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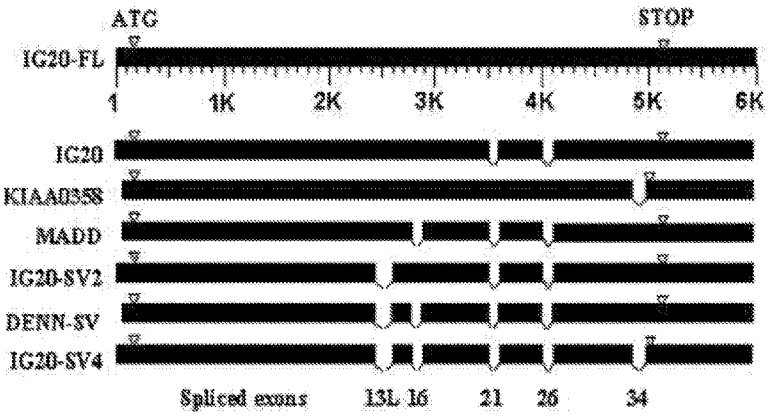
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Figure 1.



(57) Abstract: The invention provides compositions and methods for treating cancers comprising administering a combination of antineoplastic agents, wherein the combination comprises alkylating agent chemotherapeutics and one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene, and wherein not all splice variants of the IG20 gene are down-regulated. In an embodiment, the splice variant of the IG20 gene is a MADD splice variant and the nucleic acid molecules are siRNA, shRNA and antisense oligonucleotides which comprise a nucleic acid sequence complementary to a nucleic acid sequence of exon 13L of the MADD splice variant or to an mRNA transcript of exon 13L of the MADD splice variant. The invention encompasses methods of treating cancers which include combination therapy with nucleic acid molecules capable of down-regulating the expression of the at least one splice variant of the IG20 gene, and alkylating agent chemotherapeutics.

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SYNERGISTIC COMBINATION OF NUCLEIC ACID OLIGONUCLEOTIDES AND ALKYLATING AGENT CHEMOTHERAPEUTICS

FIELD OF THE INVENTION

[0001] The present invention is concerned with pharmaceutical compositions and methods for treating cancers comprising administering a combination of antineoplastic agents, wherein the combination comprises alkylating agent chemotherapeutics and one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the Insulinoma-Glucagonoma (*IG20)* gene, and wherein not all splice variants of the *IG20* gene are down-regulated. In an embodiment, the splice variant of the *IG20* gene is the MADD splice variant and the nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene are selected from siRNA, shRNA and antisense oligonucleotides, wherein the siRNA, shRNA and antisense oligonucleotides comprise a nucleic acid sequence which is complementary to a nucleic acid sequence of exon 13L of the MADD splice variant and/or to an mRNA transcript of exon 13L of the MADD splice variant. The invention encompasses methods of treating cancers which include combination therapy with nucleic acid molecules capable of down-regulating the expression of at least one splice variant of the *IG20* gene, and alkylating agent chemotherapeutics.

BACKGROUND OF THE INVENTION

[0002] The *IG20* gene plays an important role in cancer cell proliferation, apoptosis and survival (Chow VT, Lee SS. (1996). DNA Seq 6: 263–273, Chow VT, Lim KM, Lim D. (1998). Genome 41: 543–552; Schievella AR, Chen JH, Graham JR, Lin LL. (1997). J Biol Chem 272: 12069–12075; Brinkman BM, Telliez JB, Schievella AR, Lin LL, Goldfeld AE. (1999). J Biol Chem 274: 30882–30886; Murakami-Mori K, Mori S, Bonavida B, Nakamura S. (1999). J Immunol 162: 3672–3679; Telliez JB, Bean KM, Lin LL. (2000). Biochem Biophys Acta 1478: 280–288; Al-Zoubi AM, Efimova EV, Kaithamana S, Martinez O, El-Idrissi ME, Dogan RE et al. (2001). J Biol Chem 276: 47202–47211; Lim KM, Chow VT. (2002). Mol Carcinog 35: 110–126;

Efimova EV, Al-Zoubi AM, Martinez O, Kaithamana S, Lu SF, Arima T et al. (2004). Oncogene 23: 1076–1087; Efimova EV, Martinez O, Lokshin A, Arima T, Prabhakar BS. (2003). Cancer Res 63: 8768–8776; Lim KM, Yeo WS, Chow VT. (2004). Int J Cancer 109: 24–37; Ramaswamy M, Efimova EV, Martinez O, Mulherkar NU, Singh SP, Prabhakar BS. (2004). Oncogene 23: 6083–6094). Additionally, it plays an important role in neurotransmission (Zhang Y, Zhou L, Miller CA. (1998). Proc Natl Acad Sci USA 95: 2586–2591; Tanaka M, Miyoshi J, Ishizaki H, Togawa A, Ohnishi K, Endo K et al. (2001). Mol Biol Cell 12: 1421–1430; Yamaguchi K, Tanaka M, Mizoguchi A, Hirata Y, Ishizaki H, Kaneko K et al. (2002). Proc Natl Acad Sci USA 99: 14536–14541), neurodegeneration (Del Villar K, Miller CA. (2004). Proc Natl Acad Sci USA 101: 4210–4215) and guanine nucleotide exchange (Wada M, Nakanishi H, Satoh A, Hirano H, Obaishi H, Matsuura Y et al. (1997). J Biol Chem 272: 3875–3878; Brown TL, Howe PH. (1998). Curr Biol 8: R191; Iwasaki K, Toyonaga R. (2000). EMBO J 19: 4806–4816; Levivier E, Goud B, Souchet M, Calmels TP, Mornon JP, Callebaut I. (2001). Biochem Biophys Res Commun 287:688–695). These divergent functions are most likely mediated through alternative splicing of the *IG20* gene to provide splice variants and protein diversity (Chow VT, Lim KM, Lim D. (1998). Genome 41: 543–552; Al-Zoubi AM, Efimova EV, Kaithamana S, Martinez O, El-Idrissi ME, Dogan RE et al. (2001). J Biol Chem 276: 47202–47211; Efimova EV, Al-Zoubi AM, Martinez O, Kaithamana S, Lu, SF, Arima T et al. (2004). Oncogene 23: 1076–1087). Down-regulation of all endogenous *IG20* splice variants, using anti-sense oligonucleotides, has been shown to result in spontaneous apoptosis of cancer cells *in vitro* and *in vivo*, but not in normal cells (Lim KM, Chow VT. (2002). Mol Carcinog 35: 110–126; Lim KM, Yeo WS, Chow VT. (2004). Int J Cancer 109: 24–37).

[0003] US Patent No. 8,722,637 describes "IG20 and IG20-[SV2], and the previously reported KIAA0358, MADD, and DENN-SV are splice variants of the *IG20* gene, which is localized to chromosome 11p11 and consists of 36 exons. Differences among the above variants are due to alternative splicing of exons 13L, 16, 21, 26 and 34." The IG20, MADD, SV2 and DENN-SV isoforms may be considered to be described in the article: Contrasting Effects of IG20 and Its Splice Isoforms, MADD and DENN-SV, on Tumor Necrosis Factor α-induced Apoptosis and

Activation of Caspase-8 and -3, Adeeb M. Al-Zoubi, Elena V. Efimova, Shashi Kaithamana, Osvaldo Martinez, Mohammed El-Azami El-Idrissi, Rukiye E. Dogan, and Bellur S. Prabhakar, THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 276, No. 50, Issue of December 14, pp. 47202–47211, 2001.

[0004] Subsequent to the disclosure in Al-Zoubi, *et al.*, longer isoforms were identified, including KIAA0358. Consequently the *IG20* splice variant identified by Al-Zoubi, *et al.* as IG20 became known as IG20pa because, as the '637 patent explains, the IG20 expression protein is a pro-apoptotic signaling molecule which is distinct from the IG20-FL or full length variant. (Col 5, lns. 43-45)

[0005] Efimova describes that "all seven variants of IG20 identified to date arise from alternative splicing of exons 13L, 16, 21, 26 and 34. The full-length cDNA of IG20 (IG20-FL) (accession number AF440100) is 5995 base pairs (bps) long, consists of all 36 exons and represents the longest variant. Elena V. Efimova, *et al.*, IG20, in contrast to DENN-SV, (MADD splice variants) suppresses tumor cell survival, and enhances their susceptibility to apoptosis and cancer drugs, Oncogene (2004) 23, 1076–1087. These splice variants may be graphically represented as in Figure 1. Splicing of exon 34 alone generates KIAA0358 (accession number AB002356) that consists of 5942 bps. Splicing of exons 21 and 26, and splicing of exons 16, 21 and 26 generate 5878 bps long IG20 accession number AF440101) and 6002 bps long MADD (accession number U77352), respectively. MADD is also known as DENN (accession number U44953) that is 5844 bps long. Splicing of exons 13L, 21 and 26, and 13L, 16, 21 and 26 generate 5749 bps long IG20-SV2 (accession number AF440102) and 5689 bps long IG20-SV3 (accession number AF440103) (earlier referred to as DENN-SV). Finally, splicing of all five exons (13L, 16, 21, 26 and 34) generates IG20-SV4 (accession number AF440434), which is the shortest variant and consists of 5619 bps."

[0006] Figure 1 graphically displays human IG20 splice variants generated by alternative mRNA splicing. The cDNA sequence homology among the seven IG20 splice variants is shown. Solid bars represent regions of complete homology between all variants. Empty areas indicate exons 13L, 16, 21, 26 and 34, which, when spliced in different combinations, produce the seven splice variants shown on the left.

Splicing of exon 34 in KIAA0358 and IG20-SV4 induces an early stop codon in exon 35. Shown also are different 5' untranslated regions (UTRs) for different splice variants.

[0007] At the time of the invention described in the '637 patent, it was determined that IG20pa, MADD, IG20-SV2 and DENN-SV are expressed in human tissues and, to a greater degree, in tumors. '637 at column 5, lines 1-2. Overexpression of DENN-SV was associated with enhanced cell replication and resistance to apoptosis induced by chemotherapy. '637 at column 5, lines 4-6. Overexpression of IG20pa was shown to override endogenous DENN-SV function, resulting in apoptosis. '637 at column 5, lines 16-17.

[0008] Through the experimentation performed in the '637 patent, it was determined that, "[b]ecause DENN-SV lacks both exons 13L and 16, MADD lacks exon 16 and IG20-SV2 lacks exon 13L, these results demonstrated that expression of both exons 13L and 16, as seen in IG20[pa], is required for anti-proliferative and pro-apoptotic properties, whereas deletion of both exons, as seen in DENN-SV, is required for pro-proliferative and anti-apoptotic properties." '637 at column 17, lines 38-44. Ultimately, the '637 patent concludes that "[t]here is clear evidence to suggest that IG20[pa] and DENN-SV have contrasting effects on apoptosis and cell proliferation." '637 at column 18, lines 37-39. It is further concluded that " IG20[pa] is a pro-apoptotic protein that can interact with DR4 and DR5 and significantly enhance TRAIL induced apoptosis by facilitating DISC formation with increased recruitment of FADD and caspase-8." '637 at column 23, lines 1-4. Finally, it was concluded that "IG20[pa] can render cells more susceptible to apoptosis and suppress cell growth. This raises the possibility of using IG20[pa] to render cells that are otherwise resistant to become more susceptible to various modalities of cancer therapy." '637 at column 27, lines 20-24.

[0009] As a result of the understanding achieved through over-expression of *IG20* splice variants, it was concluded that "[c]ells transfected with IG20[pa] and DENN-SV were most susceptible and resistant to TNFα-induced apoptosis respectively, whereas cells transfected with MADD or IG20-SV2 did not show significant differences relative to cells transfected with a control plasmid. Because

DENN-SV lacks both exons 13L and 16, MADD lacks exon 16 and IG20-SV2 lacks exon 13L, these results demonstrated that expression of both exons 13L and 16, as seen in IG20[pa], is required for anti-proliferative and pro-apoptotic properties, whereas deletion of both exons, as seen in DENN-SV, is required for pro-proliferative and anti-apoptotic properties." '637 at column 17, lines 34-44.

[0010] Therefore, prior to the '637 patent, the full complexity of *IG20* splice variant expression, selective expression of different isoforms in different tissues and the unique functional attributes of each of the splice variants were not appreciated. Based on the experimentation into the over-expression of *IG20* splice variants as disclosed in the '637 patent, the impact of indiscriminant knock-down of splice variants, not taking into account variant splicing at introns 16 (MADD), 13L and 16 (DENN-SV), 21 and 26 (MADD, DENN-SV, IG20pa) and 34 (KIAA0358, IG20-SV4), could prove vital for normal neuronal function and survival of the animal (i.e. KIAA0358), cancer cell survival (i.e. MADD) and proliferation (i.e. DENN-SV). Therefore, the patent provides a functional characterization of *IG20* isoforms and explains the complexity in devising strategies which may selectively modulate various isoform expression while avoiding unintended lethal consequences.

[0011] The IG20pa splice variant is pro-apoptotic, anti-proliferative, and renders cells more susceptible to induced cell death (i.e. is a tumor suppressor). IG20pa, or a fragment thereof, may be over expressed to control cell proliferation, cell cycle, and to render cells more susceptible to chemotherapy, radiation therapy or death receptor mediated cell death.

[0012] DENN-SV expression can be down modulated to reduce cell proliferation, affect cell cycle and increase susceptibility to treatment with chemotherapy, radiation therapy and death receptor mediated cell death.

[0013] Differential expression of IG20pa and DENN-SV splice variants renders cells either more susceptible or resistant to induced cell death respectively, and the pro-apoptotic property of IG20pa variant may be exploited to render tumor cells that are otherwise chemotherapeutic resistant to become susceptible to killing by TRAIL and/or chemotherapeutic agents.

[0014] When IG20pa was over-expressed in cells, the cells showed significantly reduced proliferation and were much more susceptible to spontaneous, TNFα and TRAIL induced apoptosis. Thus, the pro-apoptotic property of the IG20pa splice variant may be exploited to render tumor cells which are otherwise resistant to become susceptible to killing by TRAIL and/or chemotherapeutic agents.

[0015] Down modulation of expression of IG20 splice variants with siRNA was evaluated using siRNA targeting the Mid region of the IG20 mRNA, specifically exon 15, having the sequence (5'-GTACCAGCTTCAGTCTTTC-3') and siRNA targeting the Death Domain (DD) region of IG20 mRNA. Both the Mid region and the DD region are present in all IG20 splice variants.

[0016] Treatment of cells with siRNA against the Mid region of IG20 mRNA, but not against the death domain (DD) of IG20 mRNA suppresses the levels of DENN-SV (completely abrogates). Cells treated with siRNA against the Mid region, i.e., exon 15, of IG20 mRNA undergo spontaneous apoptosis. Moreover, the cells that fail to undergo spontaneous apoptosis after siRNA treatment against the Mid region, i.e., exon 15, of IG20 mRNA are more susceptible to TNF-alpha induced apoptosis.

