dk/services/Netphos/) [Blom et al., 1999]. To predict potential *O*-glycosylation sites on Ser and Thr, the amino acid sequence of the above-mentioned proteins obtained from Swiss-Prot were used for YinOYang 1.2 (http://www.cbu.dtu.dk/services/yinoyang). With the use of YinOYang 1.2, a neural-network-based method for predicting *O*-glycosylation, it is possible to generate data from the NetPhos 2.0 server to predict Ser/Thr sites having potential for both *O*-GlcNAc modification and phosphorylation ("Yin Yang" sites).

Statistical and Computational Analysis of Yin Yang Sites Data

All multiply aligned sequences for BAD, BAX, and Bcl-2 from their respective alignments were used to mine association rules for potential Yin Yang sites predicted for BAD, BAX, and Bcl-2. Mining association rules involved generating peptides of 21 amino acid length in such a way that each predicted Ser/Thr for phosphorylation had 10 residues on the left and 10 residues on the right. These peptides can also serve the purpose for training/test data for Yin Yang sites to be pursued for further functional analysis, theoretically or experimentally (Supplementary data, Table S1). After preparing the peptide data of all Ser/Thr for BAD, BAX, and Bcl-2, the peptides were analyzed statistically by estimating significantly preferred amino acid residues at each position (-10 to $+10$) around potential Yin Yang sites as described earlier for *O*-glycosylation [Christlet and Veluraja, 2001] and for phosphorylation [Qazi et al., 2006].

Association analysis was performed to discover association rules among significantly preferred amino acid residues, using the association rules mining method MAPRes (Qazi et al., unpublished work, http://www.imsb.edu.pk/mapres). The MAPRes method is useful in developing association among different amino acids, which were found to be significantly preferred, around acceptor target (phosphorylated, glycosylated, etc.) amino acid residues. Association among different amino acid residues around potential Yin Yang sites will be helpful in deducing rules for kinase and OGT binding.

RESULTS

Prediction Results of Phosphorylation and *O*-Glycosylation Sites

The results of predicted phosphorylation sites of human Bcl-2, BAD, and BAX on Ser, Thr, and Tyr are summarized in Table I, while the prediction results of their *O*-glycosylation sites obtained by YinOYang 1.2 are described in Table II.

Prediction Results of Yin Yang Sites

The predicted Yin Yang sites are shown in Table III and marked by an asterisk (*); these sites show potential for interplay of

TABLE I. Prediction Results of *O*-Phosphorylation Sites of Human Bcl-2, BAD, and BAX

		Predicted phosphorylation sites		
No. of Obs	Protein name	On serine	On threonine	On tyrosine
1	Bcl-2	24, 70, 87, 167	56, 74, 96, 132	28, 180
2	BAD	10, 15, 16, 17, 25, 34, 74, 75, 91, 97, 99, 118, 124, 134, 144, 146, 153, 167	80	0
3	BAX A	15, 55, 60, 72, 87	0	0

phosphorylation and glycosylation. Potential of Ser and Thr as Yin Yang sites for Bcl-2, BAX, and BAD are shown in Figures 1–3, respectively. Many other conserved Ser and Thr residues showed potential for one of the two modifications (either for phosphorylation or for glycosylation), higher than the threshold value for one, and very close to the threshold for the other modification. These residues, identified as false negative Yin Yang sites (Yin Yang; FN) are shown in Table III, with their conservation status and modification potential. Besides Yin Yang sites, there were many conserved Ser/Thr amino acid residues without any appreciable potential for either phosphorylation and/or *O*-GlcNAc modification (Fig. 5). There were instances where nonconserved Ser/Thr possessed positive potential for *O*-GlcNAc modification but negative potential for phosphorylation and vice versa. In both these cases, it is difficult to assess the existence of a Ying Yang site because one of these predictions is either false positive or false negative.

Conserved Residues

The conserved residues were identified using CLUSTALW. The conservation status of Ser/Thr residues of Bcl-2, BAD, and BAX, and their modification potential are shown in Table III.

Statistical and Computational Analysis Results

Statistical analysis results for estimation of significantly preferred amino acids residues around potential Yin Yang sites are summarized in Table IV and the results pertaining to the association of different amino acids around Yin Yang sites are outlined in Table V.

DISCUSSION

PTMs are means of important mechanisms for the proper functioning of proteins, and altered PTMs have been associated with the development of cancer [Tomonaga et al., 2004]. Specific genes are turned on or off at the beginning, development, and progression of diseases such as cancer. PTMs are involved in the key steps of tumor progression, such as cell cycle checkpoint, and when induction of differentiation, and apoptosis are lost [Krueger and Srivastava, 2006]. PTMs are also useful tools for the investigation of the cellular responses to drugs, and to study their mechanism of action on the basis of resistance [Kolch et al., 2005]. Identification and comprehensive analysis of PTMs in proteins involved in the development of various forms of cancer have now become mandatory.

