

## The Advance of Anti-Cancer Flexible Heteroarotinoids

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### ABSTRACT

Retinoids have clinically proven anti-cancer activities. However, the toxicity of retinoids hinders their application *in vivo*. Heteroatom substitution on the ring structure in retinoids has resulted in a new class of chemical called heteroarotinoids, which have dramatically lower toxicity. Further modifications have led to flexible heteroarotinoids (Flex-Het). Flex-Hets contain a flexible linker between the heteroatom ring and aryl ring. Among all Flex-Hets, SHetA2, which contains a sulfur heteroatom and a thiourea linker, has been the most promising compound with the highest anti-cancer activity until recently. It has been shown effective against all of over 60 cancer cell

lines in the US National Cancer Institute. It is a candidate for clinical trials for the treatment of ovarian cancer. SHetA2 induces both intrinsic (mitochondrial mediated) and extrinsic (death receptor mediated) apoptosis pathways in cancer cells with low toxicity and differential activity against malignant versus normal cells. Heat shock protein HSPA9 (mortalin), which interacts with the tumor protein p53, has been found to be the receptor protein of SHetA2. Binding of SHetA2 to mortalin interrupts p53-mortalin interaction, releasing protein p53 to nucleus where it initiates apoptosis. In this article, the chemistry evolution, anti-cancer activity, biological mechanism, and development of better analogues of Flex-Hets are reviewed.

**Key words:** Cancer therapeutics; Small-molecule drugs; Toxicity; Mitochondria; Mortalin; SHetA2

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### INTRODUCTION

Retinoids are natural or synthetic vitamin A derivatives with clinically proven anti-cancer activities. However, the toxicity of retinoids has limited their application *in vivo*. Attempts to improve anticancer activities of retinoids while reducing their toxicity have resulted in modifications of retinoids. Heteroatom substitution on the ring in retinoids structure leads to generation of a new class called *heteroarotinoids* with dramatically lowered toxicity. Further modification led to generation of *flexible heteroarotinoids* (*Flex-Het*). Flex-Hets contain a flexible linker between the heteroatom ring and aryl ring. A variety of Flex-Hets has been tested and among all, *N*-(3,4-dihydro-2,2,4,4-tetramethyl-2H-1-benzothiopyran-6-yl)-*N'*-(4-nitrophenyl)thiourea, also known as *SHetA2*, has been the most promising compound with the highest anti-cancer activity until recent development of better analogues. SHetA2 is effective against all of over 60 cancer cell lines in the US National Cancer Institute<sup>[1]</sup> and it is a candidate for clinical trials for the treatment of ovarian cancer. It induces both intrinsic (mitochondrial mediated) and extrinsic (death

receptor mediated) apoptosis pathways in cancer cells<sup>[2]</sup>. It has low toxicity to normal tissues and differential activity against malignant versus normal cells<sup>[3,4]</sup>. SHetA2 has been shown to interfere and interact with the activity of the heat shock protein HSPA9 (mortalin)<sup>[5]</sup>. Mortalin on the other hand interacts with the tumor protein p53 in a concentration-dependent manner<sup>[6]</sup>. One pathway for the compounds to inhibit the growth of cancer cells *in vitro* is by displacing p53 from mortalin. Binding of SHetA2 to mortalin interrupts p53-mortalin complex, allowing p53 to migrate to the nucleus where it initiates apoptosis. However, interactions of mortalin with other client proteins, such as Bcl-2 and p66shc, may also impact cell apoptosis<sup>[5]</sup>. Here we review the chemistry evolution, anti-cancer activity, and biological mechanism of heteroarotinoids, specifically SHetA2. Mortalin-SHetA2 interactions and its role in induction of apoptotic are reviewed next. More recent efforts to design Flex-Het analogues with improved properties are also summarized.

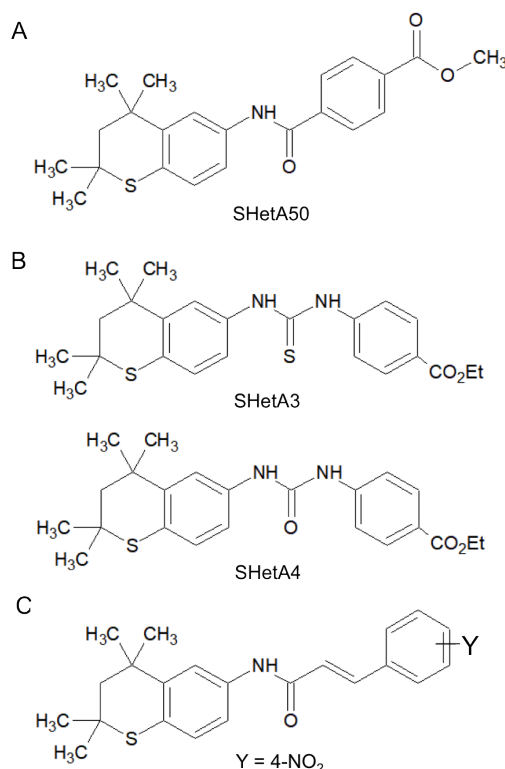
## Flex-Hets CHEMISTRY EVOLUTION

SHetA2 belongs to a relatively new chemical class, flexible heteroarotinoids, which have been evolved progressively from retinoids to arotinoids, and to heteroarotinoids.