[0017] It may be concluded that, to decrease cell replication, selection of siRNA to down modulate DENN-SV expression, specifically, without affecting expression of IG20 splice variants which are necessary for normal cell function, in particular, neuronal function and survival (KIAA0358 and IG20-SV4 splice variants), is desired.

[0018] Moreover, contrasting prior art oligodeoxynucleotide sequences which in many cases proved lethal, oligodeoxynucleotides which take into account the research of the '637 patent may be optimized to bind to particular exons which are differentially expressed in different isoforms. Knockdown using such engineered oligodeoxynucleotides may knock down only those isoforms in which that particular targeted exon is expressed, allowing for selective knockdown of intended isoforms

and reducing unintended negative effects associated with knockdown of critical isoforms.

[0019] Map kinase Activating Death Domain (MADD) containing protein, a product of the MADD splice variant of the *IG20* gene, is essential for cancer cell survival. MADD is expressed at much higher levels in cancer cells and tissues relative to their normal counterparts. MADD has been shown to bind to death receptor-4 (DR4) and death receptor-5 (DR5) and to confer resistance to TRAIL induced apoptosis in thyroid, ovarian and cervical cancer cell lines (Mulherkar N, Prasad KV, Prabhakar BS, MADD/DENN splice variant of the *IG20* gene is a negative regulator of caspase-8 activation. Knockdown enhances TRAIL-induced apoptosis of cancer cells, J Biol Chem 282: 11715-11721 (2007); Subramanian M, Pilli T, Bhattacharya P, Pacini F, Nikiforov YE, et al., Knockdown of *IG20* gene expression renders thyroid cancer cells susceptible to apoptosis, J Clin Endocrinol Metab 94: 1467-1471 (2009); Prabhakar BS, Mulherkar N, Prasad KV, Role of IG20 splice variants in TRAIL resistance,. Clin Cancer Res 14: 347-351 (2008); Li LC, Jayaram S, Ganesh L, Qian L, Rotmensch J, et al., Knockdown of MADD and c-FLIP overcomes resistance to TRAIL-induced apoptosis in ovarian cancer cells, Am J Obstet Gynecol 205: 362 e312-325 (2011)).

[0020] Abrogation of MADD, but not the other *IG20* splice variants, may render cancer cells more susceptible to spontaneous as well as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis. Spontaneous as well as TRAIL-induced apoptosis in cells devoid of MADD may be inhibited by expression of CrmA or dominant-negative FADD, thereby suggesting that endogenous MADD may interfere with caspase-8 activation. Further, it has been found that MADD can directly interact with death receptors, but not with either caspase-8 or FADD, but nonetheless inhibits caspase-8 activation. MADD has been shown to interfere with recruitment of FADD to the cytoplasmic domain of death receptors (Mulherkar N, Prasad K and Prabhakar B, MADD/DENN Splice Variant of the IG20 Gene is a Negative Regulator of Caspase-8 Activation, Journal of Biological Chemistry, Vol. 282, no. 16, 11715-11721 (2007). This demonstrates the importance of MADD in the control of cancer cell survival/death and in conferring resistance to TRAIL-induced apoptosis.

[0021] The ERK (extracellular signal-related kinase) pathway is a drug target for cancer chemotherapy since, in approximately one-third of all human cancers, there is deregulation of the mammalian mitogen-activated protein kinase (MAPK) pathways leading to ERK activation. MAPKs are serine/threonine-specific protein kinases which respond to extracellular stimuli (mitogens) and regulate several important and critical cellular functions required for cell homeostasis like metabolism, cell cycle progression, expression of cytokines, motility and adherence. Hence MAPKs influence cell survival, proliferation, differentiation, development and apoptosis. Extracellular stimuli such as cytokines, growth factors and environmental stresses lead to the sequential activation of a signaling cascade composed of MAPKs.

[0022] When activated, ERK1/2 phosphorylates several nuclear and cytoplasmic substrates involved in a multitude of cellular processes, including transcriptional factors, signaling proteins, kinases and phosphatases, cytoskeletal proteins, apoptotic proteins and proteinases. Even though the ERK pathway may be activated by numerous extracellular signals, the pathways whereby cytokines and growth factors activate ERK signaling are of particular relevance to cancer. For example, TNF-α, a cytokine rich in tumor stroma binds to TNF receptor 1 (TNFR1) which is present on cancer cells and potently activates ERK MAPKs. In the absence of MADD this pro-survival signaling pathway may be converted into an apoptotic signaling pathway leading to cancer cell death (Kurada BRVVSN, Li LC, Mulherkar N, Subramanian M, Prasad KV, Prabhakar BS, MADD, a Splice Variant of *IG20*, is Indispensable for MAPK Activation and Protection against Apoptosis upon Tumor Necrosis Factor-α Treatment, (2009), Journal of Biological Chemistry 284:13533-13541).

[0023] An extrinsic cell death inducing signaling pathway may be initiated upon death ligand (e.g. TRAIL) binding to its cognate death receptors. The death receptors undergo trimerization and recruit FADD resulting in subsequent caspase-8 activation followed by executioner caspase-3 activation leading to apoptosis. TRAIL normally binds to death receptors-4 (DR4) and -5 (DR5) on cancer cells resulting in death receptor (DR) oligomerization and subsequent recruitment of FADD and procaspase-8 to the DRs (Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider

P, et al., TRAIL receptor-2 signals apoptosis through FADD and caspase-8, Nat Cell Biol 2: 241-243 (2000); Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, et al., FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2, Immunity 12: 599-609 (2000); Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, et al., Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5, Immunity 12: 611-620 (2000)). Procaspase-8 then undergoes proximity induced cleavage and activation forming caspase-8 which then activates executioner caspase-3 which causes apoptotic cell death. However, in cancer cells where MADD is over-expressed, MADD binds to DR4 and DR5 and prevents FADD recruitment to the DRs. Upon MADD down-regulation, FADD is more readily recruited to the DRs, resulting in enhanced apoptosis (Mulherkar N, Prasad KV, Prabhakar BS, MADD/DENN splice variant of the *IG20* gene is a negative regulator of caspase-8 activation. Knockdown enhances TRAIL-induced apoptosis of cancer cells, J Biol Chem 282: 11715-11721 (2007); Mulherkar N, Ramaswamy M, Mordi DC, Prabhakar BS, MADD/DENN splice variant of the *IG20* gene is necessary and sufficient for cancer cell survival, Oncogene 25: 6252-6261 (2006)).

[0024] TRAIL is unique in that it generally does not adversely affect normal cells or tissues (Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S, Chemotherapy augments TRAIL-induced apoptosis in breast cell lines, Cancer Res 59: 734-741 (1999)). However, development of chemotherapy and TRAIL resistance due to the interference of different anti-apoptotic proteins remains a major challenge. MADD is one such anti-apoptotic protein demonstrating the utility of MADD down-regulation in rendering cancer cells susceptible to cell death (Mulherkar N, Ramaswamy M, Mordi DC, Prabhakar BS, MADD/DENN splice variant of the IG20 gene is necessary and sufficient for cancer cell survival, Oncogene 25: 6252-6261 (2006)).

[0025] MADD may have a dual function in regulating apoptosis depending on its phosphorylation by Akt. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway. MADD can act as a pro-apoptotic factor to initiate apoptosis when its phosphorylation is attenuated by

PTEN (Jayarama S, Li L, Ganesh L, Mardi D, Kanteti P, Hay N, Li P, and Prabhakar BS, J Cell Biochem. 2014, 115(2):261-270). TRAIL induces an up-regulation of PTEN with a concomitant reduction in MADD phosphorylation. Down-regulation of PTEN interferes with TRAIL-induced reduction in pMADD levels. Non-phopshorylated MADD translocates from the plasma membrane to cytoplasm where it binds to 14-3-3 protein and displaces 14-3-3 associated Bax, which Bax translocates to mitochondria resulting in cytochrome-c release. Taken together, one may conclude that PTEN can convey the death signal by preventing MADD phosphorylation by Akt.

[0026] The extrinsic apoptotic pathway may be abrogated by phosphorylated MADD. Endogenous MADD is phosphorylated at three highly conserved sites by Akt, and only the phosphorylated MADD can directly interact with the TRAIL receptor DR4 thereby preventing FADD recruitment. However, in cells susceptible to TRAIL treatment, TRAIL induces a reduction in MADD phosphorylation levels resulting in MADD dissociation from, and FADD association with DR4, which allows death-inducing signaling complex (DISC) formation leading to apoptosis (Li P, Jayarama S, Ganesh L, Mordi D, Carr R, Kanteti P, Hay N, and Prabhakar BS, J Biol Chem. 2010 July 16; 285(29): 22713–22722). Thus, the pro-survival function of MADD is dependent upon its phosphorylation by Akt. Because Akt is active in most cancer cells and phosphorylated MADD confers resistance to TRAIL-induced apoptosis, co-targeting the Akt-MADD axis is likely to increase efficacy of therapeutics which involve DR4/5 binding, including TRAIL-based therapies.

[0027] The intrinsic apoptotic pathway is initiated when a death signal induces the release of mitochondrial pro-apoptotic proteins such as cytochrome c (Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cell, 1997; 91(4):479–489), mitochondrial apoptosis-inducing factor (Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Nature. 1999; 397(6718):441–446) and Smac/Diablo (Du C, Fang M, Li Y, Li L, Wang X. Cell. 2000; 102(1):33–42; Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Cell. 2000; 102(1):43–53). Cytochrome-c forms a complex with Apaf-1 and procaspase-9 resulting in the activation of caspase-9. Smac/Diablo can associate with Inhibitor of

Apoptosis Proteins (IAPs) and counteract their caspase inhibitory effects. The intrinsic pathway is regulated by the Bcl-2 family members. For example, in response to pro-apoptotic stimuli, the cytosolic Bax and Bad translocate to mitochondria to permeabilize the outer mitochondrial membrane leading to cytochrome c release into the cytosol. In contrast, Bcl-2 and Bcl-xL can associate with Bax and Bad thereby preventing them from inducing death (Antignani A, Youle RJ. Curr Opin Cell Biol. 2006; 18(6):685–689). Interestingly, non-phopshorylated MADD translocates from the plasma membrane to cytoplasm where it binds to 14-3-3 and displaces 14-3-3 associated Bax, which translocates to mitochondria resulting in cytochrome-c release (Jayarama S, Li L, Ganesh L, Mardi D, Kanteti P, Hay N, Li P, and Prabhakar BS, J Cell Biochem. 2014, 115(2):261-270).

[0028] The MADD cDNA sequence is available on the GenBank database under accession number: NM_130470, and is represented herein by the nucleotide sequence of SEQ ID NO:11 and the polypeptide sequence of SEQ ID NO:12. Interfering RNAs which down-regulate MADD, including siRNA, shRNA and antisense oligonucleotides, are designed to target a nucleic acid sequence of exon 13L of a splice variant of the *IG20* gene, and include any allelic variants and naturally occurring mutants of MADD, and polymorphisms which occur in the MADD splice variant which may be found in a particular segment of the population. In other words, sequences which are highly similar (e.g., about 95% at the amino acid level and about 75% at the nucleic acid level) and which represent naturally occurring variations in the MADD splice variant are within the scope of the disclosure, wherein the siRNA, shRNA and antisense oligonucleotides disclosed herein are capable of down-regulating the expression of such sequences. Exon 13L of the MADD splice variant may comprise a nucleotide sequence represented by nucleotides 2699 to 2827 of SEQ ID NO:11. Nucleic acid sequences which are about 80% or 90% or 95% similar at the nucleic acid level to the MADD sequence disclosed herein may also be down-regulated. Nucleic acid sequences which generate siRNA and shRNA which comprise nucleic acid sequences complementary to a nucleic acid sequence of exon 13L of the MADD splice variant of the *IG20* gene and/or an mRNA transcript of exon 13L of the MADD splice variant, as well as nucleic acid variations which may occur within the exon 13L target region are within the scope of the instant disclosure. Moreover, antisense oligonucleotides which comprise nucleic acid sequences

complementary to a nucleic acid sequence of exon 13L of the MADD splice variant of the *IG20* gene and/or an mRNA transcript of exon 13L of the MADD splice variant, as well as nucleic acid variations which may occur within the exon 13L target region are within the scope of the instant disclosure

[0029] Methods for specifically down-regulating the expression of a splice variant of an *IG20* gene have been shown to include: (a) obtaining a nucleic acid molecule which is capable of down-regulating MADD expression, wherein the nucleic acid molecule or a transcription product thereof is capable of selectively binding to an mRNA molecule, the mRNA molecule which includes a nucleic acid sequence of a MADD splice variant of the *IG20* gene; and (b) contacting a cell which expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant. In the cytoplasm, nucleic acids selected from siRNA, expressed shRNAs, and antisense oligonucleotides bind to target exon 13L mRNA and lead to degradation of the target 13L mRNA which down-regulates expression of the MADD splice variant.

[0030] Specifically, down-regulating MADD expression has been shown to be a substantial downregulation, for example, more than 90% or 95% reduction of the endogenous MADD expression. In fact, downregulation of, for example, at least 40%, at least 50%, at least 60%, at least 70%, and at least 80% of endogenous MADD expression is desirable.

[0031] Isolated siRNA, shRNA and antisense oligonucleotides which selectively down-regulate the expression of a splice variant of an *IG20* gene, wherein the splice variant is MADD, are disclosed in US Patent No. 7,910,723. The patent describes the development of "splice variant specific" nucleic acid molecules which selectively down-regulate one or more of the splice variants of the *IG20* gene and their relative importance in cancer cell survival. Not all cancer cells express all *IG20* splice variants and some express only the MADD and the DENN splice variants. Using cells which expressed four known splice variants or only the MADD and DENN splice variants, and different nucleic acid molecules selected from shRNAs, siRNAs and antisense oligonucleotides which specifically targeted exon 13L (to down-regulate IG20pa and MADD), exon 16 (to down-regulate IG20pa and IG20-SV2) and

exon 15 (exon 15 is expressed in all splice variants of the *IG20* gene and therefore it can down-regulate expression of all splice variants) the critical requirement for MADD for cancer cell survival has been demonstrated. Although siRNA, shRNA and antisense oligonucleotides targeting exon 16 could knockdown IG20pa, only the siRNA, shRNA and antisense oligonucleotides targeting exon 13L could cause cancer cell death. This indicated for the first time that specifically MADD might be critical for cancer cell survival. An additional line of evidence which shows MADD to be essential and sufficient for cancer cell survival is demonstrated in cells transfected with Mid-shRNA resistant *IG20* splice variants in which the 3rd base of the triple codons was replaced in the Mid-shRNA targeted region of the cDNA constructs, which DNA substitutions will not alter the amino acid sequence but renders them resistant to Mid-shRNA). When the expression of all endogenous *IG20* splice variants was down-regulated using Mid-shRNA, only cells expressing a MADD splice variant could prevent cell death, whereas the other splice variants could not prevent cell death. Using cancer cells which expressed only MADD and DENN-SV splice variants, it was shown that MADD could be selectively down-regulated using siRNA, shRNA or antisense oligonucleotides which target exon 13L and it was shown that MADD, alone, is sufficient and required for cancer cell survival. (Mulherkar, N., *et al.*, Oncogene 2006; 25:6252-61.)