The PTMs are involved in proper folding and maintaining of the 3D structure of a protein

TABLE II. Prediction Results of *O*-Glycosylation Sites of Human Bcl-2, BAD, and BAX

		Predicted sites for *O*-β-GlcNAc	
No. of Obs	Protein name	On serine	On threonine
1	Bcl-2	50, 62, 87*	56*, 69, 74*
2	BAD	16*, 25*, 32, 49, 57, 58, 59, 71, 134*, 144*, 145, 146*, 163, 164, 167*	56, 139
3	BAX A	15*	56

*Yin Yang sites (Residues predicted showing potential for both *O*-GlcNAc modification and phosphorylation).

TABLE III. Conserved Ser /Thr Residues With Posttranslational Modifications of Human Bcl-2, BAD, and BAX

			Conservation status			Modification potential					
						O-phosphorylation	*O*-GlcNAc			Cross reference	
Protein name	No. of Obs	Residues	Group 1*	Group 2*	Group 3*	Pred. Pot. with fixed threshold 0.5	Pred. Pot.	Threshold	Yin Yang sites	Uni Prot	PDB
Bcl-2	1	Thr 56	NCS	N		0.727	0.5131	0.3648	Pr	NF	NF
	2	Thr 69	CR	N		0.037	0.5652	0.3847	FN	NF	NF
	3	Ser 70	CR	N		0.995	0.3400	0.3736	FN	Found (P10415)	NF
	4	Thr 74	NCS	N		0.808	0.3696	0.3635	Pr	NF	NF
	5	Ser 87	CR	NCS		0.794	0.4088	0.3939	Pr	NF	NF
BAD	6	Ser 15	CS	NCS	N	0.947	0.2693	0.3192	FP	NF	NF
	7	Ser 16	CR	NCS	CR	0.919	0.4684	0.3251	Pr	NF	NF
	8	Ser 17	NCS	N	CR	0.988	0.2818	0.3308	FP	NF	NF
	9	Ser 25	CR	N	CS	0.553	0.3801	0.3511	Pr	NF	NF
	10	Ser 74	CR	N	CS	0.997	0.3250	0.3737	FN	NF	NF
	11	Ser 75	CR	N	CS	0.986	0.2863	0.3689	FN	Found (Q92934)	NF
	12	Ser 97	CR	NCS	NCS	0.976	0.3051	0.3429	FN	NF	NF
	13	Ser 99	CR	N	N	0.930	0.2803	0.3414	FN	Found (Q92934)	NF
	14	Ser 118	CR	N	N	0.997	0.2471	0.3883	FN	Found (Q92934)	NF
	15	Ser 134	CR	N	N	0.871	0.4586	0.3388	Pr	Found (Q92934)	NF
	16	Ser 144	CR	NCS	NCS	0.996	0.5222	0.3883	Pr	NF	NF
	17	Ser 146	CS	NCS	NCS	0.943	0.5154	0.5154	Pr	NF	NF
	18	Se 167	CR	NCS	CS	0.819	0.4217	0.2993	Pr	NF	NF
BAX	19	Ser 15	CR	NCS	CR	0.885	0.4799	0.3713	Pr	NF	NF
	20	Ser 184	CR	N	NCS	0.016	0.1940	0.4693	FN	NF	NF

Group 1*: Conserved residues identified after aligning the human Bcl-2, BAD, and BAX with proteins of other mammals one by one.
Group 2*: Conserved residues identified after aligning the human Bcl-2, BAD, and BAX with each other.
Group 3*: Conserved residues identified after aligning the human BAD and BAX with each other.
Pred. Pot, predicted potential; FP, false positive; FN, false negative; Pr, predicted Ying Yang site; NCS, nonconserved substitution of residue; CS, conserved substitution of residue; CR, conserved residue; N, negative; NF, not found.

TABLE IV. Percentage of Observed Frequency of Significantly Preferred Ser and Thr

	SP Ser	Percentage of obs freq.	SP Thr	Percentage of obs freq.
1	A 10	19.44	A −9	75
2	A 9	20.83	A −2	87.5
3	E −10	15.28	A 8	100
4	G −10	25	D −5	75
5	G 5	20.83	H −7	62.5
6	G −4	34.72	L 3	75
7	H 7	11.11	P −10	75
8	K 6	16.67	P 2	100
9	L 10	26.39	P 5	87.5
10	M 7	13.89	R −6	75
11	P −7	18.06	R −1	100
12	P 1	27.78	S 1	100
13	P 3	25	V 7	75
14	P 4	26.39		
15	R −3	27.78		
16	R −2	27.78		
17	S 1	18.06		
18	T −7	16.67		
19	T −5	15.28		
20	T −1	19.44		
21	T 2	19.44		
22	V 6	22.22		

[Bork et al., 1998; Attwood, 2000]. Determination of the 3D structure in vivo is difficult to assess because of continuous changes in protein conformations as a consequence of intra- and inter-molecular interactions of proteins present in the body fluids or in the cell. Computer-assisted techniques are essential to predict multifunctional switches of proteins in vivo regulated by dynamic PTMs. Induction of apoptosis by ML-I involves triggering a series of signaling events resulting in modifications of anti- and pro-apoptotic proteins, such as Bcl-2,

TABLE V. Association Rules Among Significantly Preferred Amino Acids Around Predicted Yin Yang Site