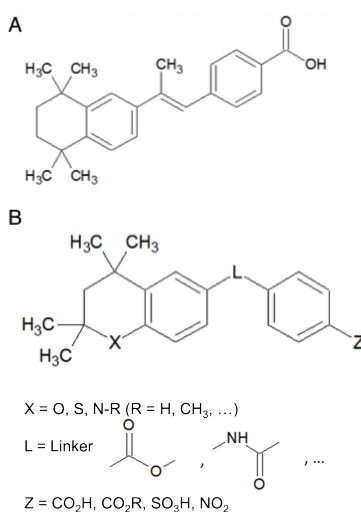
Retinoids are natural or synthetic vitamin A analogues/derivatives with at least one aromatic ring. The study on retinoids originated from the exploration of vitamin A metabolism<sup>[7]</sup>. Retinoids' mechanism of action is through their interaction with two types of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). As the regulators of these retinoid receptors, retinoids have chemotherapeutic and chemopreventive properties in a variety of cancers in animal models and human cell lines. Retinoids regulate gene expression, control developmental, metabolic, and differentiation mechanisms, and induce growth inhibition and apoptosis in cancer cells *in vivo* and *in vitro*<sup>[8]</sup>.

A retinoid molecule consists of a cyclic group, a polyene side chain, and a polar end group (Figure 1). Naturally occurring retinoids include Retinol (aka Vitamin A1), Retinal (Retinaldehyde), all-*trans*-retinoic acid (*t*-RA), 9-*cis*-retinoic acid (9-*cis*-RA), and 13-*cis*-retinoic acid (13-*cis*-RA, 2). They are shown to be potential chemotherapeutic and chemopreventive agents<sup>[9-12]</sup>.

However, the clinical applications of retinoids are limited due to innate and acquired resistance in some malignant cells and significant side effects associated with their activation of nuclear receptors, such as teratogenicity and toxicity to skin, mucous membranes, gastrointestinal system, liver, kidneys, hair, eyes, endocrine system, and bone<sup>[13,14]</sup>. Efforts to minimize retinoids toxicity, to increase the potency (i.e., to reduce the half-maximal inhibitory concentration IC<sub>50</sub>), and to overcome resistance have led to design and synthesis of novel retinoids<sup>[15]</sup>, such as 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437), N-(4-hydroxyphenyl) retinamide (Fenretinide or 4-HPR), bexarotene (LGD1069), arotinoids, heteroarotinoids, and Flex-Hets. CD437 is a RAR $\gamma$  activator that showed significant apoptosis-inducing activity in a variety of cancer cells *in vivo* and *in vitro*<sup>[16,17]</sup>. The synthetic amide of retinoic acid, Fenretinide, is a widely studied chemopreventive agent that inhibits the growth of several human cancer cell lines. Fenretinide induces apoptosis through both retinoid acid receptor-dependent and receptor-independent mechanisms<sup>[18]</sup>. It has good efficacy (maximal inhibition of cancer cell growth) compared with other retinoids, with no acute or severe toxicity and causes no liver function abnormalities. However, Fenretinide showed a few side effects in clinical trials including impaired dark adaptation (night



**Figure 1** Natural retinoids. (A) retinol/vitamin A, (B) retinal/retinaldehyde, (C) isotretinoin/13-*cis*-RA, (D) tretinoin/all-*trans*-retinoic acid (*t*-RA), and (E) alitretinoin/9-*cis*-RA.



**Figure 2** Synthetic arotinoid TTNPB (A) and heteroarotinoids (B).

blindness) resulted mainly from reduction of circulating retinol<sup>[19,20]</sup>. LGD1069 is a synthetic derivative of 9-*cis* retinoic acid that showed more potency and less toxicity than its parent compound. However, the compound showed limited therapeutic efficacy<sup>[21,22]</sup>.

To improve upon retinoids, arotinoids (Figure 2A) were synthesized by adding an aromatic ring to constrain the retinoic acid double bonds of retinoids. The first arotinoid 4-[(E)-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid known as TTNPB activates RARs instead of RXRs. Arotinoids have better anticancer activity than retinoids but intolerably higher toxicity<sup>[23,24]</sup>.

Further attempts were made to develop mimics of *trans*-retinoic acid and arotinoids with similar clinical effects but less toxicity and

side effects. To reduce toxicity, oxygen or sulfur replaced a carbon atom in the tetrahydronaphthalene ring of TTNPB. The resulting compounds were named heteroarotinoids (Hets) (Figure 2B)<sup>[25]</sup>. The partially saturated aromatic ring can be five or six-membered, with at least one heteroatom (O, S, N, etc.). The heteroatom can block the benzylic oxidation of the parent arotinoids to toxic metabolites, reducing *in vivo* toxicity by up to 1,000-fold, comparable to the level of a natural retinoid, all-*trans* retinoid (*t*-RA)<sup>[25]</sup>.

Heteroarotinoids have been tested against various cancer cell lines such as kidney, lung, head and neck, and breast cancers and showed high activity and low toxicity<sup>[26-29]</sup>. Hets have similar anti-cancer activity to retinoids by activating RARs and inducing gene regulation<sup>[25,30]</sup>. Arotinoids and *t*-RA only activate RAR receptors while 9-*cis*-RA and some heteroarotinoids activate both RAR and RXR receptors<sup>[29,31]</sup>.

The specificity for retinoid receptors could be changed by alterations in the structure of the compound. Berlin and colleagues synthesized several heteroarotinoids and studied the effect of structural characteristics like heteroatom, ring size, number of aryl groups, and terminal side chain on the retinoid receptor<sup>[32]</sup>. In general six-membered ring had greater efficacy and specificity for RAR $\beta$  than five-membered rings. Monoaryl compounds had lower RAR $\alpha$  receptor specificity but greater efficacy than diaryl compounds. The effective concentration (EC<sub>50</sub>) values of sulfur containing heteroarotinoids for RAR $\alpha$  were lower in diaryl than in monoaryl, whereas the opposite relationship was observed in oxygen containing compounds.

Dhar *et al* synthesized three heteroarotinoids with a nitrogen heteroatom and a two-atom C-O linker<sup>[26]</sup> and studied their RAR and RXR activation, biological activity, and growth inhibition against cervix, vulvar, ovarian, and head/neck tumor cell lines. In comparison with the positive control 4-HPR, the modified nitrogen heteroarotinoids had low apoptosis effect like *t*-RA and were classified as nonapoptotic retinoids.