[0032] Thus, none of the earlier research revealed the full complexity of *IG20* gene expression, the differential expression of the splice variants in different tissues, the unique functional attributes of each of the splice variants (i.e., cancer cell survival (MADD) and proliferation (DENN). Therefore, it is the discovery of various splice variants, their functional characterization and the ability to identify specific nucleic acid molecules which can selectively down-regulate expression of splice variants which allowed the inventors to develop cancer therapeutics without unintended potential negative consequences.

[0033] US Patent No. 7,910,723 describes nucleic acid molecules which target exon 13L of the *IG20* gene and the use of encoded siRNA, shRNA and antisense oligonucleotides in down-regulating expression of MADD protein. The patent describes how the nucleic acid selection may be optimized to achieve effective down-regulation of MADD expression, including selecting for nucleic acid molecules which

consist essentially of a nucleotide sequence CGGCGAATCTATGACAATC (SEQ ID NO:1), and transcribed products thereof, encoding nucleic acid molecules consisting essentially of a nucleotide sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2). Species nucleic acid molecules representative of such strategy include siRNA, shRNA and antisense oligonucleotides, which comprise less than 50% GC nucleotide content, high AU nucleotide content towards the 3' end and no inverted repeats within the siRNA region, which oligonucleotides constitute an array of nucleic acid molecules which may span the exon 13L nucleotide sequence. These encoded nucleic acid molecules and encoded siRNA, shRNA and antisense oligonucleotides are demonstrated to be sufficient to down-regulate the expression of MADD splice variants. Natural variations of MADD including specific SNPs, allelic variants, or mutations which may appear in one or more of sub-groups of cancer types may be targeted by such nucleic acid molecules.

[0034] Methods for down-regulating expression of MADD are described to include: (a) obtaining a nucleic acid molecule which selectively down-regulates MADD expression, wherein the nucleic acid molecule is capable of selectively binding to an mRNA molecule of a MADD splice variant of the *IG20* gene; and (b) contacting a cancer cell which expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant in the cancer cell.

[0035] Moreover, representative nucleic acid molecules which are shown to down-regulate the MADD splice variant of *IG20*, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, include siRNA, shRNA, and anti-sense nucleic acid molecules which may comprise a nucleotide sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2). The siRNA may be in the form of a duplex with the cognate antisense nucleic acid, which cognate antisense nucleic acid is complementary to a target MADD exon 13L nucleotide sequence and/or mRNA transcripts of MADD exon 13L.

[0036] Down-regulation of specific splice variants of the *IG20* gene is difficult because of the very short target sequences which are differentially expressed in different splice variants. US Patent No. 7,910,723 explains that, in an embodiment of

the invention, down-regulation may be achieved through the use of specially designed short hairpin RNA molecules (shRNA). Short hairpin RNA (shRNA) comprises complementary sense and antisense sequences of the target gene linked by a loop structure. A dsDNA may be cloned into an expression vector for transfection which may then be transcribed to form shRNA, which is cleaved into siRNA, which may inhibit gene expression through RNA interference (RNAi). In an embodiment, nucleic acids encoding such an shRNA, including the structure:

$$X_{sense} - \text{hairpin loop} - X_{anti\text{-}sense},$$

wherein X (encoding siRNA) includes or consists essentially of a nucleic acid having the sequence CGGCGAATCTATGACAATC (SEQ ID NO:1). The nucleic acid is transcribed to form shRNA and may be cleaved to form siRNA which may ultimately inhibit MADD expression. Thus, RNA molecules which are transcribed *in vitro* or *in vivo*, e.g., in a cancer cell or tumors to form shRNA and siRNA are also included.

[0037] An exemplary dsDNA nucleic acid sequence encoding an shRNA inhibiting MADD expression may be CGGCGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG (SEQ ID NO:3), wherein the hairpin loop region between X_{sense} and $X_{anti\text{-}sense}$ is from positions 20-28 of the sequence. The hairpin loop region may contain any suitable sequence. To construct shRNA vectors, see McIntyre and Fanning, Design and cloning strategies for constructing shRNA expression vectors, BMC Biotechnol. 2006; 6: 1 (2006), incorporated herein by reference in its entirety.

[0038] In an embodiment, double stranded RNA or dsRNA refers to a double stranded RNA which matches a predetermined gene sequence which is capable of activating cellular enzymes which degrade corresponding messenger RNA transcripts of the gene. These dsRNAs comprise nucleic acid molecules which may be short interfering RNA (siRNA) and may be used to inhibit gene expression. The term "double stranded RNA" or "dsRNA" as used herein refers to a double stranded RNA molecule capable of RNA interference "RNAi," including short interfering RNA "siRNA."

[0039] siRNA, shRNA and antisense oligonucleotide molecules as described herein may also include nucleic acid modifications known in the art to enhance stability and to enhance cleavage destruction of the target mRNA. With respect to the embodied nucleic acid molecules, a representative example may comprise a 19-base core which includes 2 or 3 nucleotide overhanding 3' ends, such as a 3' terminal thymidines (TT). The overhangs may play a structural role for presenting a symmetrical duplex to the RISC. Often dTdT is selected because they can confer nuclease resistance to nucleic acid molecules. Nevertheless, some investigators prefer UU overhangs or overhangs that are complementary to the authentic mRNA target. What is most important is the identity of the 19-base core of the nucleic acid molecules towards a unique mRNA target.

[0040] The siRNA, shRNA and antisense oligonucleotide molecules may also be chemically synthesized *de novo*. The synthesized nucleic acid molecules may be in the form of a single-stranded nucleic acid molecule or may be in the form of a duplex with the cognate antisense nucleic acid molecule. The siRNA, shRNA and antisense oligonucleotides comprise a nucleic acid sequence which is complementary to a target MADD exon 13L nucleotide sequence and/or mRNA transcripts of MADD exon 13L.

[0041] RNA interference is a conserved pathway found in most eukaryotes where double-stranded RNAs (dsRNAs) down-regulate expression of genes with complementary sequences. Long dsRNAs are degraded by the endoribonuclease Dicer into small effector molecules called siRNAs (small interfering RNAs). siRNAs are usually around 21 base pairs (bp) long with a central 19 bp duplex and 2 -base 3' –overhangs (this could be TT) (Elbashir, SM, Lendeckel W and Tuschl T, RNA interference is mediated by 21- and 23-nucleotide RNAs, Genes & Development, 2001, 15:188-200). In mammals, Dicer processing occurs in a multiprotein complex with the RNA -binding protein TRBP. The nascent siRNA associates with Dicer, TRBP, and Argonaute 2 (Ago2) to form the RNA -Induced Silencing Complex (RISC). Once in RISC, one strand of the siRNA (the passenger strand/the strand that has the same sequence as the target mRNA) is degraded or discarded while the other strand (the guide strand/strand that is complementary to the targeted mRNA) remains to

direct sequence specificity of the silencing complex. The Ago2 component of RISC is a ribonuclease that will cleave the target RNA under direction of the guide strand. Once the RISC complex is activated, it can move on to target additional mRNA targets. This effect amplifies gene silencing and allows the therapeutic effect to last for 3-7 days in rapidly dividing cells. Many researchers today employ synthetic RNA duplexes as their RNAi reagents, which mimic the natural siRNAs that result from Dicer processing of long substrate RNAs. These synthetic siRNA duplexes are transfected into cell lines where they mimic *in vivo* Dicer products.

[0042] The modulation of MADD expression and/or downregulation of MADD expression utilizing siRNA, shRNA or antisense oligonucleotides may augment traditional cancer therapies.

[0043] From a chemotherapeutic perspective, cancer chemotherapy has advanced dramatically in recent years. Numerous cancer chemotherapy substances have been identified which are effective in treating cancer. Nonetheless, many cancer chemotherapies are characterized by toxic side effects which are often encountered with administration of particular chemotherapeutics.

[0044] For example, the administration of many established chemotherapeutics is well known to result in unwanted side-effects including nausea and vomiting, loss of appetite, change in taste, thinned or brittle hair, joint pain, nail change, and tingling in the hands or toes. More serious side effects such as impaired bowel movement, difficulty in swallowing, dizziness, shortness of breath, severe exhaustion, and chest pain may also occur. Consequently, there is an unmet need to provide care givers with a treatment regimen which is possessed of the advantages of the chemotherapeutic potential of various known chemotherapeutics, but without the unwanted side effects which limit compliance and efficacy.

[0045] Chemotherapeutics, such as alkylating agents, act by attaching an alkyl group to the guanine base of DNA, thereby disrupting the normal functioning. They can be used on a wide range of cancers due to their ability to have this effect on a cancer cell on any phase of its life cycle. They can be used in the treatment of lymphoma, leukemia, testicular cancer, melanoma, brain cancer and breast cancer.

[0046] Alkylating agents act by irreversibly binding to DNA. Once bound, the altered molecule leads to disruption of normal action and replication of DNA, ultimately leading to apoptosis or arrest of cellular replication. Therefore, upon treatment with alkylating agents, they can limit tumor growth and lead to tumor destruction.

[0047] The most beneficial effect of alkylating agents is that it can be used to treat slow growing solid tumors and leukemia. They can also be used to treat lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, and Hodgkin's disease.

[0048] Representatives of alkylating agents can be classified as classical alkylating agents such as cyclophosphamide, mechlorethane; alkylating-like agents such as cisplatin, carboplatin; and agents such as procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine. Mechlorethamine, marketed under the trade name Mustargen®, is given by injection to treat Hodgkin's disease and non-Hodgkin's lymphoma, and as a palliative therapy for breast and lung cancers, and given as a topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma). Ifosfamide, sold under the trade name Ifex®, is used to treat both Hodgkin's and non-Hodgkin's lymphoma, as well as recurrent testicular cancer and germ cell tumors, sarcomas, lung cancer, bladder cancer, head and neck cancer, and cervical cancer. It is administered intravenously. Melphalan is a chemotherapy drug sold under the brand name Alkeran®, and is also referred to as L-PAM or phenylalanine mustard. It is used to treat multiple myeloma, ovarian cancer, neuroblastoma, rhabdomyosarcoma, and breast cancer. Chlorambucil is sold by the trade name Leukeran®, and is most widely used to treat chronic lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma, and Hodgkin's disease. It has also been successfully used to treat non-Hodgkin's lymphoma, breast, ovarian and testicular cancer, Waldenstrom's macroglobulinemia, thrombocythemia, and choriocarcinoma. It comes in coated tablet form. Cyclophosphamide is marketed as Cytoxan® or Neosar®, and is used to treat Hodgkin's and non-Hodgkin's lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma,

multiple myeloma, neuroblastoma, retinoblastoma, rhabdomyosarcoma, Ewing's sarcoma; breast, testicular, endometrial, ovarian, and lung cancers. Streptozocin is sold under the trade name Zanosar®, and is used to treat islet cell pancreatic cancer. Carmustine is also known as BiCNU® or BCNU, and is used for some kinds of brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors. It is also used in treatment for multiple myeloma, Hodgkin's disease, non-Hodgkin's lymphoma, melanoma, lung cancer, and colon cancer. Lomustine, also known as CCNU or CeeNU®, is used to treat primary and metastatic brain tumors, Hodgkin's disease and non-Hodgkin's lymphoma, and has also been used for melanoma, lung, and colon cancer. Busulfan, sold under trade names Busulfex® and Myleran®, is used to treat chronic myelogenous leukemia. Dacarbazine is sold under the trade name DTIC-Dome® and is used to treat metastatic malignant melanoma, Hodgkin's disease, soft tissue sarcomas, neuroblastoma, fibrosarcomas, rhabdomyosarcoma, islet cell carcinoma, and medullary thyroid carcinoma. Temozolomide is sold under the trade name Temodar®, and is used to treat the specific types of brain tumors anaplastic astrocytoma and glioblastoma multiforme. Thiotepa, known under the trade name Thioplex®, is an alkylating agent used to treat breast cancer, ovarian cancer, Hodgkin's disease, and non-Hodgkin's lymphoma. It is given by intravenous infusion. Altretamine is sold under the trade name Hexalen®, and is also called hexamethylmelamine or HMM. It is used to treat ovarian cancer.

[0049] Alkylating agents are also associated with side effects. They are toxic to normal cells as well, particularly rapidly dividing cells such as those in gastrointestinal tract and bone marrow. Long term use of alkylating agents can result in permanent infertility by reducing sperm production in males and leading to cessation of menstruation in females. Many alkylating agents can also lead to secondary cancers like acute myeloid leukemia.

[0050] Moreover, while the life expectancy under alkylating agent therapy is extended, it is not without undesirable side-effects effects of the drug. Thus, there is an unmet need for providing a treatment which would include the administration of a new class of drug with a different mechanism of action, and which would result in a more complete remission profile without undesirable side effects.

THE PRESENT INVENTION

[0051] The present inventors have now identified novel methods of administering alkylating agents which, when administered in combination with down-regulation of the MADD splice variant, result in dramatic synergistic effects including increased efficacy as well as reduced side-effects.

[0052] The present inventors have conceived and demonstrate for the first time that the clinical combination of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene, wherein not all splice variants of the IG20 gene are down-regulated, with conventional chemotherapeutics such as alkylating agents, is an unexpectedly valuable pharmacotherapeutic approach to treating various forms of cancer. The present inventors demonstrate that, when administered in combination to subjects suffering from cancers such as those of ovary, breast, lung, pancreas, bladder, cervix, prostate, melanoma, esophageal and other solid tumors including Kaposi's sarcoma, the effects of siRNA, shRNA and antisense oligonucleotides, wherein the siRNA, shRNA and antisense oligonucleotides comprise a nucleic acid sequence which is complementary to a nucleic acid sequence of exon 13L of the MADD splice variant or to an mRNA transcript of exon 13L of the MADD splice variant, and chemotherapeutics such as alkylating agents is of unexpected benefit and, at least over a period of time results in an unexpectedly superadditive relief of symptoms, evidenced by dramatic reduction in, or absence of, symptoms, tumor growth and tumor survival, and in this way will be particularly beneficial in the treatment of multiple cancers. Moreover, the combination of at least one siRNA, shRNA and antisense oligonucleotides, wherein the siRNA, shRNA and antisense oligonucleotides comprise a nucleic acid sequence which is complementary to a nucleic acid sequence of exon 13L of the MADD splice variant or to an mRNA transcript of exon 13L of the MADD splice variant, and chemotherapeutics such as alkylating agents may, for the first time, show promise in providing complete remission from multiple cancers, as well as enhanced margin of safety and tolerance.

OBJECTS OF THE INVENTION

[0053] It is an object of the present invention to provide novel combination antineoplastic treatments comprising administering representative chemotherapeutics in combination with nucleic acid molecules capable of down-regulating the expression of the MADD splice variant, which combination antineoplastic treatments are effective in treating various cancers, and pharmaceutical compositions comprising such combination antineoplastic. It is a further object of the invention to provide a novel method of treating various cancers which include administering combination antineoplastic treatments comprising nucleic acid molecules capable of down-regulating the expression of the MADD splice variant and chemotherapeutics such as alkylating agents.