Association rules	Conf. %	Support level %
G −10, P −7, G −5, G −4, T −1, S 1, K 6, A 9, L 10 = S	100	5
P −10, A −9, H −7, R −6, D −5, A−2, R 1, S 1, P 2, L 3, P 5, V 7, A 8 = T	100	5
G −4, P 1, P 3, P 4, V 6, H 7, L 10 = S	100	10
P 1, P 4, V 6 = S	100	15
P 1, P 4 = S	100	20
P 1, V 6 = S	100	20
P −10, A −9, H −7, R −6, D −5, A −2, R −1, S 1, P 2, L 3, P 5, V 7, A 8 = T	100	20
G −10 = S	100	25
G −4 = S	100	25
L 10 = S	100	25
P 1 = S	100	25
P 3 = S	100	25
P 4 = S	100	25
R −2 = S	100	25
R −3 = S	100	25
G −4 = S	100	30
P −10, A −9, H −7, R −6, D −5, A −2, R −1, S 1, P 2 L 3, P 5, V 7, A 8 = T	100	60
P −10, A −9, R −6, D −5, A −2, R−1, S 1, P 2, L 3, A 8 = T	100	65
P −10, A −9, R −6, D −5, A −2, R −1, S 1, P 2, L 3, A 8 = T	100	70
P −10, A −9, R −6, D −5, A −2, R −1, S 1, P 2, L 3, A 8 = T	100	75
A −2, R −1, S 1, P 2, A 8 = T	100	80
R −1, S 1, P 2, P 5, A 8 = T	100	80
A −2, R −1, S 1, P 2, A 8 = T	100	85
R −1, S 1, P 2, P 5, A 8 = T	100	85
R −1, S 1, P 2, A 8 = T	100	90
R −1, S 1, P 2, A 8 = T	100	100