Zacheis *et al* evaluated growth inhibition activity of 14 heteroarotinoids against two HNSCC cell lines *in vitro* and compared their activities to that of 9-*c*-RA<sup>[29]</sup>. The results suggested that heteroarotinoid induced growth inhibition through the regulation of gene expression via the nuclear retinoic acid receptors. For most tested heteroarotinoids, the tumor size had statistically significant reduction while no significant toxicity was observed.

Brown *et al.* ranked the antibacterial activity of 15 heteroarotinoids against *Mycobacterium bovis* according to their MIC values, which are defined as the lowest concentration of drug in  $\mu\text{g/mL}$  of drug that results in 99% reduction in the number of bacterial colonies compared to the control drug free plate<sup>[33]</sup>. The result revealed the inhibitory ability of these first examples of heteroarotinoids against the growth of *M. bovis*.

Further exploration led to a more potent class of Hets, the flexible heteroarotinoids (Figure 3). Flex-Hets have a three-atom flexible linker of urea or thiourea linker group between the heterocyclic ring and the aryl ring.

*In vitro* experiments on cancer cells have shown that Flex-Hets regulate growth, differentiation and apoptosis, similar to conventional Hets. However, unlike conventional Hets with conformationally restricted two-atom linkers, Flex-Hets induce apoptosis independent of retinoic acid receptors RAR/RXR, hence not causing toxicity like skin irritation, genotoxicity, and teratogenicity<sup>[1,3,28,34,35]</sup>. The National Cancer Institute's Rapid Access to Intervention Development (RAID) program (Application 196, Compound NSC 721689) and Rapid Access to Preventive Intervention Development (RAPID) program

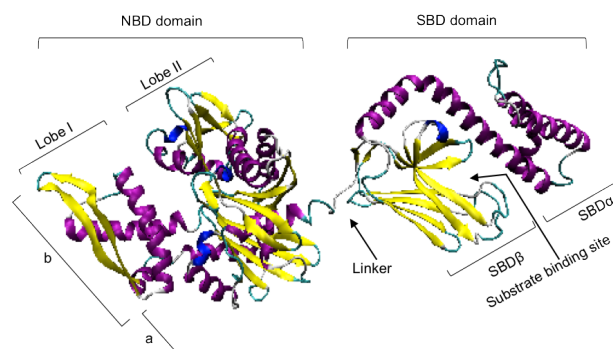


Figure 3 (A) Structure of flexible heteroarotinoids (Flex-Hets)<sup>[3]</sup>. (B) For SHetA2, X=H, Z'=H, and Y=S.

completed preclinical testing on SHetA2 (Figure 3B). Not showing toxicity effects in preclinical trials, SHetA2 is now in Phase-0 clinical trial.

## BIOLOGICAL ACTIVITIES OF Flex-Hets

Although Flex-Hets regulate growth and induce differential apoptosis in cancer cells, in normal cells they induce growth inhibition only<sup>[1]</sup>. Differential effect of SHetA2 has been observed *in vivo*, e.g. in ovarian cancer xenografts<sup>[1]</sup>, in ovarian cancer cell lines versus normal HOSE cells<sup>[36]</sup>, and in the Caki-1 kidney cancer cell lines versus normal kidney cells<sup>[37]</sup>. Study of the role of RNA and protein synthesis in SHetA2's mitochondria mechanism of action has showed that SHetA2 works independently from regulation of gene expression<sup>[4]</sup>. Similar to other Flex-Hets, SHetA2 induces differential cell apoptosis independent of the retinoic acid receptor-signaling pathway and by directly targeting mitochondria<sup>[35]</sup>. Resistance of mitochondria to SHetA2 in normal cells has been confirmed by lack of cardiac toxicity in treated wild type mice versus untreated mice at the highest dose used<sup>[38]</sup>.

Myers and colleagues<sup>[39]</sup> demonstrated anti-angiogenic activity of SHetA2 through microarray analysis of ovarian cancer organotypic cultures and showed that Flex-Hets regulate expression of angiogenic cytokines. Thus by blocking cytokine release from cancer cells, SHetA2, inhibits angiogenesis. In this study, 44 genes were significantly regulated by SHetA2. SHetA2 reduces the expression of thymidine phosphorylase (anti-angiogenic growth factor) in cancer cells but not normal cells. Angiogenic inhibition of SHetA2 *in vivo* has been confirmed in Caki-1 renal cancer xenografts.

### 1. Apoptosis Pathways

There are two major apoptosis pathways: extrinsic death receptor (DR)-induced pathway and intrinsic mitochondria-mediated pathway, which involve activation of caspase-8 and caspase-9, respectively<sup>[40]</sup>. SHetA2 induces both intrinsic and extrinsic apoptosis<sup>[2]</sup>.

Lin *et al*<sup>[41,42]</sup> studied the effect of SHetA2 on expression levels of some apoptosis regulator proteins. They found that in human NSCLC cells, SHetA2 down-regulates both cellular FLICE-inhibitory protein (c-FLIP) (a major inhibitor of extrinsic apoptosis) and Survivin (an inhibitor of both extrinsic and intrinsic apoptosis) in all 6 tested cell lines and that it reduces the levels of several other apoptosis regulator proteins like XIAP, Bcl-2, Bcl-xL in some tested cell lines.