[0054] An additional object of the invention is the provision of a process for producing targeted formulations and therapeutic delivery procedures for the combination antineoplastic treatments. Yet additional objects will become apparent hereinafter, and still further objects will be apparent to one skilled in the art.

SUMMARY OF THE INVENTION

[0055] What we therefore believe to be comprised by our invention may be summarized *inter alia* in the following words:

[0056] A combination of antineoplastic agents useful for treating cancer comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene, wherein not all splice variants of the *IG20* gene are down-regulated, and one or more alkylating agent chemotherapeutic.

[0057] Such a combination, wherein the at least one splice variant of the *IG20* gene is selected from a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof.

[0058] Such a combination, wherein the at least one splice variant of the *IG20* gene is a MADD splice variant which exhibits exon 13L.

[0059] Such a combination, wherein the one or more nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene is selected from siRNA, shRNA and antisense oligonucleotides.

[0060] Such a combination, wherein the siRNA, shRNA and antisense oligonucleotides comprise nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, and/or an mRNA transcript thereof.

[0061] Such a combination, wherein the nucleic acid molecule capable of down-regulating expression of at least one splice variant of the *IG20* gene is comprised in an siRNA or shRNA.

[0062] Such a combination, wherein the siRNA and shRNA is encoded by a nucleic acid molecule which includes the structure:

$$X_{sense} - \text{hairpin loop} - X_{anti\text{-}sense},$$

wherein X includes or consists essentially of a nucleic acid sequence CGGCGAATCTATGACAATC (SEQ ID NO:1).

[0063] Such a combination, wherein the siRNA or shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2).

[0064] Such a combination, wherein the siRNA or shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2) and is in the form of a duplex with a cognate nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

[0065] Such a combination, wherein the shRNA and siRNA is encoded by a nucleic acid having the sequence CGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG (SEQ ID NO:3), wherein a hairpin loop region is from positions 20-28 of the sequence.

[0066] Such a combination, wherein the siRNA, shRNA and antisense oligonucleotide comprising nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant of the *IG20* gene and/or an mRNA transcript thereof comprises a nucleic acid having the sequence selected from GAUUGUCAUAGAUUCGCCGTT (SEQ ID NO:4) and GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

[0067] Such a combination, wherein the one or more nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene is comprised in a drug delivery system.

[0068] Such a combination, wherein the drug delivery system is a targeted liposome formulation or a lentivirus vector.

[0069] Such a combination, wherein the one or more alkylating agent chemotherapeutic is selected cyclophosphamide, mechlorethane, cisplatin, carboplatin, procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine.

[0070] Such a combination, wherein the one or more alkylating agent chemotherapeutic is in the form of a pharmaceutically acceptable salt.

[0071] Such a combination, wherein the cancer is selected from slow growing solid tumors and leukemia, lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, Hodgkin's disease, topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma), both Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, germ cell tumors, bladder cancer, head and neck cancer, cervical cancer, multiple myeloma, neuroblastoma, rhabdomyosarcoma, lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant

follicular lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma, multiple myeloma, retinoblastoma, Ewing's sarcoma, endometrial cancer, islet cell pancreatic cancer, brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors, colon cancer, fibrosarcomas, anaplastic astrocytoma, and glioblastoma multiforme

[0072] Moreover, a method of treating cancers selected from slow growing solid tumors and leukemia, lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, Hodgkin's disease, topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma), both Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, germ cell tumors, bladder cancer, head and neck cancer, cervical cancer, multiple myeloma, neuroblastoma, rhabdomyosarcoma, lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma, multiple myeloma, retinoblastoma, Ewing's sarcoma, endometrial cancer, islet cell pancreatic cancer, brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors, colon cancer, fibrosarcomas, anaplastic astrocytoma, and glioblastoma multiforme in a subject in need thereof, comprising administering an effective amount of a combination of antineoplastic agents comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene, wherein not all splice variants of the *IG20* gene are down-regulated, and one or more alkylating agent chemotherapeutic.

[0073] Such a method, wherein the cancers exhibit expression of at least one splice variant of the *IG20* gene.

[0074] Such a method, wherein the cancers exhibit expression of the MADD splice variant of the *IG20* gene, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof.

[0075] Such a method, wherein MADD splice variant exhibits exon 13L of the *IG20* gene.

[0076] Such a method, wherein the one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene is selected from siRNA, shRNA and antisense oligonucleotides.

[0077] Such a method, wherein the siRNA, shRNA and antisense oligonucleotides comprise nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, of the IG20 gene and/or an mRNA transcript thereof.

[0078] Such a method, wherein the nucleic acid molecule capable of down-regulating expression of at least one splice variant of the *IG20* gene is comprised in siRNA or shRNA.

[0079] Such a method, wherein the siRNA and shRNA is encoded by a nucleic acid molecule which includes the structure:

$$X_{sense} - \text{hairpin loop} - X_{anti\text{-}sense},$$

wherein X includes or consists essentially of a nucleic acid sequence CGGCGAATCTATGACAATC (SEQ ID NO:1).

[0080] Such a method, wherein the siRNA and shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2).

[0081] Such a method, wherein the siRNA and shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2) and is in the form of a duplex with a cognate nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

[0082] Such a method, wherein the shRNA is encoded by a nucleic acid having the sequence CGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG (SEQ ID NO:3), wherein a hairpin loop region is from positions 20-28 of the sequence.

[0083] Such a method, wherein the siRNA, shRNA and antisense oligonucleotides comprising nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant of the *IG20* gene and/or an mRNA transcript thereof comprise a nucleic acid having the sequence selected from GAUUGUCAUAGAUUCGCCGTT (SEQ ID NO:4) and GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

[0084] Such a method, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered in the form of a liposomal formulation or by lentivirus transfection.

[0085] Such a method, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered as an adjuvant.

[0086] Such a method, wherein the one or more alkylating agent chemotherapeutic is selected from cyclophosphamide, mechlorethane, cisplatin, carboplatin, procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine.

[0087] Such a method, wherein the one or more alkylating agent chemotherapeutic is administered in the form of a pharmaceutical composition further comprising one or more pharmaceutically acceptable diluents, excipients, or carriers.

[0088] Such a method, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered prior to the one or more alkylating agent chemotherapeutic, or is administered simultaneously with the one or more alkylating agent chemotherapeutic.

[0089] Such a method, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered in the form of a pharmaceutical composition further comprising one or more pharmaceutically acceptable diluents, excipients, or carriers.

[0090] Such a method, wherein the one or more siRNA, shRNA, and antisense oligonucleotides and the one or more alkylating agent chemotherapeutic are formulated in a dosage pack and are administered according to a selected treatment regime.

BRIEF DESCRIPTION OF THE FIGURES

[0091] **Figure 1.** Human IG20 splice variants generated by alternative mRNA splicing. The cDNA sequence homology among the seven IG20 splice variants is shown. Solid bars represent regions of complete homology between all variants. Empty areas indicate exons 13L, 16, 21, 26 and 34, which, when spliced in different combinations, produce the seven splice variants shown on the left. Splicing of exon 34 in KIAA0358 and IG20-SV4 induces an early stop codon in exon 35. Shown also are different 5' untranslated regions (UTRs) for different splice variants.

DETAILED DESCRIPTION OF THE INVENTION

Combination of the Invention

[0092] As specified above, in one aspect, the instant invention provides a novel drug combination useful for treating, preventing, arresting, delaying the onset of and/or reducing the risk of developing, or reversing at least one symptom of a cancer selected from ovarian, breast, lung, pancreas, bladder, cervical, prostate, melanoma, esophageal and other solid tumors including Kaposi's sarcoma, in a mammal comprising administering to said mammal an amount of siRNA, shRNA and/or antisense oligonucleotides capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein the splice variant is MADD, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, and wherein not all splice variants of *IG20* are down-regulated, and alkylating agents at therapeutically effective dosages which, when combined, provide a beneficial effect.

Definitions

[0093] The term "combination" applied to active ingredients is used herein to define a single pharmaceutical composition (formulation) comprising both drugs of the invention (*i.e.*, one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of *IG20* are down-regulated, and one or more

alkylating agents) or two separate pharmaceutical compositions (formulations), each comprising a single drug of the invention (*i.e.*, one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of *IG20* are down-regulated and one or more alkylating agents), to be administered conjointly or in a pretreatment/treatment protocol.

[0094] Within the meaning of the present invention, the term "conjoint administration" is used to refer to administration of the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of *IG20* are down-regulated and one or more alkylating agents simultaneously in one composition, or simultaneously in different compositions, or sequentially in different compositions. For the sequential administration to be considered "conjoint", however, the administration of the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of *IG20* are down-regulated and one or more alkylating agents must be administered separated by a time interval which still permits the resultant beneficial effect of conjoint treatment for treating, preventing, arresting, delaying the onset of and/or reducing the risk of developing a cancer in a mammal. For example, the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of *IG20* are down-regulated and one or more alkylating agents may be administered sequentially. For example, siRNA may be administered 8-72 hours (hrs) before the administration of the alkylating agent so as to have the MADD expression down-modulated prior to treatment with the chemotherapeutic.

[0095] According to still another embodiment of the invention, the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene is used as an adjuvant. An "adjuvant" in the context of the present description refers to an enhancer of the specific alkylating agent response. "Using the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD

splice variant of an *IG20* gene" means including the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene in a pre-treatment prior to the chemotherapy agent, or in combination with the chemotherapeutic agent for simultaneous delivery. An adjuvant such as the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene in combination with a chemotherapeutic agent provides synergistic cell death.

[0096] Hence, in a further embodiment of the invention, the one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene are administered to mammals exhibiting cancers, including humans, activate the death of cancerous cells. The one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and one or more chemotherapeutic agent may also be used to support apoptotic pathways in a situation of increased susceptibility to developing tumors, such as in the case of patients in remission.

[0097] The term "treat" is used herein to mean to relieve or alleviate at least one symptom of a disease in a subject. For example, in relation to cancer, the term "treat" may mean to relieve or alleviate tumor growth or symptoms associated with the cancer and/or cause tumor regression. Within the meaning of the present invention, the term "treat" may also denote to arrest, delay the onset (*i.e.*, the period prior to clinical manifestation of a disease) and/or reduce the risk of developing or worsening a disease. The term "protect" is used herein to mean prevent delay or treat, or all, as appropriate, development or continuance or aggravation of a disease in a subject. Within the meaning of the present invention, the cancer is associated with clinical manifestations, including without limitation drug induced undesirable side-effects. For example, as disclosed herein, a prophylactic administration of one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents may protect a recipient subject to risk of developing cancer (*e.g.*, individuals having elevated levels of CA125, individuals, who exhibit histopathologic cancer markers; *see* also genetic screening and clinical analysis described in oncology literature for

standard screening for various cancers). Similarly, according to the present invention, a therapeutic administration of one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents can lead to slow-down in the development of clinical symptoms or even regression of symptoms.

[0098] The term short interfering RNA (siRNA) refers to RNA molecules which are capable of interfering with a particular gene transcription, thereby silencing the gene expression of a target protein. Representative mechanisms of this process may comprise administration of nucleic acid molecules comprising siRNAs, dsRNAs, short hairpin RNAs (shRNA) and/or antisense oligonucleotides complementary to a nucleic acid sequence of exon 13L of the MADD splice variant mRNA transcript. dsRNA and short hairpin RNAs are cleaved by an endo-ribonuclease Dicer, which cuts the dsRNA or shRNA into constituent siRNA. The siRNA operates through the formation of RNA-induced Silencing Complexes or RISCs. The RISC complex unwinds the siRNA to form single stranded siRNA. The RISC, comprising single stranded siRNAs binds to the target mRNA, cleaving the mRNA, rendering it unrecognizable and thereby silencing the production of the intended protein.

[0099] Within the meaning of the present invention, the terms "siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene" are used to refer to drugs, which target messenger RNAs. Embodiment siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene of the invention may be siRNA derivatives such as modified siRNAs, shRNAs and/or antisense oligonucleotides comprise nucleic acids which are complementary to the nucleic acid sequence of exon 13L of the MADD splice variant mRNA transcript. Particular embodiments include those substances described in US Patent No. 7,910,723.

[00100] Design of siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene may be accomplished through techniques which have been established. The siRNA, shRNA and antisense oligonucleotides used to target MADD mRNA transcripts were

obtained from Dharmacon (Lafayette, Colo.). The most suitable sequences were sorted out based on less than 50% GC nucleotide content, high AU nucleotide content towards the 3' end and no inverted repeats within the siRNA region (Reynolds et al. (2004), Nat Biotechnol, 22(3), 326-30).

[00101] Antisense oligonucleotides refers to a nucleic acid molecule which binds to target mRNA by means of RNA-RNA or RNA-DNA or RNA-PNA (peptide nucleic acid) interactions and alters the activity of the target mRNA. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule.

[00102] In another embodiment, antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region, which is capable of activating RNAse H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof.

[00103] In another aspect nucleic acid molecules or antisense molecules which interact with target RNA molecules and down-regulate MADD activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors may be DNA plasmids or viral vectors. Enzymatic nucleic acid molecule or antisense expressing viral vectors can be constructed based on adeno-associated virus, retrovirus, adenovirus, or alphavirus. In an embodiment, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of shRNA/siRNA/anti-sense nucleic acid molecules. Such vectors can be repeatedly administered as necessary. After being expressed, the interfering nucleic acid molecules bind to the target RNA and down-regulate its function or expression. Delivery of nucleic acid molecule or antisense expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells explanted from the patient or subject followed by reintroduction into the patient or subject, or by any other means that

would allow for introduction into the desired target cell. Antisense DNA can also be expressed via the use of a single stranded DNA intracellular expression vector.

[00104] siRNA and shRNA nucleic acid molecules and antisense oligonucleotides are designed accordingly based on the nucleotide sequence of the exon 13L of the MADD splice variant. Exon 13L of the MADD splice variant exhibits the nucleotide sequence such as that defined as nucleotides 2699 to 2827 of SEQ ID NO:11. The siRNA, shRNA and antisense oligonucleotides comprise oligonucleotides having a 19-base core nucleotide sequence which specifically targets exon 13L of the MADD splice variant. One skilled in the art may construct siRNA, shRNA and antisense oligonucleotides which comprise less than 50% GC nucleotide content, high AU nucleotide content towards the 3' end and no inverted repeats, which oligonucleotides constitute an array of oligonucleotides which may span exon 13L nucleotide sequence. The siRNA, shRNA and antisense oligonucleotides may further comprise 2 or 3 nucleotide overhanding 3' ends, such as a terminal TT to enhance cleavage destruction of the target mRNA. Often dTdT is selected because it can confer nuclease resistance to oligonucleotides. Moreover, UU overhangs or overhangs that are complementary to the authentic mRNA target may be added to the 19-base core of the oligonucleotides.