TABLE VI. Training/Test Data for all Proposed Yin Yang Sites

Protein name	Ser no.	Predicted Yin Yang site	Species	Peptides
Bcl-2	1	Thr 69	*Homo sapiens*	PAASRDPVARTSPLQTPAAPG
			Canis familiaris (Thr 69)	QPGRTPAPARTSPPPPPVAPA
			Mus musculus (Thr 69)	PAVHRDMAARTSPLRPLVATA
			Oryctolagus cuniculus (Thr 69)	PAVHRDTAARTSPLRPLVANA
			Rattus norvegicus (Thr 69)	PAVHRDMAARTSPLRPLVATA
			Felis cattus (Thr 62)	QPGRTPAPARTSPPPPPVAPA
			Bos taurus (Thr 62)	QPGRTPAPSRTSPPPPPAAAA
	2	Ser 70	*Homo sapiens*	AASRDPVARTSPLQTPAAPGA
			Cricetulus longicaudatus (Ser 70)	AVHRDMAARTSPLRTIVATTG
			Canis familiaris (Ser70)	AVHRDMAARTSPLRPIVATTG
			Mus musculus (Ser 70)	AVHRDMAARTSPLRPLVATAG
			Oryctolagus cuniculus (Ser 70)	AVHRDTAARTSPLRPLVANAG
			Rattus norvegicus (Ser 70)	AVHRDMAARTSPLRPLVATAG
			Felis cattus (Ser 63)	QPGRTPAPARTSPPPTPVAPA
			Bos taurus (Ser 63)	QPGRTPAPSRTSPPPTPAAAA
	3	Ser 87	*Homo sapiens*	APGAAAGPALSPVPPVVHLTL
			Cricetulus longicaudatus (Ser 84)	PIVATTGPTLSPVPPVVHLTL
			Canis familiaris (Ser 84)	PIVATTGPTLSPVPPVVHLTL
			Mus musculus (Ser 84)	PLVATAGPALSPVPPVVHLTL
			Oryctolagus cuniculus (Ser 84)	PLVANAGPALSPVPPVVHLTL
			Rattus norvegicus (Ser 84)	PLVATAGPALSPVPPVVHLTL
			Felis cattus (Ser 83)	AAAAAAGPALSPVPPVVHLTL
			Bos taurus (Ser 77)	PPAAAAGPAPSPVPPVVHLTL
BAD	1	Ser 16	*Homo sapiens*	EFEPSEQEDSSSAERGLGPSP
			Canis familiaris (Ser16)	EFEPSEQEDSSSANRGLGPSP
			Mus musculus (Ser 58)	EFEPSEQEDASSTDRGLGPSL
			Rattus norvegicus (Ser 58)	EFEPSEQEDASTTDRGLGPSL
	2	Ser 25	*Homo sapiens*	SSSAERGLGPSPAGDGPSGSG
			Canis familiaris (Ser 25)	SSPANRGLGPSPTGDRPPSPG
			Mus musculus (Ser 67)	ASATDRGLGPSLTEDQPGPYL
			Rattus norvegicus (Ser 67)	ASTTDRGLGPSLTEDQPGPYL
	3	Ser 74	*Homo sapiens*	AGAVEIRSRHSSYPAGTEDDE
			Canis familiaris (Ser 73)	GAGAETRSRHSSFPAGTDEDE
			Mus musculus (Ser 111)	AGAMETRSRHSSYPAGTEEDE
			Rattus norvegicus (Ser 112)	AGTMETRSRHSSYPAGTEEDE
	4	Ser 75	*Homo sapiens*	GAVEIRSRHSSYPAGTEDDEG
			Canis familiaris (Ser 74)	AGAETRSRHSSFPAGTDEDEG
			Mus musculus (Ser 112)	GAMETRSRHSSYPAGTEEDEG
			Rattus norvegicus (Ser 113)	GTMETRSRHSSYPAGTEEDEG
	5	Ser 97	*Homo sapiens*	GEEPSPFRGRSRSAPPNLWAA
			Canis familiaris (Ser 98)	EELSPFRGRSSSAPPNLCAAR
			Mus musculus (Ser 134)	EEELSPFRGRSRSAPPNLWAA
			Rattus norvegicus (Ser 135)	EEELSPFRGRSRSAPPNLWAA
	6	Ser 99	*Homo sapiens*	EPSPFRGRSRSAPPNLWAAQR
			Canis familiaris (Ser 100)	LSPFRGRSSSAPPNLCAARRY
			Mus musculus (Ser 136)	ELSPFRGRSRSAPPNLWAAQR
			Rattus norvegicus (Ser 137)	ELSPFRGRSRSAPPNLWAAQR
	7	Ser 118	*Homo sapiens*	QRYGRELRRMSDEFVDSFKKG
			Canis familiaris (Ser 119)	RRYGRELRRMSDEFQGSFKGL
			Mus musculus (Ser 155)	QRYGRELRRMSDEFEGSFKGL
			Rattus norvegicus (Ser 156)	QRYGRELRRMSDEFEGSFKGL
	8	Ser 134	*Homo sapiens*	SFKKGLPRPKSAGTATQMRQS
			Canis familiaris (Ser133)	GSFKGLPRPKSAGTATQMRQS
			Mus musculus (Ser 170)	GSFKGLPRPKSAGTATQMRQS
			Rattus norvegicus (Ser 171)	GSFKGLPRPKSAGTATQMRQS
	9	Ser 144	*Homo sapiens*	SAGTATQMRQSSSWTRVFQSW
			Canis familiaris (Ser143)	SAGTATQMRQSPSWTRVIQSW
			Mus musculus (Ser 180)	SAGTATQMRQSAGWTRIIQSW
			Rattus norvegicus (Ser 181)	SAGTATQMRQSAGWTRIIQSW
	10	Ser 146	*Homo sapiens*	GTATQMRQSSSWTRVFQSWWD
			Canis familiaris (Ser 145)	GTATQMRQSPSWTRVIQSWWD
			Mus musculus (Ser 182)	GTATQMRQSASWTRIIQSWWD
			Rattus norvegicus (Ser 183)	GTATQMRQSASWTRIIQSWWD
	11	Ser 167	*Homo sapiens*	RNLGRGSSAPSQ———
			Canis familiaris (Ser 166)	RNLGRGGSAPSQ———
			Mus musculus (Ser 203)	RNLGKGGSTPSQ———
			Rattus norvegicus (Ser 204)	RNLGKGGSTPSQ———
BAX	1	Ser 15	*Homo sapiens*	GEQPRGGGPTSSEQIMKTGAL
			Canis familiaris (Ser 15)	GEQPRGGGPTSSEQIMKTGAL
			Bos taurus (Ser 15)	GEQPRGGGPTSSEQIMKTGAL
			Felis cattus (Ser 15)	GEQPRGGGHTSSEQIMKTGAL
			Mus musculus (Ser 15)	GEQLGSGGPTSSEQIMKTGAF
			Rattus norvegicus (Ser 15)	GEQLGGGGPTSSEQFMKTGAF
	2	Ser 184	*Homo sapiens*	TIFVAGVLTASLTIWKKMG–
			Canis familiaris (Ser 184)	TIFVAGVLTASLTIWKKMG–
			Bos taurus (Ser 184)	TIFVAGVLTASLTIWKKMG–
			Felis cattus (Ser 184)	TIFVAGVLTASLAIWKKMG–
			Mus musculus (Ser 184)	TIFVAGVLTASLTIWKKMG–
			Rattus norvegicus (Ser 184)	TIFVAGVLTASLTIWKKMG–