SHetA2 can induce extrinsic caspase-8-dependent apoptosis by enhancing death receptor expression and differentially sensitizing cancer cells to DRLs (Death Receptor Ligands) such as Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ )<sup>[2,43]</sup> or other DRLs<sup>[42]</sup>. Lin *et al*<sup>[42]</sup> showed for the first time that SHetA2 induced a caspase-8-dependent

apoptosis mechanism, in which SHetA2 enhances CAAT/enhancer-binding protein (CHOP) expression and up-regulated death receptor 5 (DR5) in human lung cancer in cell cultures and in mice. The induction of DR5 also increased TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Moxley *et al.*<sup>[43]</sup> studied the effect of chemotropic agents cisplatin, paclitaxel, and SHetA2 with TNF $\alpha$  and TRAIL on ovarian cell lines A2780 and SKOV3 and normal human endometrial cells. Normal and cancer cells are resistant to both death receptor ligands. Combination of cisplatin and paclitaxel with TNF $\alpha$  and TRAIL did not eliminate this resistance. However, dose responsive apoptosis effect of SHetA2 was enhanced when combined with TNF $\alpha$  and TRAIL. SHetA2 when combined with TNF $\alpha$  and TRAIL sensitized cancer cells and induced an extrinsic caspase-8 and -3 apoptotic pathways. Moreover, SHetA2 did not affect the sensitivity of normal cells. Moreover, SHetA2 enhances caspase 9-dependent intrinsic apoptosis activity as a single agent<sup>[2,43]</sup>.

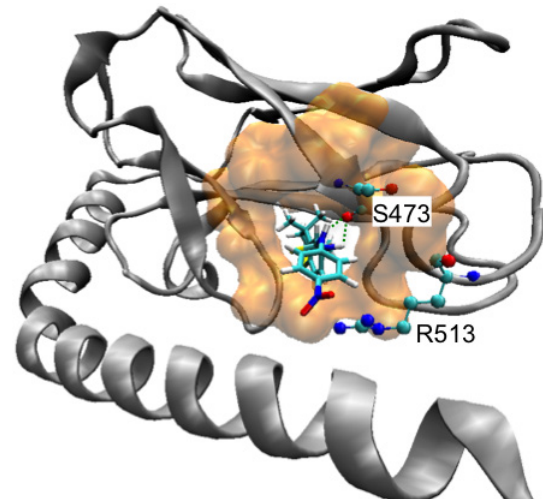
The intrinsic apoptotic pathway of SHetA2 is through direct targeting of mitochondria<sup>[4,35,38,44]</sup>. This may be due to SHetA2 mediated release of p66shc from mortalin, which can cause opening of mitochondria pores<sup>[5]</sup>. Moreover, SHetA2 and mortalin conflictingly regulate B-cell lymphoma 2 (Bcl-2) family members. Bcl-2 family of proteins reside on the mitochondria outer membrane and are responsible for maintaining the integrity of mitochondria. The balance between members of Bcl-2 family is important in mitochondria stabilization and regulating cell apoptosis. Flex-Hets decrease expression of anti-apoptotic Bcl-2 family members in cancer cells and increase their expression level in normal cells in a time dependent manner, while not changing the pro-apoptotic expression levels<sup>[4,37]</sup>. Alteration in the mitochondrial membrane potential and suppression of mitochondrial membrane permeability transition enhances the release of cytochrome *c* from mitochondria to the cytoplasm and generation of reactive oxygen species (ROS). The released cytochrome *c* interacts with cytosolic factors like Apaf-1 and dATP, thus activates caspase-9 and caspase-3-like activity in a time dependent manner.

The major concerns regarding the toxicity of any mitochondria-targeting drug are effect on the mitochondria in normal cells and increased ROS levels. Increase of ROS levels exceeding the capacity of detoxifying enzymes can lead to ROS-induced cellular damage and cause potentially carcinogenic change in cells. A good drug candidate should have least effect on normal cells, and maximum effect on cancer cell. Normal cell lines show more resistance to Flex-Hets than cancer cell lines. Interestingly, Liu and colleagues tested the hypothesis that SHetA2 induces mitochondrial swelling through ROS generation and found that ROS generation is a consequence of Flex-Het action on mitochondria<sup>[4,37]</sup>.

## 2. Mortalin as a Receptor for SHetA2

Benbrook *et al* applied affinity chromatography combined with mass spectroscopy analysis to identify SHetA2 target proteins<sup>[5]</sup>. Three heat shock protein family members, HSPA5, HSPA8, and HSPA9 (mortalin), were identified. Among them mortalin was significantly present and it was considered as the receptor protein for SHetA2. Moreover, western blot analysis of co-immunoprecipitates of mortalin with its client proteins in protein extracts from A2780 and SK-OV-3 human ovarian cancer cells revealed that SHetA2 binds to mortalin and disturbs its interaction with p66shc and p53.

Mortalin is a 74-kDa protein with 679 amino acids and a member of the heat shock protein (HSP) family. Heat shock proteins are ubiquitous, highly conserved proteins that are expressed



**Figure 4** Structure model of full-length human mortalin in ADP state, constructed with I-TASSER server [53] by using known structures of NBD (PDB code 4HBO) and SBD (PDB code 3N8E).

constitutively and located in all cell compartments<sup>[45]</sup>. According to their molecular weight, HSPs are classified into several subfamilies, among which HSP70 is the most important heat shock proteins involved in protein folding and tumor progression. Therefore HSP70 proteins are good targets for cancer therapy<sup>[46]</sup>. HSP70 subfamily consists of at least eight homologous chaperone proteins, among which six are mainly located in the cytosol and nucleus and the other two in endoplasmic reticulum (Grp78) and mitochondria (mortalin).

Mortalin is also known as glucose-regulating protein 75 (Grp75), mot-2, hmot-2, p66mot-1, mitochondria stress-70 protein (mtHSP70), HSPA9/HSPA9B, peptide-binding protein 74 (PBP74), CSA, Mot, Mot2, and MGC4500. Human mortalin is translated in the cytoplasm and transported to mitochondria. Although mortalin is primarily found in mitochondria, it has also been found in several other subcellular sites including endoplasmic reticulum, Golgi apparatus, plasma membranes, cytoplasmic vesicles, and cytosol<sup>[45]</sup>. Immunocytochemical studies using antibody against mortalin showed the immunofluorescence in perinuclear region of immortal cells and pancytoplasmic distribution in normal cells<sup>[47]</sup>. Thus, immunostaining pattern is a sensitive mortalin marker of normal and cancerous cell types.