[00105] In an embodiment, the siRNA, shRNA and antisense oligonucleotide comprises a nucleic acid having the sequence GAUUGUCAUAGAUUCGCCGTT (SEQ ID NO:4). The siRNA and shRNA may be in the form of a duplex.

[00106] In an embodiment, the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, wherein not all splice variants of the IG20 gene are down-regulated, comprises a nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10). The siRNA or shRNA may be in the form of a duplex.

[00107] In an embodiment, the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice

variant of an *IG20* gene, wherein not all splice variants of the IG20 gene are down-regulated, consists essentially of a nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10). The siRNA or shRNA may be in the form of a duplex.

[00108] The antisense oligonucleotide capable of down-regulating expression of at least one splice variant of the *IG20* gene, wherein not all splice variants are down-regulated, may be a single-stranded nucleic acid molecule which exhibits a nucleic acid sequence which is complementary to the nucleic acid sequence, or a portion thereof, of exon 13L of the MADD splice variant and/or an mRNA transcript thereof. The single-stranded nucleic acid molecule may be in the form of RNA or DNA. In an embodiment, the antisense oligonucleotide comprises the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

[00109] The term alkylating agent is used herein to refer to a drug which exhibits an anti-cancer chemotherapeutic effect. As used herein, alkylating agents act by attaching an alkyl group to the guanine base of DNA, thereby disrupting the normal functioning. Alkylating agents act by irreversibly binding to DNA. Once bound, the altered molecule leads to disruption of normal action and replication of DNA, ultimately leading to apoptosis or arrest of cellular replication. Therefore, upon treatment with alkylating agents, they can limit tumor growth and lead to tumor destruction. The term encompasses alkylating agents which can be classified as classical alkylating agents such as cyclophosphamide, mechlorethane; alkylating-like agents such as cisplatin, carboplatin; and agents such as procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine.

[00110] The term “analog” or “derivative” is used herein in the conventional pharmaceutical sense, to refer to a molecule which structurally resembles a reference molecule (such as alkylating agents), but has been modified in a targeted and controlled manner to replace one or more specific substituents of the referent molecule with an alternate substituent, thereby generating a molecule which is structurally similar to the reference molecule. Synthesis and screening of analogs

(*e.g.*, using structural and/or biochemical analysis), to identify slightly modified versions of a known compound which may have improved or biased traits (such as higher potency and/or selectivity at a specific targeted receptor type, greater ability to penetrate mammalian blood-brain barriers, fewer side effects, etc.) is a drug design approach which is well known in pharmaceutical chemistry.

[00111] Various salts and isomers (including stereoisomers and enantiomers) of the drugs listed herein may be used. The term "salts" can include addition salts of pharmaceutically acceptable free acids or free bases. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include inorganic acids such as hydrochloric, sulfuric, or phosphoric acid, and organic acids such as acetic, maleic, succinic, or citric acid, etc. All of these salts (or other similar salts) may be prepared by conventional means. The nature of the salt or isomer is not critical, provided that it is non-toxic and does not substantially interfere with the desired pharmacological activity.

[00112] The term "therapeutically effective" applied to a dose or amount refers to that quantity of a compound or pharmaceutical composition which is sufficient to result in a desired activity upon administration to a mammal in need thereof. As used herein with respect to the pharmaceutical compositions comprising an siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents, the term "therapeutically effective amount/dose" is used interchangeably with the term "chemotherapeutically effective amount/dose" and refers to the amount/dose of a compound or pharmaceutical composition which is sufficient to produce an effective chemotherapeutic response upon administration to a mammal. Note that when a combination of active ingredients is administered the effective amount of the combination may or may not include amounts of each ingredient which are individually therapeutically effective.

[00113] The term "subthreshold" referring to the amount of an active ingredient means an amount inadequate to produce a response, *i.e.*, an amount below the minimum effective amount. The term "suboptimal" in the same context means an amount of an active ingredient which produces a response but not to its full extent,

which would be achieved with a higher amount. This methodology is particularly interesting in the instant context wherein the combination therapy described provides for the administration of "subthreshold" quantities of, for example, a alkylating agent, wherein the administration is otherwise "subthreshold," but through the combination with siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene, provides for otherwise fully effective treatment with the alleviation of unwanted side effects associated with the alkylating agent.

[00114] The phrase "synergistic effect", "synergistic", "synergy", "synergism", as used in connection with the combination therapy of the invention, refers to the cooperative action of two or more stimuli that when combined produce an effect which is greater than the sum of the effect of the contributions of each individual stimulus, i.e., more than an additive effect. The stimuli, for example, may be an agent which down-regulates expression of MADD and at least one therapeutic agent.

[00115] The phrase "pharmaceutically acceptable", as used in connection with compositions of the invention, refers to molecular entities and other ingredients of such compositions which are physiologically tolerable and do not typically produce untoward reactions when administered to a mammal (*e.g.*, human). In an embodiment, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans.

[00116] The term "carrier" applied to pharmaceutical compositions of the invention refers to a diluent, excipient, or vehicle with which an active compound (*e.g.*, an siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents) is administered. Such pharmaceutical carriers may be sterile liquids, such as water, saline solutions, aqueous dextrose solutions, aqueous glycerol solutions, and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Furthermore, suitable carriers

may comprise liposomal formulations. Suitable pharmaceutical carriers may also be described in "Remington's Pharmaceutical Sciences" by E.W. Martin, 18th Edition.

[00117] The term "subject" as used herein refers to a mammal (*e.g.*, rodent such as mouse or rat). In particular, the term refers to humans.

[00118] The term "about" or "approximately" usually means within 20%, within 10%, and optionally within 5% of a given value or range. Alternatively, especially in biological systems, the term "about" means within about a log (*i.e.*, an order of magnitude) optionally within a factor of two of a given value.

[00119] The term "consisting of" excludes any element, step, or ingredient not specified in the claim. In re Gray, 53 F.2d 520, 11 USPQ 255 (CCPA 1931). The term "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. *In re Herz,* 537 F.2d 549, 551-52, 190 USPQ 461, 463 (CCPA 1976) (emphasis in original).

Pharmaceutical Compositions

[00120] In conjunction with the methods of the present invention, also provided are pharmaceutical compositions comprising a therapeutically effective amount of an siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and/or a therapeutically effective amount of an alkylating agent as well as, optionally, an additional carrier or excipient (all pharmaceutically acceptable). Said siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents may be either formulated as a single composition or as two separate compositions, which may be administered conjointly. In an embodiment, they are formulated as a single composition or as two separate compositions, which are optionally administered sequentially or simultaneously. The compositions may be formulated for once-a-day administration or twice-a-day administration, as well as dosage regimens typical in the respective therapies.

[00121] In a further embodiment, the instant combinations may be formulated such that they may be administrated in a titration regimen such that the patient may acclimate to the effects of the alkylating agent and/or the clinician may titrate up the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene so that the alkylating agent may be down titrated to significantly lower dosages so as to minimize toxic side effects of the alkylating agent and/or accommodate for dosing difficulties associated with the alkylating agent.

[00122] In the disclosed compositions, optionally, both the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents are present in therapeutically effective amounts. The optimal therapeutically effective amount should be determined experimentally, taking into consideration the exact mode of administration, form in which the drug is administered, the indication toward which the administration is directed, the subject involved (*e.g.*, body weight, health, age, sex, etc.), and the preference and experience of the physician or veterinarian in charge. As disclosed herein, for human administration, both the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents are administered in suitable form in doses ranging from those understood in the art. In an embodiment, the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene are administered at therapeutic doses; the alkylating agents are administered at suboptimal or lowered doses. It may also be desirable in certain cases to administer one or the other of the active ingredients in a suboptimal or subthreshold amount, and such administration would also be within the scope of the invention.

[00123] The invention also provides a method for preparing pharmaceutical compositions comprising admixing an siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents in therapeutically effective amounts, and optionally one or more physiologically acceptable carriers and/or excipients and/or auxiliary substances.

Administration

[00124] The active agents of the present invention may be administered intradermally, parenterally, intranasally or intra-tumorally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers with or without a targeting molecule which would selectively deliver the active agent to a particular cell type or tissue. The intradermal administration could involve using transdermal patches, microabrasion or nanoemulsions. The parenterally administered medicaments may be administered in the form of an injection, a time-controlled release vehicle, including diffusion-controlled systems, osmotic devices, dissolution-controlled matrices, and erodible/degradable matrices.

[00125] For oral administration in the form of a tablet or capsule, the active drug component may be combined with non-toxic, pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, sucrose, glucose, mannitol, sorbitol and other reducing and non-reducing sugars, microcrystalline cellulose, calcium sulfate, or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc, or silica, steric acid, sodium stearyl fumarate, glyceryl behenate, calcium stearate, and the like); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate), coloring and flavoring agents, gelatin, sweeteners, natural and synthetic gums (such as acacia, tragacanth or alginates), buffer salts, carboxymethylcellulose, polyethyleneglycol, waxes, and the like. For oral administration in liquid form, the drug components may be combined with non-toxic, pharmaceutically acceptable inert carriers (*e.g.*, ethanol, glycerol, water), suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats), emulsifying agents (*e.g.*, lecithin or acacia), non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils), preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid), and the like. Stabilizing agents such as antioxidants (BHA, BHT, propyl gallate, sodium ascorbate, citric acid) can also be added to stabilize the dosage forms.

[00126] The tablets may be coated by methods well known in the art. The compositions of the invention may be also introduced in microspheres or microcapsules, *e.g.*, fabricated from polyglycolic acid/lactic acid (PGLA) (*see, e.g.,* U.S. Patents No. 5,814,344; 5,100,669 and 4,849,222; PCT Publications No. WO95/11010 and WO93/07861). Liquid preparations for oral administration can take the form of, for example, solutions, syrups, emulsions or suspensions, or they may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Preparations for oral administration may be suitably formulated to give controlled or postponed release of the active compound.

[00127] Drug delivery systems known in the art are specialized technologies for the targeted delivery and/or controlled release of therapeutic agents.

[00128] The drug delivery systems deploy medications intact to specifically targeted parts of the body through a medium that can control the therapy's administration. Such drug delivery systems may include micro- and nanotechnology.

[00129] The nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene selected from siRNA, shRNA and antisense oligonucleotides complementary to the nucleotide sequence the MADD splice variant of the *IG20* gene mRNA transcript, may be incorporated into drug delivery systems known in the art and which may include polymeric microspheres, polymer micelles, and hydrogel-type materials, which drug delivery systems are understood in the art to be effective in enhancing drug targeting specificity, lowering systemic toxicity, improving treatment absorption rates, and providing protection for pharmaceuticals against biochemical degradation. In addition, several other drug delivery systems are contemplated, including biodegradable polymers, dendrimers (so-called star polymers), electroactive polymers, and modified C-60 fullerenes (also known as "buckyballs").

[00130] Moreover, drug delivery systems may include lentivirus-mediated transduction of nucleic acids encoding nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene are selected

from siRNA, shRNA and antisense oligonucleotides complementary to the nucleotide sequence the MADD splice variant of the *IG20* gene mRNA transcript.

[00131] The active drugs can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes may be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines, as is well known.

[00132] The siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene may be bound in the form of a targeted liposome formulation. Representative assemblies may include encapsulation within self-assembled engineered proteins which provide for efficient packaging, binding, assembly and delivery of such oligonucleotides. The constituents of such engineered proteins may be selected from peptides which actively target tumor cells through attachment to selected cell surface receptors, peptides which facilitate receptor-mediated endocytosis and peptides which provide for active release of the transported oligonucleotides. Such self-assembled protein transport molecules, comprising the oligonucleotides of the instant invention, may be assembled in the form of nanoparticles (<50nm) comprising two components: the engineered polypeptide (targeting peptide, membrane penetration peptide, oligonucleotide capturing peptide) and the oligonucleotide therapeutic payload.

[00133] Drugs of the invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. Active drugs may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxy-propyl methacrylamide-phenol, polyhydroxy-ethyl-aspartamide-phenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, active drug may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxybutyric acid, polyorthoesters, polyacetals, polyhydropyrans, polycyanoacrylates, and cross-linked or amphipathic block copolymers of hydrogels.

[00134] For administration by inhalation, the therapeutics according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[00135] The formulations of the invention may be delivered parenterally, *i.e.*, by intravenous (i.v.), intracerebroventricular (i.c.v.), subcutaneous (s.c.), intraperitoneal (i.p.), intramuscular (i.m.), subdermal (s.d.), intratumoral (i.t.) or intradermal (i.d.) administration, by direct injection, via, for example, bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as excipients, suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for reconstitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[00136] Compositions of the present invention can also be formulated for rectal administration, *e.g.*, as suppositories or retention enemas (*e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides).

[00137] As disclosed herein, siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents may be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredients. In addition, if desired, the preparations may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or agents which enhance the effectiveness of the pharmaceutical composition. These auxiliary molecules may be delivered systemically or locally as proteins or by expression of a vector which codes

for expression of the molecule. The techniques described above for the delivery of siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents can also be employed for the delivery of auxiliary molecules.

[00138] Although the active agents of the present invention may be administered in divided doses, for example, two or three times daily, a single daily dose of each of the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents, with a single daily dose of both agents in one composition or in two separate compositions administered simultaneously is an embodiment.

[00139] The instant invention also encompasses a process for preparing pharmaceutical compositions comprising combining siRNA, shRNA and antisense oligonucleotides capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents with a pharmaceutically acceptable carrier and/or excipient.

[00140] Specific amounts of the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene which may be used in unit dosage amounts of the invention may be readily ascertainable to those skilled in the art. Specific amounts of the alkylating agent which may be used in reduced unit dosage amounts of the invention include, for example, the alkylating agent used at 3/4, 1/2, 1/3rd, 1/4th, 1/5th 1/10th 1/20th of the recommended dose.

[00141] The invention also provides a pharmaceutical pack or kit comprising one or more containers containing one or more of the ingredients of the formulations of the invention. In a related embodiment, the present invention provides a kit for the preparation of the pharmaceutical compositions of the invention, said kit comprising a formulation of one or more siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene in a first container or multiple containers, and one or more alkylating agent in a second

container or multiple containers, and, optionally, instructions for admixing the two drugs and/or for administration of the drugs in therapeutically meaningful regimens. Each container of the kit may also optionally include one or more physiologically acceptable carriers and/or excipients and/or auxiliary substances. Associated with such container(s) may be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00142] The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Effective Dose and Safety Evaluations

[00143] According to the methods of the present invention, the pharmaceutical compositions described herein are administered to a patient at therapeutically effective doses, in an embodiment, with minimal toxicity other than required for the therapeutic purpose of the combination. The Section entitled "Definitions" provides definitions for the terms "chemotherapeutically effective dose" and "therapeutically effective dose". In an embodiment, the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents are each used at a dosage which, when combined, provide an enhanced effect, for example, an effect not observed upon administration of each agent alone. It is an embodiment of the instant invention that both the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents are administered at "suboptimal" or "subthreshold" doses, which doses, in combination, provide for a superadditive effect with surprising reduction in unwanted side effects.