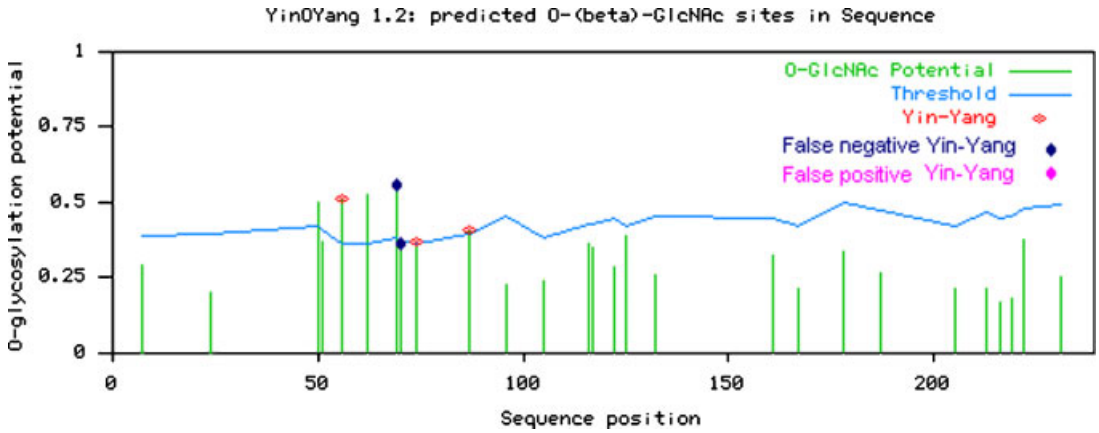

Fig. 1. Graphic presentation of potential for *O*-GlcNAc modification for Ser/Thr residues in human Bcl-2, BAX, and BAD sequences. Green vertical lines show the potential of Ser/Thr residue for *O*-GlcNAc modification and the light blue horizontal wavy line shows the threshold for modification potential. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BAD, and BAX [Lyu et al., 2002]. Modifications of anti- and pro-apoptotic proteins ultimately result in the disruption of mitochondria and subsequent activation of the caspase protease cascade [Lyu et al., 2002], a process that is tightly regulated by PTMs of the Bcl-2 family of proteins [Breitschopf et al., 2000]. When ML-1 is endocytosed, it activates a variety of intracellular signaling pathways leading to the expression of tumor necrosis factor-alpha (TNF-α) [Boneberg and Hartung, 2001], which activates c-jun *N*-terminal kinase (JNK) [Valladares et al., 2000]. TNF-α dephosphorylates Bcl-2 [Breitschopf et al., 2000], while JNK promotes translocation of BAD and BAX to mitochondria (Fig. 4) [Sunayama et al., 2005]. Phosphorylated BAD and BAX are sequestered by the 14-3-3 protein (cytosolic anchor of BAD and BAX), and are present in the cytosol in their inactive form, whereas dephosphorylated BAD and BAX are targeted to the mitochondria, where they bind to Bcl-2 [Zha et al., 1996; Putcha et al., 1999] (Fig. 4). This results in mitochondrial dysfunction followed by cytochrome *c* release, caspase activation, and finally apoptotic cell death (Fig. 4).

Activation of JNK promotes translocation of BAD and BAX to mitochondria through phosphorylation of the 14-3-3 protein which, once phosphorylated, releases BAD and BAX (Fig. 4) [Zha et al., 1996; Putcha et al., 1999]. This dissociation results in dephosphorylation of

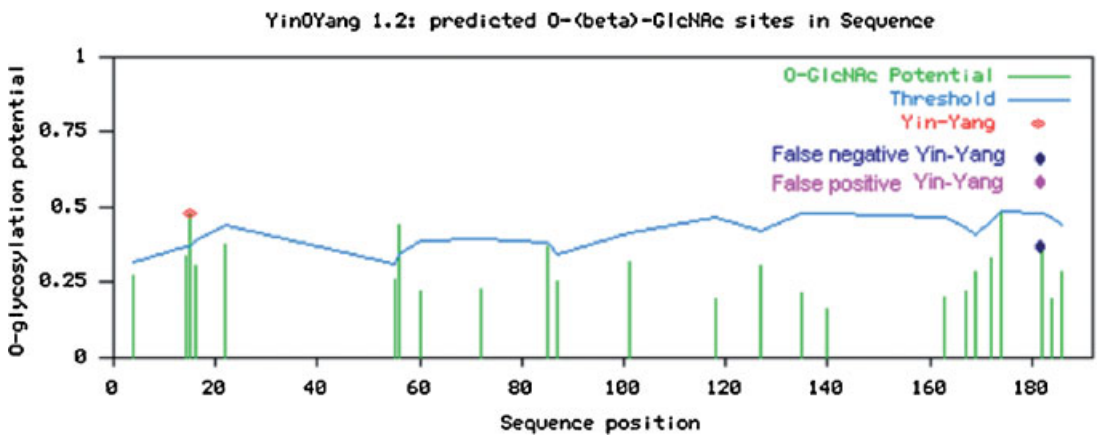

Fig. 2. Graphic presentation of potential for *O*-GlcNAc modification for Ser/Thr. Green vertical lines show the potential of Ser/Thr residue for *O*-GlcNAc modification and light blue horizontal wavy line shows threshold for modification potential. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