Mortalin is not a heat activated protein and it can be induced by various stressors such as accumulation of unfolded or misfolded proteins, low levels of ionizing radiation, glucose deprivation, loss of calcium homeostasis, and metabolic stress<sup>[48-50]</sup>. Mortalin is also found overexpressed in several transformed and tumor cell lines<sup>[51,52]</sup>.

The three-dimensional structure of full-length mortalin is not available yet. Evolutional conservation suggests that mortalin has a similar structure to other HSP70s, possessing a conserved ~42 kD nucleotide-binding domain (NBD, aka ATPase domain, residues 1-431), a highly conserved protease sensitive linker (residues 432-441), and a ~25 kD substrate-binding domain (SBD, aka peptide-binding domain, residues 442-679) (Figure 4). The NBD consists of subdomains I and II, each with a and b regions. The SBD can be divided into two subdomains, the ~13 kD  $\beta$ -sandwich domain (SBD $\beta$ ) consisting of 2 sets of 4-stranded antiparallel beta sheets and the ~12 kD C-terminal helical 'lid' domain (SBD $\alpha$ ) consisting of five helices. The substrate-binding site is a hydrophobic region between the two  $\beta$ -sheets forming a deep binding pocket for hydrophobic residues.

### 3. Protein p53 and Its Interaction with Mortalin

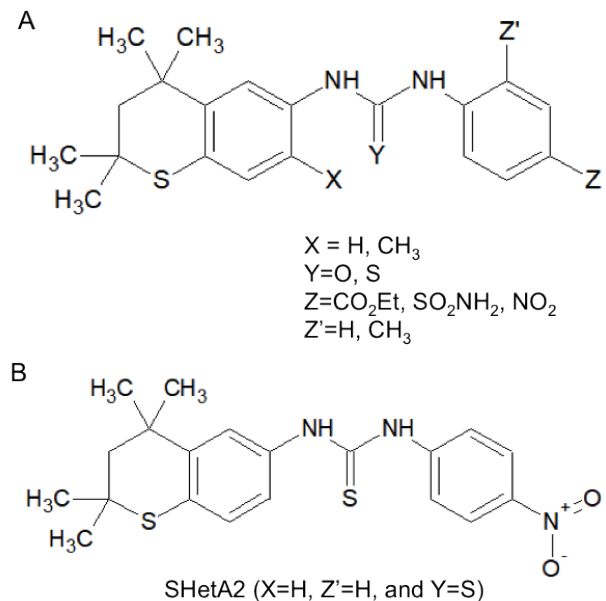
Protein p53 is known as the “guardian of the genome”. It is a nuclear transcription factor that controls cell cycle. To prevent genetic instability p53 induces cell cycle arrest (G1 arrest) and apoptosis in response to endogenous and exogenous stress signals, through transcriptional regulation or direct interaction with apoptotic proteins<sup>[54]</sup>. Through its interaction with Bcl2 and mortalin, p53 regulates mitochondrial membrane potential<sup>[55]</sup>. Protein p53 is inactive in more than half of human cancers. Inactivation of tumor suppressor p53 is involved in transformation and immortalization of cells, and it is the most common genetic abnormality in human cancer. Three main mechanisms responsible for inactivation of p53 are mutations, post-translational modifications, and cytoplasmic sequestration/nuclear exclusion<sup>[56,57]</sup>.

Cell cycle arrest was proposed to explain the apoptosis-independent growth inhibition observed in normal kidney cell cultures<sup>[37]</sup>. It was found in both normal and cancer cells that the binding of SHetA2 to mortalin leads to cyclin D1 degradation and G1 cell cycle arrest, with greater effect on cancer cells than normal cells<sup>[37,58,59]</sup>. Whether and how p53 is involved in this process is not clear yet.

The amino acid sequence of p53 consists of an amino-terminus transactivation domain (TAD, residues 1-42), a proline rich domain (PRD, 60-93), a sequence specific DNA binding domain (DBD or core domain CD, 100-300), a nuclear localization signal domain (NLS, 283-325), a tetramerization domain (TD, 326-356), and a negative regulatory domain (NRD, 361-393) (Figure 8). Binding of proteins to p53 NRD activates the DNA-binding of p53<sup>[60]</sup>.

Protein p53 is synthesized in the cytoplasm and translocated to the nucleus in order to perform its transcription factor function<sup>[63]</sup>. Cytoplasmic sequestration of p53 causes loss of the function, which can lead to immortalization of cells and tumor resistance to radiotherapy and chemotherapy. The residues 323-337 of the carboxyl terminus of p53 were shown to be required for its cytoplasmic translocation from nucleus<sup>[64]</sup>. Cytoplasmic sequestration of p53 can be due to overexpression of Parc<sup>[65]</sup> or mortalin<sup>[66-70]</sup>. Mortalin and p53 have been shown to colocalize in perinuclear region in several cancer cells, e.g. NIH 3T3, MCF7, COS7, NT-2, A172, A2182, HeLa, Balb/3T3, YKG-1, SY-5Y, and U2OS. Kaul *et al* showed for the first time that overexpression of mortalin in normal human diploid fibroblasts (MRC-5) leads to decreased p53 function, thus temporary escape from senescence<sup>[71]</sup>. Also in human foreskin fibroblasts (HFF5) overexpression of mortalin together with telomerase hTERT extended lifespan substantially<sup>[72]</sup>, indicating reduced p53 function. Life span extension of human lung fibroblasts (MRC-5), increased malignancy of human cancer cells<sup>[51]</sup>, malignant transformation of NIH3T3 cells in mice<sup>[73]</sup>, attenuation of HL-60 leukemia cells by mot-2<sup>[74]</sup> are some evidences that suggest the inactivation of p53 by overexpression of mortalin<sup>[71,73,75]</sup>. By interacting with the mitochondrial proteins Bcl2, mortalin, and HSP60, p53 regulates mitochondrial potential<sup>[55]</sup>. The stress-induced mitochondrial p53 localization is followed by changes in mitochondrial membrane potential, cytochrome *c* release, procaspase-3 activation and apoptosis. The presence of mortalin-p53 complexes in mitochondria during p53-induced apoptosis suggests the transcriptionally independent role of p53 in apoptotic signaling<sup>[76]</sup>. Different western blot patterns for mutant and wild-type p53 suggested that loss of p53 function in human diploid fibroblasts transfected by mortalin is not caused by mutation<sup>[71]</sup>. It is reported by the same group<sup>[77]</sup> that by sequestering p53 in the cytoplasm, mortalin inhibits transcriptional activation function of the protein in mouse and human transformed cells.