[00144] The efficacy of the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene of the invention may be determined using such *in vitro* pharmacological tests such as measurements of the levels of mRNA using quantitative Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR) or RT-PCR, etc. The efficacy of the alkylating agents of the invention may be determined *in vitro* using methods known to those skilled in the art, for example, cell cytotoxicity assays, MTT assay, apoptosis assays, cell migration assays, etc.

[00145] Following methodologies which are well-established in the art, effective doses and toxicity of the compounds and compositions of the instant invention, which perform well in *in vitro* tests, may then be determined in preclinical studies using small animal models (*e.g.*, mice or rats) in which both the siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents have been found to be therapeutically effective.

[00146] For any pharmaceutical composition used in the methods of the invention, the therapeutically effective dose may be estimated initially from animal models to achieve a circulating plasma concentration range which includes the IC_{50} (*i.e.*, the concentration of the test compound which achieves a half-maximal). Dose-response curves derived from animal systems may then be used to determine testing doses for the initial clinical studies in humans. In safety determinations for each composition, the dose and frequency of administration should meet or exceed those anticipated for use in the clinical trial.

[00147] As disclosed herein, the dose of the components in the compositions of the present invention is determined to ensure that the dose administered continuously or intermittently will not exceed an amount determined after consideration of the results in test animals and the individual conditions of a patient. A specific dose naturally varies depending on the dosage procedure, the conditions of a patient or a subject animal such as age, body weight, sex, sensitivity, feed, dosage period, drugs used in combination, seriousness of the disease. The appropriate dose and dosage times under certain conditions may be determined by

the test based on the above-described indices but may be refined and ultimately decided according to the judgment of the practitioner and each patient's circumstances (age, general condition, severity of symptoms, sex, etc.) according to standard clinical techniques. As disclosed herein, an appropriate dose of an siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene is generally ascertainable to those skilled in the art, and an appropriate dose of alkylating agent is generally ascertainable to those skilled in the art. In an embodiment, the dosage of siRNA to be administered may range from 1 to 10 mg per kilogram of body weight. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. In an embodiment, a single dose of each drug may be administered daily.

[00148] Toxicity and therapeutic efficacy of the compositions of the invention may be determined by standard pharmaceutical procedures in experimental animals, *e.g.*, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index and it may be expressed as the ratio ED_{50}/LD_{50}. Formulations/combinations which exhibit large therapeutic indices are preferred.

[00149] The data obtained from animal studies may be used in formulating a range of doses for use in humans. The therapeutically effective doses of siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene and alkylating agents in humans lay within a range of circulating concentrations which include the ED_{50} with little or no toxicity other than therapeutically necessary. For example, such therapeutically effective circulating concentration for siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene generally ascertainable to those skilled in the art. The dosage of siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene to be administered may range from 1 to 10 mg per kilogram of body weight. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. Ideally, a single dose of each drug should be used daily.

[00150] The drug combination of the invention is not only highly effective at relatively low doses but also possesses low toxicity other than therapeutically necessary and produces few side effects. Indeed, the only common side effects for the alkylating agents of the invention are those for which the instant combination therapy has been designed to alleviate, while the most common side effect resulting from the use of siRNA, shRNA and antisense oligonucleotide capable of down-regulating the expression of the MADD splice variant of an *IG20* gene of the invention is that associated with injection of RNA such as transient heightened inflammatory response including increased interferon production.

PHARMACOLOGY - SUMMARY

[00151] The active principles of the present invention, and pharmaceutical compositions thereof and method of treating therewith, are characterized by unique advantageous and unpredictable properties, rendering the "subject matter as a whole", as claimed herein, unobvious. The combinations and pharmaceutical compositions thereof exhibit, in standard accepted reliable test procedures, the following valuable properties and characteristics.

[00152] The results demonstrate a synergistic effect of the combination of siRNA knockdown of MADD and Cisplatin treatment on the cell death of thyroid cancer cells, wherein the amount of cell death from the combination of siRNA knockdown of MADD and Cisplatin treatment exceeds the additive effect of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy.

[00153] The results demonstrate a synergistic effect of the combination of siRNA knockdown of MADD and Cisplatin treatment on the cell death of hepatic cancer cells, wherein the amount of cell death from the combination of siRNA knockdown of MADD and Cisplatin treatment exceeds the additive effect of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy.

Example 1: Determination of alkylating agent chemotherapeutic cytotoxicity dosage curves

[00154] Cancer cell lines representative of various cancers which may be susceptible to alkylating agent treatment include 8505C (thyroid carcinoma), HTH7 (thyroid carcinoma), C643 (human thyroid carcinoma), AsPC-1 (pancreatic cancer), SU.86.86 (pancreatic cancer), CFPAC-1 (pancreatic cancer), MCF7 (adenocarcinoma breast cancer), SK-BR-3 (breast cancer), OVCAR3 (ovarian cancer), SKOV3 (ovarian cancer), NCI-H522 (non-small cell lung cancer), NCI-H2122 (non-small cell lung cancer), NCI-H2227 (small cell lung cancer), HepG2 (liver hepatocellular carcinoma), PLC/PRF/5 (liver hepatoma), JIMT1 (breast cancer) and K562 (chronic myelogenous leukemia). Cell lines may be obtained from the National Cancer Institute, Bethesda, MD or American Type Culture Collection, or similar organizations in other countries. These cell lines may be selected because they all express higher levels of the MADD splice variant, are derived from different types of cancers, all which are in need of new modalities of treatment, exhibit unique growth properties and have differential susceptibility to different modalities of treatment with chemotherapeutic agents due to underlying different mutations. In spite of their heterogeneity, these cell lines may be rendered susceptible to therapeutic treatment upon MADD knockdown, and thus show the potential beneficial effects of MADD knockdown in a range of different cancers. Because of their unique growth properties these cell lines are cultured in culture media which have been formulated to support their optimum growth in culture.

[00155] 8505C cells are cultured in RPMI-1640 culture medium and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00156] HTH7 cells are cultured in RPMI-1640 culture medium and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00157] C643 cells are cultured in RPMI-1640 culture medium and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00158] AsPC-1 cells are cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO_2 in air. Additional sodium bicarbonate may be required for use in incubators containing higher

percentages of CO_2. Base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00159] SU.86.86 cells are cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO_2 in air. Additional sodium bicarbonate may be required for use in incubators containing higher percentages of CO_2. Base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00160] CFPAC-1 cells are cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 4 mM L-glutamine, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. This base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00161] MCF7 cells are cultured in Eagle's minimum essential medium modified to contain Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. This base medium is supplemented with 0.01 mg/ml human recombinant insulin; fetal bovine serum to a final concentration of 10%.

[00162] JIMT1 cells were cultured in complete RPMI media (GIBCO) with 10% fetal bovine serum and 1X Antibiotic-Antimycotic solution (Gibco). The Antibiotic-Antimycotic solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B.

[00163] SK-BR-3 cells are cultured in McCoy's 5A Medium modified to contain 1.5 mM L-glutamine and 2200 mg/L sodium bicarbonate. This base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00164] OVCAR3 cells are cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO_2 in air. This base

medium is supplemented with 0.01 mg/ml bovine insulin, antibiotic and anti-mycotic agents and fetal bovine serum to a final concentration of 20%.

[00165] SKOV3 cells are cultured in McCoy's 5A Medium is modified to contain 1.5 mM L-glutamine and 2200 mg/L sodium bicarbonate. This base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00166] NCI-H522 cells are cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO_2 in air. This base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00167] NCI-H2122 cells are cultured in RPMI-1640 medium modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO_2 in air. This base medium is supplemented with 10% fetal bovine serum and antibiotics and anti-mycotic agents.

[00168] NCI-H2227 cells are cultured in DMEM:F12 Medium containing 0.005 mg/ml Insulin, 0.01 mg/ml Transferrin, 30nM Sodium selenite, 10 nM Hydrocortisone, 10 nM beta-estradiol, extra 2mM L-glutamine (for final conc. of 4.5 mM), 5% fetal bovine serum.

[00169] HepG2 cells are cultured in Eagle's Minimum Essential Medium (EMEM) and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00170] PLC/PRF/5 cells are cultured in Eagle's Minimum Essential Medium (EMEM) and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00171] K562 cells are cultured in Iscove's Modified Dulbecco's Medium (IMDM) and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

[00172] HCT116 cells are cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% pencillin/streptomycin.

[00173] N87 cells are cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% pencillin/streptomycin.

[00174] AGS cells are cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% pencillin/streptomycin.

[00175] All cell lines are maintained at 37°C in a humidified atmosphere with 5% CO_2.

[00176] Alkylating agent chemotherapeutics may be obtained commercially as FDA approved drugs from pharmacies with a doctor's prescription.

All cells are cultured in their respective culture medium with appropriate supplements as described above.

Table 1.

Overview of Protocol – Adherent cell lines

Day 0	Cell seeding in 96 well plates
Day 1	Chemotherapeutic drugs addition
Day 2	Cell viability analysis/MTT assay for 24 h
Day 3	Cell viability analysis/MTT assay for 48 h

Overview of Protocol – Suspension cell line

Day 0	Cell seeding and chemotherapeutic drugs addition in 96 well plates
Day 1	MTT assay, followed by addition of equal volume of solubilization agent (20% SDS in 50% Dimethylformamide) and overnight incubation

Day 2	Cell viability analysis for 24 h
Day 3	Cell viability analysis for 48 h

[00177] On Day 0, adherent cells are seeded in 96-well plates (Corning; Cat number: 353072) and incubated overnight.

[00178] On Day 0, suspension cells are seeded in 96-well plates (Corning; Cat number: 353072) along with the alkylating agent chemotherapeutic as indicated in Table 1.

[00179] After overnight incubation (Day 1), six different concentrations of each chemotherapeutic drug are added in triplicates. The stock and working concentrations of each drug are as follows:

Table 2.

Drugs	Stock concentrations (mM)	Working concentrations
Cisplatin	170	1.25, 1.875, 2.5, 5 (μM)

Stock solutions of Cisplatin are prepared in DMSO. Working concentrations of these drugs are prepared in the culture medium.

[00180] Cytotoxicity of the respective cell line models may be evaluated based on the compared cytotoxicity as measured utilizing the MTT assay.

[00181] **MTT Straining for Metabolic Activity**: MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) may be purchased from Sigma Aldrich (Cat number: M5655) and dissolved in sterile DPBS at a concentration of 5mg/ml.

[00182] In adherent cells, after 24 and 48 hrs of drug exposure (Days 2 and 3 respectively), 10μl of prepared MTT solution is added per well of a 96-well plate. Plates are incubated for 2 hours at 37^0 C in the humidified CO_2 incubator. After 2 hours, media is aspirated using vacuum inside the biosafety hood. Purple colored

formazan crystals which form in viable cells are dissolved by adding 100µl of Dimethyl sulfoxide (Thermo Fisher Scientific; Cat number D128-500). Plates are kept on shaker for 10 minutes for compete dissolution of crystals and absorbance is recorded at 595 nm using Bio-Rad iMark microplate reader.

[00183] In suspension cells, after 24 and 48 hrs of drug exposure (Days 1 and 2 respectively), 10µl of prepared MTT solution is added per well of a 96-well plate. Plates are incubated for 2 hours at 37^0 C in the humidified CO_2 incubator. After 2 hrs incubation, equal volume of solubilization agent (20% SDS in 50% Dimethylformamide) is added and the plates are incubated overnight at 37^0 C in the humidified CO_2 incubator. After overnight incubation, plates are kept on shaker for 10 minutes for compete dissolution of crystals and absorbance is recorded at 595 nm using Bio-Rad iMark microplate reader.

[00184] Percent cell survival is calculated using MS excel. GraphPad prism software is used to calculate EC_{50}. The MTT assay indicates the overall cell death.

[00185] The studies demonstrate that treatment of cancer cells with a alkylating agent chemotherapeutic exhibits a dose response profile measurable based on the death of the cancer cells.

Example 2: Map kinase Activating Death Domain containing protein (MADD) siRNA Transfection.

[00186] siRNA, shRNA and/or antisense oligonucleotides comprising nucleic acid molecules which target exon 13L of the MADD splice variant may be designed. siRNAs comprising a central 19 bp duplex with 2 –base 3'-overhangs are synthesized. Moreover, a non-specific Scramble siRNA having a 19 bp duplex with 2 –base 3'-overhangs is synthesized to provide a negative control siRNA. The sequences of siRNAs (double stranded duplex) used are:

MADD siRNA: 5' – GAUUGUCAUAGAUUCGCCGTT – 3' (SEQ ID NO:4)
Scramble siRNA: 5' – UUGCUAAGCGUCGGUCAAUTT – 3' (SEQ ID NO:5)

[00187] Lipofectamine RNAimax (transfection mediating reagent) is a cationic unilamellar liposomal structure with a positive surface charge in water. The lipid cationic charges interacts with negative phosphate group of nucleic acids in siRNA and forms a liposome/siRNA transfection complex with the cell membrane. This complex can easily fuse with a cell membrane and siRNA is delivered inside the cell via endocytosis.

[00188] Dosing of siRNA and transfection reagents needs to be determined for each cell line. The manufacturer's recommended volume of transfection reagent per mm^3 area of tissue culture plate is used, generally, and the siRNA concentration may vary. For most cells, 10 nM of siRNA is sufficient to cause MADD knock-down within 48 hours of transfection.

Transfection:

[00189] Cells are cultured and maintained in a humidified atmosphere containing 5% CO_2 in ATCC recommended media with 10% fetal bovine serum (Gibco; Cat number 26140-079) and 1x anti-biotic/anti-mycotic (Gibco; Cat number:15240096) at 37^0C, 5% CO_2 incubator. For transfection, cells are seeded in to 96-well plates (24h before transfection to reach 60-80% confluence).

[00190] The details of the transfection reaction for each cell line tested is given below.

[00191] Transfection reaction mix is prepared as recommended by manufacturer's instructions in triplicate. Briefly, an aliquot of a 100 µM siRNA stock solution is diluted in 5µl OPTI-MEM in tube A to give a final concentration of 10 nM siRNA per well. 0.3 µl of RNAiMax (Thermo Fisher Scientific) reagent is mixed in 4.7 µl OPTI-MEM in tube B. Contents from tube A and B are mixed and incubated for 15 minutes at room temperature. Reaction mix is added to the plates and incubated for 24h, 48h and 72h at 37^0 C. Media is changed after 48 hours.