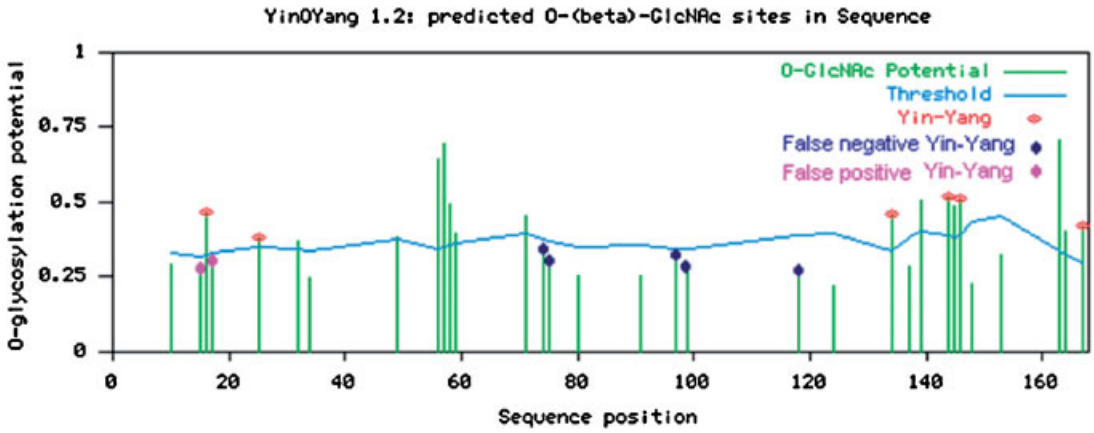

Fig. 3. Graphic presentation of potential for *O*-GlcNAc modification for Ser/Thr. Green vertical lines show the potential of Ser/Thr residue for *O*-GlcNAc modification and light blue horizontal wavy line shows threshold for modification potential. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BAD and BAX as well. Similarly, phosphorylated Bcl-2 inhibits apoptosis [Ruvolo et al., 2001]. ML-1 activates TNF-α [Pae et al., 2000], which induces dephosphorylation of Ser87 of Bcl-2 (Fig. 4) [Breitschopf et al., 2000]. Phosphorylation of Bcl-2 on Ser 70 by protein kinase C (PKC) promotes Bcl-2 anti-apoptotic activity [Breitschopf et al., 2000]. Increased levels of

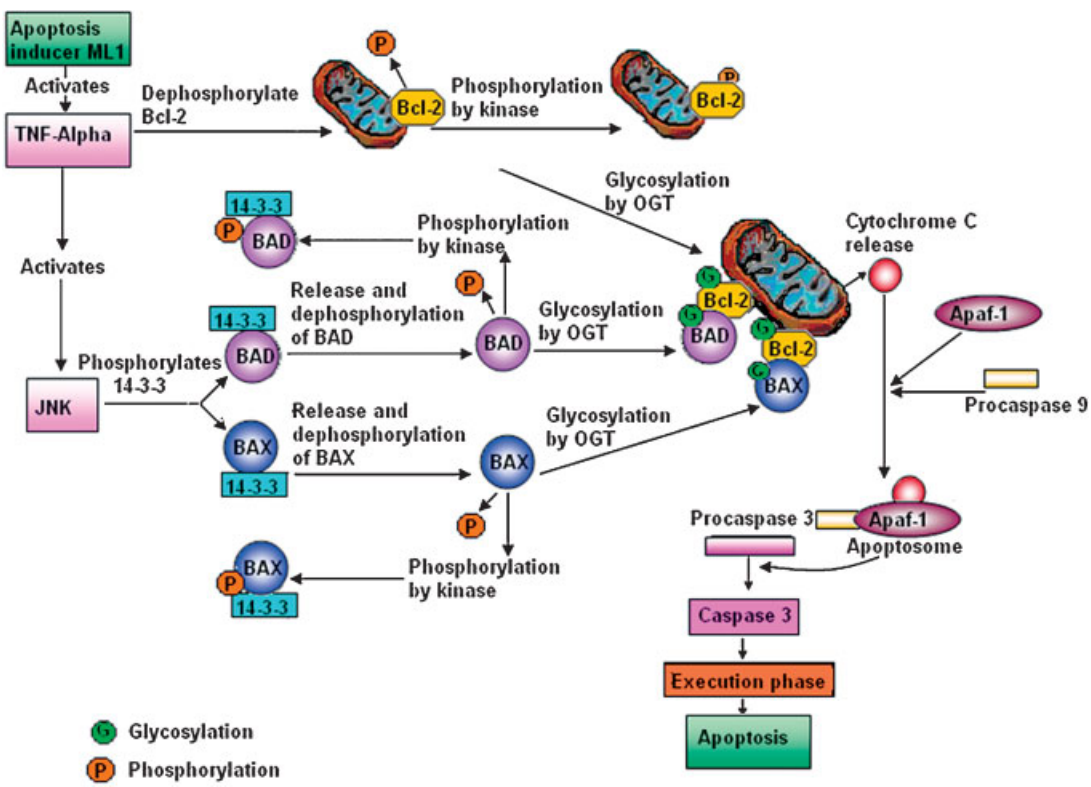

Fig. 4. Schematic presentation of apoptosis induced by ML-1. ML-1 induces apoptosis through the intrinsic mitochondrial pathway. ML-1 endocytosis activates TNF-α, which induces dephosphorylation of Bcl-2. TNF-α then activates JNK which phosphorylates the 14-3-3 protein (cytosolic anchor of BAD and BAX). BAD and BAX dissociate from dephosphorylated 14-3-3, and become themselves dephosphorylated. BAD, BAX, and Bcl-2 are then *O*-GlcNAc modified by OGT, which blocks the phosphorylation sites. These *O*-GlcNAc modified BAD and BAX are then translocated to mitochondria where heterodimerizations of BAD and BAX with Bcl-2 occur. Leakage of cytochrome *c* from the mitochondrion follows, activating caspases and apoptotic cell death pathways.