Regulatory function of p53, which is independent of its



**Figure 5** (A) Structure model of full-length human p53, constructed with I-TASSER server [53]. (B) Domain structure of p53 [61]. (C) Tetramer model of p53 obtained by aligning the predicted full-length model to the tetramer crystal structure (PDB code 2AC0) of four p53 DNA-binding domains in complex with two DNA half-sites [62].

localization, is performed by interaction of p53 with mortalin and Bcl2. Mortalin regulates the DNA-binding ability of p53<sup>[6]</sup>. When binding to p53 in cancer cells, mortalin inhibits apoptotic properties of p53 and leads to survival of cancer cells<sup>[48]</sup>. It sequesters p53 in the cytoplasm fraction through physical contact and blocks p53-mediated transcription, downregulates p53-target genes expression, e.g. p21<sup>SD11/WAF</sup>, and enhances degradation of p53 by MDM2 in cytoplasm, disturbs the G1-associated nuclear translocation of p53, that leads to uncontrolled cell proliferation, a hallmark of cancer cells<sup>[48,64,68]</sup>. Mortalin breaks the p53-Bcl-xL/2 complex and causes proteasomal degradation pathway of p53 by its association with E3 ligase CHIP (Carboxy terminus of Hsc70 Interacting Protein)<sup>[78]</sup>. Moreover, mortalin-p53 interaction causes deregulation of centrosome duplication, which is an indicator for cancer. Overexpression of mortalin can reverse the p53-dependent suppression of centrosome duplication indicating the interaction was localized to centrosomes<sup>[79]</sup>.

It is shown that mortalin-p53 interaction depends on the stress levels in cells. In unstressed or weakly stressed cells, p53 and mortalin do not interact<sup>[70]</sup>. Thus, knock down of mortalin does not induce apoptosis. In contrast, in stressed cancer cells, whether physiologically stressed or under the effect of stress-inducing chemicals, mortalin-p53 interaction exists and knock down of mortalin induces p53-mediated apoptosis.

Many studies have been performed to determine the binding sites of p53 and mortalin with conflicting conclusions. Carboxyl terminus of p53 was considered to be its cytoplasmic sequestration domain<sup>[75,80]</sup>. This is in agreement with the observation that breaking mortalin-p53 complex for example by p53 C-terminal peptides leads to restoration of p53 activities, abrogation of this cytoplasmic sequestration and relocation of p53 to the nucleus. Thus, the sequestration is reversible.

Gabizon *et al* identified and characterized ten binding peptides by screening proteins that interact with tetrameric p53 C-terminal region<sup>[61]</sup>. They identified residues 266-280 in mortalin as the binding

site for NRD (negative regulatory domain) of p53. *In vivo* co-immunoprecipitation of mortalin with p53 and its mutants in human osteosarcoma (U2OS) and breast carcinoma (MCF7) cells revealed carboxyl terminus of p53 (residues 312-352) as the binding site for N-terminal region of mortalin<sup>[68,75,81]</sup>. Peptides consisting of p53 residues 323-337 interfere with p53-mortalin binding and activate p53 by relocating it to the nucleus. Using His-tagged deletion mutants of mortalin, the co-immunoprecipitation of mot-1 and mot-2 with p53 and *in vitro* pull-down assay identified N-terminal residues 253-282, which is common in both mot1 and mot2, of mortalin to be necessary for binding<sup>[81]</sup>. Interestingly this segment overlaps with chemical MKT-077 binding pocket of mortalin residues 252-310.

Kaul *et al* studied the effect of mot-1 mutants in binding to p53<sup>[77]</sup>. Mot-1 mutants used in the study either lacked one or more predicted motifs (hsp70, EF-hand, and leucine zipper) or 30 residues from the C-terminus, or single-site substitution at either one of the two unique residues 618 or 624 to their mot-2 counterparts. C-terminus deleted mot-1 mutants repressed p53 activity, suggesting that C-terminus of mot-1 is not needed for interaction with p53. But each of the three predicted motifs and two unique amino acids of mot-1 are essential in interaction with p53.

On the other hand, Iosefson *et al* used pull-down assay to track p53-mortalin interaction with purified proteins<sup>[6]</sup>. In contrast with results from cell lysates, mortalin SBD binds to p53 in a concentration-dependent manner. They suggested that the contrast between *in vitro* results and results from cell lysate can be attributed to modifications or additional proteins in cell lysates that might affect p53-mortalin interaction. They also found two sites on p53 for mortalin binding, TD and NRD (Figure 5B), with either one being sufficient for binding. The tetramerization does not seem to be necessary for mortalin binding since the L344P mutant of p53, which fails to form tetramer, binds to mortalin similar to the wild-type tetrameric p53.

The mutation of V482F in the substrate-binding pocket abrogated the binding between mortalin and p53<sup>[6]</sup>, consistent with a similar mutation in DnaK, a bacterial homolog of mortalin, which also disrupts the substrate binding<sup>[82]</sup>. Accordingly, pre-incubation of mortalin with a short substrate peptide greatly reduced its binding to p53<sup>[64,82]</sup>. The interaction between mortalin and full-length p53 can be abrogated by adding ATP<sup>[6]</sup>, similar to ATP activated C-terminal lid opening and substrate release. All these evidences suggest that mortalin binds to p53 through its substrate-binding pocket on SBD.