RNA isolation:

[00192] Total RNA is extracted using Trizol reagent (Ambion; Cat number: 15596018). For each well of 96-well plate, 150 µl of Trizol is added to suspend and homogenize the cell suspension. Triplicate samples are pooled together for further

processing. Cells are incubated for 10 minutes at room temperature. 250µl of Chloroform (Fisher; Cat number: C606-1) is added to the cell suspension. Samples are incubated at room temperature for 3 minutes. Tubes are centrifuged at 10,000 rpm for 15 minutes at 4^0C. The top layer is transferred to a fresh tube and 600 µl of isopropanol (Fisher; Cat number: A451-1) is added. The tube is incubated at room temperature for 10 minutes. The tube is subsequently centrifuged at 10,000 rpm for 10 minutes at 4^0C. Supernatant is discarded and the pellet is washed with 1 ml of 75% ethanol (Decon; Cat number: DSP-AZ-1). The tube is again centrifuged at 7500 rpm for 5 minutes at 4^0 C. The pellet is air dried and dissolved in 20 µl of DEPC water (Fisher; Cat number: BP561-1). RNA is quantified by using Thermo Fisher Scientific NanoDrop One. A260/280 and A260/230 are used to validate the purity of sample.

Reverse-transcriptase PCR:

[00193] For RT-PCR, MADD and β-actin (internal loading control) primers are used. The sequences of primers used are:

MADD 13L Forward: 5'- AGC CCC AAT ATG GCT TTC CC-3' (SEQ ID NO:6)

MADD 13L Reverse: 5'- CTG ATC CAC TAA CGC CCT CC-3'(SEQ ID NO:7)

β-actin forward: 5'- ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' (SEQ ID NO:8)

β-actin reverse: 5'- CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (SEQ ID NO:9)

[00194] For amplification, Qiagen One step RT_PCR kit (Cat number: 210212) and BioRaD T100 thermocycler are used. The description of RT-PCR is given below.

Table 3.

Components/Tube	Volume (µL)
5x buffer	5
dNTP	1
Enzyme	1
Primers (Forward and Reverse;	1.5

10µm each)	
H_2O	15.25(variable)
Template	1µg (MADD) 100ng (β-actin)
Total Volume	25

Amplification protocol for RT-PCR is:

1. 50 ^{0}C for 30 minutes
2. 95 ^{0}C for 15 minutes
3. 94 ^{0}C for 50 seconds
4. 55 ^{0}C for 50 seconds
5. 72 ^{0}C for 60 seconds
6. Repeat Step 3-5, 39 times
7. 72 ^{0}C for 7 minutes
8. Hold at 4 ^{0}C

5% Polyacrylamide gel electrophoresis:

[00195] Polyacrylamide gels are casted manually and following recipe is used to prepare the gel. All components are added sequentially.

Table 4.

Components/gel of 1mm thickness	**Volume**
H_2O	19.4 ml
10x TBE (BioRad; Cat number: 161-0741)	1.25 ml
30% Acrylamide (BioRad; Cat number: 161-0156)	4.2 ml
10% APS (Fisher; Cat number: BP179-25)	150 µl
TEMED (BioRad; Cat number: 161-0801)	15µl
All components are mixed together, poured and allowed to polymerize for an hour at room temperature.	

[00196] The entire sample volume (25µl) is mixed with 5µl of 6x nucleic acid buffer (Thermo Fisher Scientific; Cat number: RO611) before loading on to the gel. Gel electrophoresis is carried out at 200 volts for two hours using BioRad Protean II Xi Cell. Gel is stained for 30 minutes using 100ml of working staining solution (1µg/ml Ethidium Bromide in 0.5x TBE). Ethidium bromide is procured from BioRad (Cat

number 161-0433). Images are captured using BioRad ChemiDoc MP Imaging System.

[00197] These studies demonstrate effective transfection of the instant experimental nucleic acids as well as distinguish the effect of the transfection reagent on MADD expression (MADD knockdown) and cell survival.

Example 3: Effect of alkylating agent chemotherapeutics on cell death in cancer cells with and without MADD knockdown

[00198] The cytotoxic effects of alkylating agent chemotherapeutics on cancer cells in the presence or absence of MADD down-regulation may be determined.

[00199] SU.86.86, H2227, C643, PLC/PRF/5, JIMT1, HTH7, 8505C and H522 cells are cultured in their respective culture medium with appropriate supplements as described above.

Table 5.

Overview of Protocol

Day 0	Cell seeding in 100 mm^3 plates and 96 well plates
Day 1	Transfection with Scrambled (Scr) or MADD siRNA
Day 2	Media change
Day 3	Cells Reseeding in 96-well plates Cell viability analysis in 96-well plate before reseeding
Day 4	Chemotherapeutic drugs addition

[00200] Cells are cultured and maintained at 37°C in a humidified atmosphere containing 5% CO_2 as described above. On Day 0, Cells are seeded in 100 mm^3 tissue culture dishes (Corning; Cat Number: 353003) to attain 60-70 % confluence. In parallel, cells are also seeded in one 96-well plate (Corning; Cat number: 353072) for analysis of survival before reseeding.

[00201] After 12-18 hours (Day 1), cells are transfected with Scramble or MADD siRNA at 10nM concentration. The sequences of siRNAs (double stranded) used are:

MADD siRNA: 5' – GAUUGUCAUAGAUUCGCCGTT – 3' (SEQ ID NO:4)
Scramble siRNA: 5' – UUGCUAAGCGUCGGUCAAUTT – 3' (SEQ ID NO:5)

[00202] Stock siRNAs are reconstituted to 100µM concentration in 1x siRNA dissolution buffer (GE Healthcare; Cat number: B-002000-UB-700) and stored at -20^0 C. For transfection, reaction mix per plate is prepared by adding 30 µl lipofectamine RNAimax (Invitrogen; Cat number 13778-150), 1ml Opti-MEM (Gibco; Cat number: 31985062), and an aliquot of the stock siRNA to obtain a final concentration of 10 nM siRNA per plate or wells of a plate containing 70% confluent cells. Reaction mix is incubated at room temperature for 10 minutes. Transfection mixture is added dropwise on to the plate containing 70% confluent cells in RPMI media with 10% FBS but no antibiotics. The transfection mix is added in parallel to cells plated in a 96-well plate.

[00203] Cells are incubated at 37^{0}C in humidified CO_2 incubator for 24 hours and the next day (Day 2) the media is replaced with complete RPMI (10% FBS and 1x antibiotic/antimycotic). Media is changed for all the cells.

[00204] After 48 hours of transfection (Day 3), cells are harvested by 0.05% trypsin (Gibco; Cat number: 25-300-054) treatment. Briefly, cells are washed with DPBS (Gibco; Cat number: 14190250) and treated with trypsin for 5 minutes. Subsequently, cells are detached and collected in 10 ml complete media. Cells suspension is centrifuged at 1500 rpm for 10 minutes. Supernatant is discarded and cell pellet is suspended in 5 ml complete RPMI. All cells [Control (untreated), Scramble and MADD siRNA transfected] are counted using hemocytometer before reseeding. For cell counting, equal volumes of cell suspension and Trypan Blue 0.4% (Lonza; Cat number: 17-942E) are mixed. 10 µl of cell suspension (with trypan blue) are placed on hemocytometer and cells are counted. Counting is performed twice and average is used to determine the cell number.

[00205] Equal number of cells [Control (untreated), Scramble and MADD siRNA transfected] are seeded in 96-well plates. On the same day (after 48 hours of transfection), an MTT assay is carried out to determine the relative cell survival before reseeding.

[00206] On Day 4, when cells are properly adhered and retained their shape in 96-well plates, drugs are added. All drugs are diluted in complete media on the same day prior to the addition to the cells. In untreated cells, media is replaced with complete media. 200µl volume of media (with or without drugs) is used for the drug treatment assay. Plates are incubated 24h, 48h and 72 hours at 37^0C in humidified CO_2 incubator.

[00207] An MTT assay is performed to determine relative survival after drug treatment as described above.

[00208] **MTT Staining for Metabolic Activity**: MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) is purchased from Sigma Aldrich (Cat number: M5655). MTT is dissolved in sterile DPBS at the concentration of 5mg/ml and filtered through 0.45 µM syringe filter (Corning; Cat number: 431220). 10µl of MTT solution is added per well of 96-well plate. Plates are incubated for 2 hours at 37^0 C in the humidified CO_2 incubator. After 2 hours, media is aspirated using vacuum inside the biosafety hood. Purple colored crystals formed in viable cells are dissolved by adding 100µl of Dimethyl sulfoxide (Thermo Fisher Scientific; Cat number D128-500). Plates are kept on shaker for 10 minutes for compete dissolution of crystals and absorbance is recorded at 595 nm using Bio-Rad iMark microplate reader.

[00209] Data is analyzed in MS excel software. Relative survival is calculated with respect to absorbance of control (untreated cells) to determine the effect of transfection as well as combination treatment.

[00210] Transfection with Scramble siRNA results in little or no spontaneous cell death. Spontaneous cell death may be attributed to the sensitivity of the individual cell lines to the transfection reagent. A Scramble siRNA transfection is necessarily

included as a transfection control in each experiment and establishes a baseline which reflects the amount of cell death which may occur due to the transfection reaction.

[00211] Each data set takes into account any spontaneous cell death which is observed upon transfection with Scramble siRNA; the amount of spontaneous cell death from Scramble siRNA transfection (baseline) is subtracted from the amount of cell death observed in each siRNA transfection reaction to yield a Net Percent (Net %) cell death after accounting for Scrambled siRNA-induced cell death.

[00212] In an embodiment, 2.5 μM Cisplatin was added as monotherapy to 8505C thyroid cancer cells. Moreover, cells were transfected with MADD siRNA as monotherapy. In combination, 2.5 μM Cisplatin was added to cells transfected with MADD siRNA. The amount of cell death in the cell cultures of the treatment paradigm represented by untreated control cells, control cells transfected with Scrambled siRNA, cells transfected with MADD siRNA, cells treated with 2.5 μM Cisplatin, and cells transfected with MADD siRNA and treated with 2.5 μM Cisplatin, was evaluated at 48 hours post Cisplatin treatment.

Table 6.

Treatment	Observed % Cell death	Net % cell death after accounting for Scrambled (non-specific siRNA)
Control (untreated)	0	0
Scrambled siRNA (alone)	10	10
MADD siRNA (alone)	49	49-10=39
Cisplatin (alone)	19	19
Cisplatin + MADD siRNA	73	73-10=63
Additive: Cisplatin + MADD siRNA = 19 + 39 = 58% cell death		

[00213] The results, as reported in Table 6, demonstrate that siRNA knockdown of MADD resulted in 39% cell death, Cisplatin monotherapy at 2.5 μM concentration

resulted in 19% cell death, and addition of 2.5 μM Cisplatin to cells transfected with MADD siRNA resulted in 63% cell death. All results accommodated for transfection effect utilizing Scramble siRNA cell death data. Taken together, the results demonstrate synergy of the combination of siRNA knockdown of MADD and Cisplatin treatment, resulting in 63% cell death which exceeds the additive effect of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy, the additive effect of each monotherapy being 58% cell death.

[00214] In another embodiment, 5 μM Cisplatin was added as monotherapy to HTH7 thyroid cancer cells. Moreover, cells were transfected with MADD siRNA as monotherapy. In combination, 5 μM Cisplatin was added to cells transfected with MADD siRNA. The amount of cell death in the cell cultures of the treatment paradigm represented by untreated control cells, control cells transfected with Scrambled siRNA, cells transfected with MADD siRNA, cells treated with 5 μM Cisplatin, and cells transfected with MADD siRNA and treated with 5 μM Cisplatin, was evaluated at 24 hours post Cisplatin treatment.

Table 7.

Treatment	Observed % Cell death	Net % cell death after accounting for Scrambled (non-specific siRNA)
Control (untreated)	0	0
Scrambled siRNA (alone)	0	0-0=0
MADD siRNA (alone)	6	6-0=6
Cisplatin (alone)	13	13
Cisplatin + MADD siRNA	31	31-0=31
Additive: Cisplatin + MADD siRNA = 13 + 6 = 19% cell death		

[00215] The results, as reported in Table 7, demonstrate that siRNA knockdown of MADD resulted in 6% cell death, Cisplatin monotherapy at 5 μM concentration resulted in 13% cell death, and addition of 5 μM Cisplatin to cells transfected with MADD siRNA resulted in 31% cell death. All results accommodated for transfection

effect utilizing Scramble siRNA cell death data. Taken together, the results demonstrate synergy of the combination of siRNA knockdown of MADD and Cisplatin treatment, resulting in 31% cell death which far exceeds the additive effect of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy, the additive effect of each monotherapy being 19% cell death.

[00216] In another embodiment, 1.25 μM Cisplatin was added as monotherapy to HTH7 thyroid cancer cells. Moreover, cells were transfected with MADD siRNA as monotherapy. In combination, 1.25 μM Cisplatin was added to cells transfected with MADD siRNA. The amount of cell death in the cell cultures of the treatment paradigm represented by untreated control cells, control cells transfected with Scrambled siRNA, cells transfected with MADD siRNA, cells treated with 1.25 μM Cisplatin, and cells transfected with MADD siRNA and treated with 1.25 μM Cisplatin, was evaluated at 72 hours post Cisplatin treatment.

Table 8.

Treatment	Observed % Cell death	Net % cell death after accounting for Scrambled (non-specific siRNA)
Control (untreated)	0	0
Scrambled siRNA (alone)	0	0-0=0
MADD siRNA (alone)	54	54-0=54
Cisplatin (alone)	0	0
Cisplatin + MADD siRNA	67	67-0=67
Additive: Cisplatin + MADD siRNA = 0 + 54 = 54% cell death		

[00217] The results, as reported in Table 8, demonstrate that siRNA knockdown of MADD resulted in 54% cell death, Cisplatin monotherapy at 1.25 μM concentration resulted in 0% cell death, and addition of 1.25 μM Cisplatin to cells transfected with MADD siRNA resulted in 67 % cell death. All results accommodated for transfection effect utilizing Scramble siRNA cell death data. Taken together, the results demonstrate synergy of the combination of siRNA knockdown of MADD and Cisplatin

treatment, resulting in 67% cell death which far exceeds the additive effect of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy, the additive effect of each monotherapy being 54% cell death.

[00218] In another embodiment, 1.875 μM Cisplatin was added as monotherapy to PLC/PRF/5 hepatic cancer cells. Moreover, cells were transfected with MADD siRNA as monotherapy. In combination, 1.875 μM Cisplatin was added to cells transfected with MADD siRNA. The amount of cell death in the cell cultures of the treatment paradigm represented by untreated control cells, control cells transfected with Scrambled siRNA, cells transfected with MADD siRNA, cells treated with 1.875 μM Cisplatin, and cells transfected with MADD siRNA and treated with 1.875 μM Cisplatin, was evaluated at 24 hours post Cisplatin treatment.

Table 9.