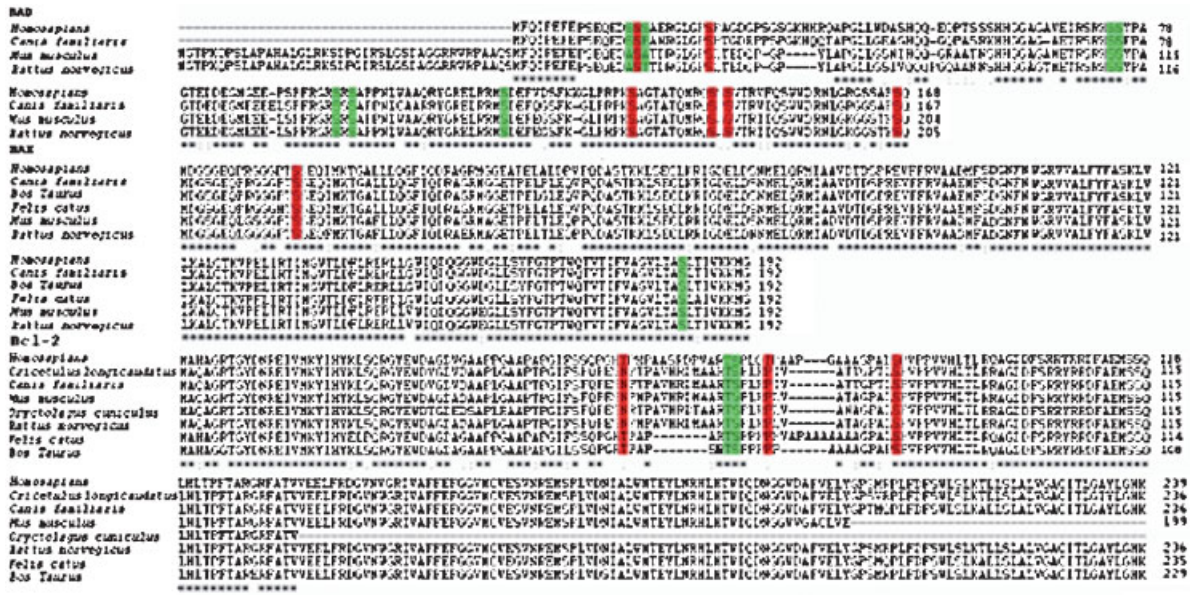

Fig. 5. Multiple alignment of human BAD, BAX, and Bcl-2 sequences with proteins of other mammals. Multiple alignment of human BAD, BAX, and Bcl-2 sequences with proteins of other mammals was used to identify the conserved Ser/Thr residues of human BAD, BAX, and Bcl-2. The consensus sequence is represented by (*), conserved substitution by double dot and semi-conserved substitution by single dot. The conserved Ser/Thr residues highlighted in red are predicted Yin Yang sites by YinOYang 1.2, whereas green highlighted Ser/Thr residues are proposed as false negatively predicted Yin Yang sites. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

O-GlcNAc incorporation to PKC has been shown to result in its inactivation and decreased membrane attachment [Griffith and Schmitz, 1999]. Inactivation of PKC may result in occupation of Ser 70 by *O*-GlcNAc through the action of *O*-GlcNAc transferase (OGT), thus blocking the anti-apoptotic activity of Bcl-2 for cancer cell apoptosis. The known phosphorylated sites of Bcl-2 include Thr 69, Ser 70, and Ser 87 [Deng et al., 2004]. The prediction results and conservation status suggest that Thr 56, Thr 74, and Ser 87 are positive Yin Yang sites (Fig. 5; Table III) of Bcl-2. While Thr 69 and Ser 70 are known to be phosphorylated [Deng et al., 2004], no data are known for their GlcNAc modification. Prediction results indeed show that these two residues, conserved throughout all mammals, are false negative prediction sites (Table III). Thr 69 and Ser 70 can possibly serve as residues for interplay of phosphorylation and *O*-GlcNAc modification, thereby regulating important steps of Bcl-2-mediated cancer cell apoptosis.

Dephosphorylated BAD promotes cell death by binding to and inactivating Bcl-2 [Yang et al., 1995]. Four phosphorylation sites have been reported for BAD. Phosphorylation on one or more residues of Ser 75, 99, 118, and 134, all conserved in mammals (Table III), in response to survival stimuli, blocks its pro-apoptotic activity [Eisenmann et al., 2003]. This has also been reported to be the case in melanomas but not in normal melanocytes [Eisenmann et al., 2003]. Among these four known phosphorylation sites, Ser 134 is the only one predicted as Yin Yang site (Table III), whereas the other three sites (Ser 75, 99, and 118) are proposed as false negative Yin Yang sites (Table III). Though all three sites, by YinOYang 1.2, show lower potential for *O*-β-GlcNAc modification than the threshold, we propose these sites as possible Yin Yang sites on the following basis, that is, if a kinase can access these sites for phosphorylation, then OGT may have equal chances to modify these residues by GlcNAc. Consequently, the *O*-GlcNAc modification of the dephosphorylated form of BAD on these sites is proposed as a means of promoting apoptotic activity of cancerous cells. In the case of normal cells, however, dephosphorylated BAD has less possibility to be modified by *O*-GlcNAc. Thus the complex interplay of phosphorylation and *O*-GlcNAc modification on these sites may control apoptosis of cancerous cells and survival of normal cells. Besides these, other Ser residues at positions 16, 25, 144, 146, and 167 are

predicted Yin Yang sites (Table III). All these predicted Yin Yang sites are conserved in pro- and anti-apoptotic proteins (BAD, BAX, and Bcl-2) and are also conserved in BAD of other mammals (Table III) when aligned independently. As yet, there is no evidence of any of these sites being modified either by phosphorylation or by *O*-GlcNAc. However, experimental verification of these modifications by phosphorylation or by *O*-glycosylation may be useful in defining the regulation of functional switches. Ser 15 and 17 are nonconserved in mammals (Fig. 5) and have less potential as possible Yin Yang sites; they are false positive prediction sites for phosphorylation (Table III).