These data are still ambiguous. Deletion might cause partially structure unfold and result in non-specific interactions. In another computer modeling study, it was shown that a p53 peptide (residues 326 to 356, tetramerization domain) binded to mortalin SBD and the linker between NBD and SBD<sup>[83]</sup>. Residues 326 to 341 on p53 were reported to form the mortalin-binding site.

#### 4. Other Inhibitors of Mortalin-p53 Complex

Mortalin-p53 complex is a selective target for cancer therapy<sup>[70]</sup>. Some short peptides<sup>[64,80]</sup>, small molecules, small interfering RNA (siRNA)<sup>[84]</sup>, mortalin small hairpin shRNA (in hepatocellular carcinoma), UBXN2A (Ubiquitin-like (UBX)-domain-containing protein) can hinder p53-mortalin interaction, leading to p53 reactivation and apoptosis in cancer cells.

Several small-molecule mortalin inhibitors include withanone<sup>[85]</sup> (a withanolide, one of the major components of the alcoholic extract of Ashwagangha leaves) and MKT-077<sup>[86-88]</sup> (an anti-tumor compound, a water soluble delocalized lipophilic cationic/rhodacyanine dye analogue). Withaferin A (Wi-A) and Withanone (Wi-N) are two

structurally similar withanolides. Wi-A is toxic to both cancer and normal cells, Wi-N however has milder effect and does not have toxic effects on normal cells. It suggests that Wi-N can be a safer cancer drug but higher doses are needed for the same effect as Wi-A. Mortalin was docked with both of these compounds and it was found that Arg513 in carboxyl terminus of mortalin interacts with them<sup>[89]</sup>. Data shows stronger binding of Wi-A to mortalin. Wi-A and Wi-N also bonds with Arg282 and Leu111 residues of mortalin. Recently attempts have been made to enhance the production of withanolides and elucidate their molecular mechanisms<sup>[90]</sup>.

MKT-077 is selectively toxic to cancer cells and IC<sub>50</sub> of MKT077 for normal cells is more than 100 times greater than that of cancer cell lines thus it was considered as cancer chemo-therapeutic in a Phase I clinical trial. However, the trial was stopped due to excessive renal toxicity in phase I clinical trials against solid tumors<sup>[91, 92]</sup>. MKT-077 interacts with HSPA8<sup>[86]</sup> and HSPA9<sup>[87]</sup>. The binding site of MKT-077 (positively charged) to HSPA8 ADP-state has been determined to be a negatively charged pocket close to residues in nucleotide binding site in the interface of subdomain Ia and IIa (Figure 4), which are 100% conserved between 13 human HSAs<sup>[86]</sup>. The binding site of MKT-077 to HSPA9 is within residues 252-310, which in some studies were shown to include residues needed for binding of p53<sup>[87]</sup>. Immunoaffinity studies showed that MKT-077 binds to mortalin at alpha helices within sub-domain IIb of catalytic cleft and abrogates mortalin function, causing pancytoplasmic distribution of mortalin (characteristic of normal cells) and nuclear translocation and activation of p53. Thus induces growth arrest of cancer cells<sup>[87]</sup>.

Molecular dynamic simulations and co-immunoprecipitation studies for both MKT-077 and withanone ligands showed the abrogation of p53-mortalin interaction and re-activation of p53 upon treatment with the ligands<sup>[85,87]</sup>. There are several potential ways for MKT-077 to inhibit mortalin binding to p53. MKT-077 may inhibit binding of Tid1 by destabilizing the ATP-bound conformation. Or it might inhibit binding of p53 by perturbing the conformation of mortalin at its  $\beta$  strand (residues 267-271)<sup>[93]</sup>. It is shown that growth arrest function of MKT-077 is mediated by a p53-dependent pathway. In cancer cells, and not in normal cells, abrogation of p53-mortalin interaction leads to release of p53 from cytoplasm and translocation of p53 to nucleus and restoration of its transcriptional activation<sup>[87]</sup>. Similar effect for MKT-077 has been observed in a nonmammalian cancer model in soft shell clam *Mya arenaria* W. Guo *et al*<sup>[69]</sup>. suggested that a combination therapy with mortalin inhibitor MT-077 and HSP90 inhibitor 17-AAG could lead to enhanced killing of HCC cells. They showed 17-AAG increases expression of p53 and stabilized it<sup>[94]</sup>.

## STRUCTURE-ACTIVITY RELATIONSHIP AND DESIGN OF BETTER ANALOGS

The low toxicity of SHetA2 to normal cells could be attributed to SHetA2's specific binding to its receptor mortalin without activating nuclear receptors RARs and RXRs<sup>[25,34]</sup>, which are responsible to the many side effects of retinoids<sup>[13,14]</sup>. The three-atom flexible linker of Flex-Hets appeared to be the key structure feature to the reduced toxicity. Studies have shown that toxicity of retinoids and heteroarotinoids is associated with activation of nuclear RARs and RXRs<sup>[25,34]</sup>. SHet50 (Figure 6A, with 2-atom amide linker) is both RAR and RXR receptor pan-agonist. Flex-Hets SHetA2 (Figure 3B), SHetA3, and SHetA4 (Figure 6B) show no activity towards nuclear retinoid receptors when used at doses up to 10  $\mu$ M and they show