Treatment	Observed % Cell death	Net % cell death after accounting for Scrambled (non-specific siRNA)
Control (untreated)	0	0
Scrambled siRNA (alone)	5	5-5=0
MADD siRNA (alone)	25	25-5=20
Cisplatin (alone)	41	41
Cisplatin + MADD siRNA	91	91-5=86
Additive: Cisplatin + MADD siRNA = 41 + 20 = 61% cell death		

[00219] The results, as reported in Table 9, demonstrate that siRNA knockdown of MADD resulted in 20% cell death, Cisplatin monotherapy at 1.875 μM concentration resulted in 41% cell death, and addition of 1.875 μM Cisplatin to cells transfected with MADD siRNA resulted in 86% cell death. All results accommodated for transfection effect utilizing Scramble siRNA cell death data. Taken together, the results demonstrate synergy of the combination of siRNA knockdown of MADD and Cisplatin treatment, resulting in 86% cell death which far exceeds the additive effect

of monotherapy with either MADD siRNA knockdown or Cisplatin monotherapy, the additive effect of each monotherapy being 61% cell death.

[00220] Considering the results of the foregoing experiments, the instant assay demonstrates that combination of alkylating agent chemotherapeutic treatment and MADD knock-down results in surprisingly synergistic effects on inducing cancer cell death. The results demonstrate that the combination alkylating agent chemotherapy and MADD knock down results in more than an additive effect on cell death. As a result, dosages of the alkylating agent chemotherapeutic may be lowered to a level which was previously considered to be non-therapeutic, thereby providing a cancer therapy which exhibits an unexpected margin of safety and reduction of unwanted side effects.

* * * * *

[00221] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

[00222] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference.

* * * * *

CLAIMS

We claim:

1. A combination of antineoplastic agents useful for treating cancer comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene, wherein not all splice variants of the *IG20* gene are down-regulated, and one or more alkylating agent chemotherapeutic.

2. The combination of Claim 1, wherein the at least one splice variant of the *IG20* gene is selected from a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof.

3. The combination of Claim 2, wherein the at least one splice variant of the *IG20* gene is a MADD splice variant which exhibits exon 13L.

4. The combination of Claim 2, wherein the one or more nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene is selected from siRNA, shRNA and antisense oligonucleotides.

5. The combination of Claim 4, wherein the siRNA, shRNA and antisense oligonucleotides comprise nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, and/or an mRNA transcript thereof.

6. The combination of Claim 4, wherein the nucleic acid molecule capable of down-regulating expression of at least one splice variant of the *IG20* gene is comprised in an siRNA or shRNA.

7. The combination of Claim 6, wherein the siRNA and shRNA is encoded by a nucleic acid molecule which includes the structure:

$$X_{sense} - \text{hairpin loop} - X_{anti\text{-}sense},$$

wherein X includes or consists essentially of a nucleic acid sequence CGGCGAATCTATGACAATC (SEQ ID NO:1).

8. The combination of Claim 6, wherein the siRNA or shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2).

9. The combination of Claim 6, wherein the siRNA or shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2) and is in the form of a duplex with a cognate nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

10. The combination of Claim 7, wherein the shRNA and siRNA is encoded by a nucleic acid having the sequence CGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG (SEQ ID NO:3), wherein a hairpin loop region is from positions 20-28 of the sequence.

11. The combination of Claim 4, wherein the siRNA, shRNA and antisense oligonucleotide comprising nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant of the *IG20* gene and/or an mRNA transcript thereof comprises a nucleic acid having the sequence selected from GAUUGUCAUAGAUUCGCCGTT (SEQ ID NO:4) and GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

12. The combination of Claim 4, wherein the one or more nucleic acid molecules capable of down-regulating expression of the at least one splice variant of the *IG20* gene is comprised in a drug delivery system.

13. The combination of Claim 12, wherein the drug delivery system is a targeted liposome formulation or a lentivirus vector.

14. The combination of Claim 1, wherein the one or more alkylating agent chemotherapeutic is selected from cyclophosphamide, mechlorethane, cisplatin, carboplatin, procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine.

15. The combination of Claim 1, wherein the one or more alkylating agent chemotherapeutic is in the form of a pharmaceutically acceptable salt.

16. The combination of Claim 1, wherein the cancer is selected from slow growing solid tumors and leukemia, lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, Hodgkin's disease, topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma), both Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, germ cell tumors, bladder cancer, head and neck cancer, cervical cancer, multiple myeloma, neuroblastoma, rhabdomyosarcoma, lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma, multiple myeloma, retinoblastoma, Ewing's sarcoma, endometrial cancer, islet cell pancreatic cancer, brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors, colon cancer, fibrosarcomas, anaplastic astrocytoma, and glioblastoma multiforme.

17. A method of treating cancers selected from slow growing solid tumors and leukemia, lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, Hodgkin's disease, topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma), both Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, germ cell tumors, bladder cancer, head and neck cancer, cervical cancer, multiple myeloma, neuroblastoma, rhabdomyosarcoma, lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma, Burkitt's lymphoma, chronic

lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma, multiple myeloma, retinoblastoma, Ewing's sarcoma, endometrial cancer, islet cell pancreatic cancer, brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors, colon cancer, fibrosarcomas, anaplastic astrocytoma, and glioblastoma multiforme in a subject in need thereof, comprising administering an effective amount of a combination of antineoplastic agents comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene, wherein not all splice variants of the *IG20* gene are down-regulated, and one or more alkylating agent chemotherapeutic.

18. The method of Claim 17, wherein the cancers exhibit expression of at least one splice variant of the *IG20* gene.

19. The method of Claim 17, wherein the cancers exhibit expression of the MADD splice variant of the *IG20* gene, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof.

20. The method of Claim 19, wherein MADD splice variant exhibits exon 13L of the *IG20* gene.

21. The method of Claim 17, wherein the one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the *IG20* gene is selected from siRNA, shRNA and antisense oligonucleotides.

22. The method of Claim 21, wherein the siRNA, shRNA and antisense oligonucleotides comprise nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant, SNPs, allelic variations thereof, polymorphisms thereof, and genetic mutations thereof, of the IG20 gene and/or an mRNA transcript thereof.

23. The method of Claim 22, wherein the nucleic acid molecule capable of down-regulating expression of at least one splice variant of the *IG20* gene is comprised in siRNA or shRNA.

24. The method of Claim 23, wherein the siRNA and shRNA is encoded by a nucleic acid molecule which includes the structure:

$$X_{sense} - \text{hairpin loop} - X_{anti\text{-}sense},$$

wherein X includes or consists essentially of a nucleic acid sequence CGGCGAATCTATGACAATC (SEQ ID NO:1).

25. The method of Claim 23, wherein the siRNA and shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2).

26. The method of Claim 25, wherein the siRNA and shRNA comprises a nucleic acid having the sequence CGGCGAAUCUAUGACAAUC (SEQ ID NO:2) and is in the form of a duplex with a cognate nucleic acid having the sequence GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

27. The method of Claim 22, wherein the shRNA is encoded by a nucleic acid having the sequence CGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG (SEQ ID NO:3), wherein a hairpin loop region is from positions 20-28 of the sequence.

28. The method of Claim 22, wherein the siRNA, shRNA and antisense oligonucleotides comprising nucleic acids which are complementary to a nucleic acid sequence of exon 13L of a MADD splice variant of the *IG20* gene and/or an mRNA transcript thereof comprise a nucleic acid having the sequence selected from GAUUGUCAUAGAUUCGCCGTT (SEQ ID NO:4) and GAUUGUCAUAGAUUCGCCG (SEQ ID NO:10).

29. The method of Claim 22, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered in the form of a liposomal formulation or by lentivirus transfection.

30. The method of Claim 22, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered as an adjuvant.

31. The method of Claim 17, wherein the one or more alkylating agent chemotherapeutic is selected from cyclophosphamide, mechlorethane, cisplatin, carboplatin, procarbazine, ifosfamide, melphalan, chlorambucil, cyclophosphamide, streptozocin, carmustine, lomustine, busulfan, and dacarbazine.

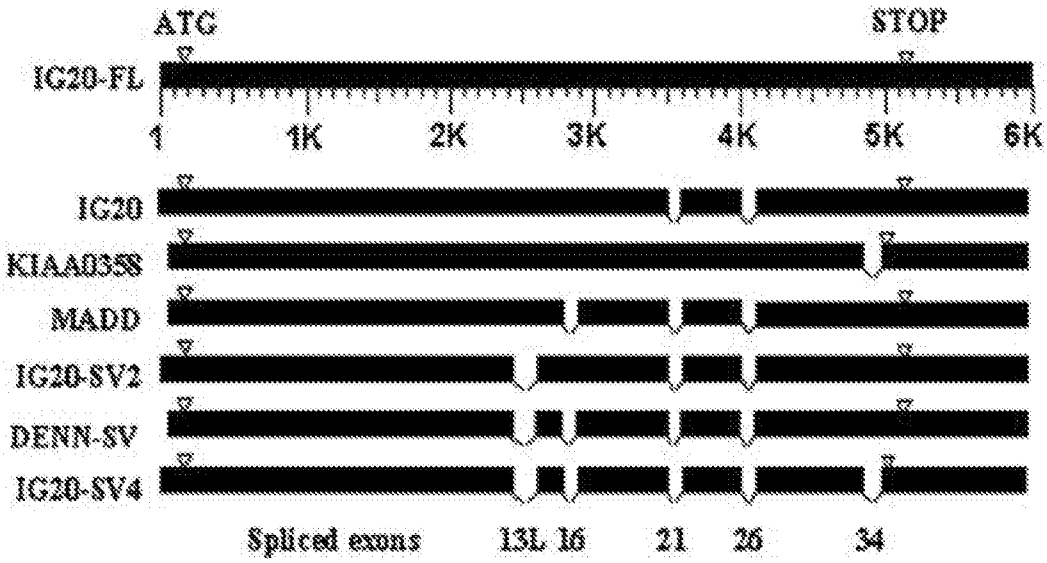
32. The method of Claim 17, wherein the one or more alkylating agent chemotherapeutic is administered in the form of a pharmaceutical composition further comprising one or more pharmaceutically acceptable diluents, excipients, or carriers.

33. The method of Claim 22, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered prior to the one or more alkylating agent chemotherapeutic, or is administered simultaneously with the one or more alkylating agent chemotherapeutic.

34. The method of Claim 22, wherein the one or more siRNA, shRNA and antisense oligonucleotides is administered in the form of a pharmaceutical composition further comprising one or more pharmaceutically acceptable diluents, excipients, or carriers.

35. The method of Claim 22, wherein the one or more siRNA, shRNA, and antisense oligonucleotides and the one or more alkylating agent chemotherapeutic are formulated in a dosage pack and are administered according to a selected treatment regime.

Figure 1.


ATG
STOP
IG20-FL
1
1K
2K
3K
4K
5K
6K
IG20
KIAA0358
MADD
IG20-SV2
DENN-SV
IG20-SV4
Spliced exons
13L
16
21
26
34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/26182

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12N 15/113, 15/63; C07K 14/82 (2018.01)
CPC - C12N 15/1135, 15/63; C07K 14/82; A61K 47/6913

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0075929 A1 (PRABHAKAR, BS et al.) 19 March 2009; paragraphs [0019], [0031], [0033]-[0035], [0072], [0092], [0103], [0108], [0117]-[0119], [0126], [0146], [0178]; claims 1-3, 11	1-7, 10, 12-24, 27, 29-35
A	US 2004/0096436 A1 (CARSON, DA et al.) 20 May 2004; paragraphs [0152], [0161]-[0162], [0164]-[0165]	35
A	US 2011/0117627 A1 (PRABHAKAR, BS et al.) 19 May 2011; whole document	1-7, 10, 12-24, 27, 29-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 2018 (30.07.2018)	10 AUG 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/26182

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***- Please See Within the Next Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group I+, Claims 1-35; SEQ ID NO: 1 (nucleic acid sequence); SEQ ID NO: 3 (RNA molecule)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/26182

-***-Continued from Box III Observations where unity of invention is lacking -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+, Claims 1-35; SEQ ID NO: 1 (nucleic acid sequence); SEQ ID NO: 3 (RNA molecule) are directed toward compositions and methods of treating cancers which include combination therapy with nucleic acid molecules capable of down-regulating the expression of at least one splice variant of the IG20 gene, and alkylating agent chemotherapeutics.

The compositions and methods will be searched to the extent that they encompass SEQ ID NO: 1 (first exemplary nucleic acid sequence); SEQ ID NO: 3 (first exemplary RNA molecule). Applicant is invited to elect additional sequence(s) and/or RNA molecule(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional sequence(s) and/or RNA molecule(s) will be searched upon the payment of additional fees. It is believed that claims 1-7, 10, 12-24, 27, and 29-35 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (nucleic acid sequence); SEQ ID NO: 3 (RNA molecule). Applicants must specify the claims that encompass any additionally elected sequence(s) and/or RNA molecule(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (nucleic acid sequence).

No technical features are shared between the nucleic acid sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a combination of antineoplastic agents useful for treating cancer comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene, wherein not all splice variants of the iG20 gene are down-regulated, and one or more alkylating agent chemotherapeutic; a method of treating cancers selected from slow growing solid tumors and leukemia, lung cancer, ovarian cancer, breast cancer, lymphomas, sarcomas, myelomas, Hodgkin's disease, topical treatment for skin lesions of mycosis fungoides (cutaneous T-cell lymphoma), both Hodgkin's and non-Hodgkin's lymphoma, testicular cancer, germ cell tumors. bladder cancer, head and neck cancer, cervical cancer, multiple myeloma, neuroblastoma, rhabdomyosarcoma, lymphocytic leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, t-cell lymphoma, multiple myeloma, retinoblastoma, Ewing's sarcoma, endometlial cancer, islet cell pancreatic cancer, brain tumors, glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors, colon cancer, fibrosarcomas, anaplastic astrocytoma, and glioblastoma rnultiforme in a subject in need thereof, comprising administering an effective amount of a combination of antineoplastic agents comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene, wherein not all splice variants of the IG20 gene are down-regulated, and one or more alkylating agent chemotherapeutic; these shared technical features are previously disclosed by US 7,910,723 B2 to Prabhakar et al. (hereinafter 'Prabhakar').

Prabhakar discloses a combination of antineoplastic agents useful for treating cancer (a combination of antineoplastic agents useful for treating cancer; column 5, lines 43-51) comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene (comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene; abstract; column 5, lines 43-51; column 25, lines 52-67), wherein not all splice variants of the IG20 gene are down-regulated (claim 1), and one or more alkylating agent chemotherapeutic (and cyclophosphamide (one or more alkylating agent chemotherapeutic); column 24, lines 49-51); a method of treating cancers selected from lymphoma in a subject in need thereof (a method of treating cancers selected from circulatory cancers such as lymphoma in a subject in need thereof; column 24, lines 31-43), comprising administering an effective amount of a combination of antineoplastic agents comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene (comprising administering an effective amount of a combination of antineoplastic agents comprising an effective amount of one or more nucleic acid molecules capable of down-regulating expression of at least one splice variant of the IG20 gene; column 5, lines 43-51; column 24, lines 31-43), wherein not all splice variants of the IG20 gene are down-regulated (claim 1), and one or more alkylating agent chemotherapeutic (and cyclophosphamide (one or more alkylating agent chemotherapeutic); column 24, lines 49-51).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Prabhakar reference, unity of invention is lacking.