Dephosphorylated BAX is another pro-apoptotic protein [Putcha et al., 1999]. The mechanism of its apoptotic behavior is incompletely understood; however, it is known that phosphorylation of Ser 184 of BAX regulates its activity, and that phosphorylation requires phosphatidylinositol 3-kinase/Akt activation [Gardai et al., 2004]. Ser 184 of BAX is a false negative prediction site for phosphorylation and a Yin Yang site as well (Table III). Ser15, 55, 60, 72, and 87 also showed potential for phosphorylation (Table I), while Ser 15 is the only residue which has potential for both glycosylation and phosphorylation and is also conserved in all mammals; hence it is a potential Yin Yang site (Table II). The Ser 15 is comparable to Ser 16 of BAD (Fig. 5), and it shows positive potential for *O*-GlcNAc modification (Table III). Therefore, there is a strong possibility that Ser 15 is a Yin Yang site. Ser 184 of BAX is known to be phosphorylated [Gardai et al., 2004], but it is a negative prediction site for both phosphate and *O*-GlcNAc modification. In the context of accessibility of kinase and OGT, as discussed above, Ser 184 is a false negative prediction site. Phosphorylation of BAX promotes cell survival by inhibiting its effects on the mitochondria, retaining the protein in the cytoplasm [Gardai et al., 2004], but dephosphorylation of Ser 184 results in its translocation to mitochondria. We propose that dephosphorylated Ser 184 can be blocked by *O*-GlcNAc modification, preventing re-phosphorylation and facilitating its translocation to mitochondria, ultimately resulting in triggering the downstream cascade of the apoptosis process (Fig. 4).

Statistical and computational analysis of all positively predicted Yin Yang sites and false negatively predicted Yin Yang sites showed that, in pro- and anti-apoptotic proteins, the amino acid preference around predicted Yin Yang sites is not much different for Ser and Thr (Table IV). A higher percentage of observed frequency of significantly preferred Ser was observed for Pro at +1, +3, and +4 positions (Table IV), which is in close accordance with the results described earlier for phosphorylation [Qazi et al., 2006], for *O*-glycosylation [Christlet and Veluraja, 2001] and for Yin Yang sites [Ahmad et al., 2006]. On the other hand, Arg at −2 and −3 was found to be preferred for kinase binding (Table IV); indeed, as described earlier, kinases prefer basic residues in vicinity of a Ser/Thr to be phosphorylated [Barsyte-Lovejoy et al., 2002; Croce, 2005]. Similarly, the highest percentage of observed frequency of significantly preferred amino acids around Thr was for Pro at position +2, Ser at +1 and Arg at −1 (Table IV), which is again in accordance with earlier reports, as described above for Ser with an exception of Arg at −1. But there are instances where basic residues are present at −1 position in some known phosphorylation sites, as in the case of histone H3.

The association rules described in Table V as possible motifs or patterns may be helpful in designing experiments for verification of the involvement of neighboring residues of a Yin Yang site in the complex interplay of phosphorylation and glycosylation in the modulation of functions of pro- and anti-apoptotic proteins. The peptide data, used for the analysis of all proposed Yin Yang sites, is provided in the Supplementary data table (Table VI) and will serve the purpose of training/test data for further theoretical or experimental investigations in analyzing OGT and kinase specificities.

Induction of tumor cell apoptosis by ML-1 is triggered by a signaling cascade through the complex interplay of phosphorylation and *O*-GlcNAc modification in pro- and anti-apoptotic proteins; the interplay of phosphorylation and glycosylation at Ser and Thr may initially alter the balance between pro- and anti-apoptotic proteins within a cell, thereby initiating the apoptotic machinery and, consequently, induce tumor cell apoptosis. We further propose that the signaling cascade of phosphorylation and dephosphorylation by ML-I in the Bcl-2 family of proteins is associated with *O*-GlcNAc modification. ML-1 initiation of the signaling cascade through interplay of these PTMs in Bcl-2

proteins contributes significantly to disrupt the cell and promote cell death or apoptosis. This study should be useful in the subsequent verification of the predicted results and further promote ML-1 as a potentially chemotherapeutic agent in different cancers.

A better understanding of PTMs and their interplay in these proteins should help designing precise inhibitors of a specific PTM at a particular site. Proteins from other sources—besides ML-1, etc.—might be used as potential chemotherapeutic agents to trigger downstream signaling events and control of apoptosis, using several PTM inhibitors in combination. Cancer chemotherapy will greatly benefit from the design of drugs specifically targeted to the control of PTMs in different proteins involved in cancer.

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