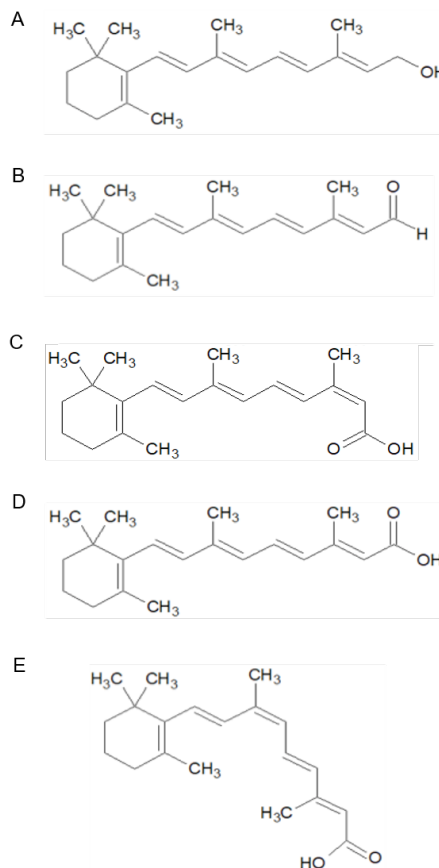
much greater growth inhibitory effects towards cancer cells than normal or benign cells. Gnanasekaran *et al.*<sup>[95]</sup> synthesized a series of 11 compounds with a 4-atom acrylamide linker (Figure 6C) and studied the biological activity against the A2780 ovarian cancer cell line. In general, electron-withdrawing groups on the linker, showed enhanced activity compared to electron donating groups. However, even the most active candidate is still only at comparable level to SHetA2.

To better understand the structure-activity relationship of Flex-Hets, SHetA2 was docked *in silico* to the structure of mortalin substrate-binding domain (Figure 7) using Autodock 4.2<sup>[96]</sup>. There are three important structural features contributing to the ligand-protein interaction. The negatively charged NO<sub>2</sub> group at the phenyl ring has electrostatic interaction with the positively charged side-chain group of amino acid R513. The two NH groups of the thiourea linker form hydrogen bonds with the backbone carbonyl group of S473. The double-ring chroman unit with two pairs of germinal methyls resides in a hydrophobic pocket.

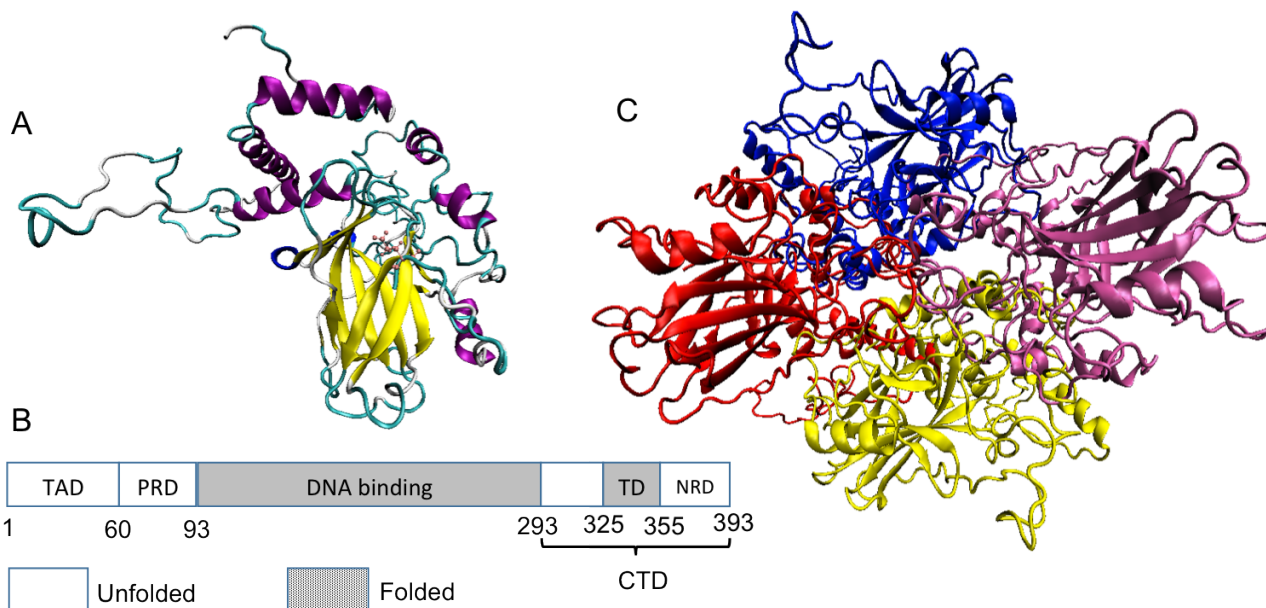
Careful inspection of the hydrophobic pocket revealed that more room was available, and it was reasonable to hypothesize that the receptor binding affinity and cancer inhibitory activity would be enhanced by increasing the size of the hydrophobic groups of the ligand compound. Therefore, several series of SHetA2 analogues were designed to test the hydrophobicity hypothesis (Figure 8)<sup>[97]</sup>. In addition, to determine if certain fragments of the lead molecule SHetA2 could be modified to enhance aqueous solubility, oxygen analogs of SHetA2 were synthesized. Both urea and thiourea containing compounds with oxygen-containing chroman unit in place of the thiochroman in presence of germinal dimethyl/diethyl in the double ring, and different substituents on phenyl ring (NO<sub>2</sub>, CF<sub>3</sub>, CN, OCF<sub>3</sub>, CO<sub>2</sub>Et) were studied (Figure 8). Their potency and efficacy against human A2780 ovarian cancer cells *in vitro*, and binding free energy values *in silico* were assessed.

The hydrophobicity dependence was confirmed by cellular dose-response data that indicated increasing the hydrophobicity leads to higher affinity to the SBD mortalin. Removing the germinal dimethyl group at C2 (Figure 8A) led to a significantly lower efficacy

in growth inhibition. Analogs with a set of dimethyl replaced for diethyl (Figure 8B-C), had higher efficacy and IC<sub>50</sub> value compared to SHetA2. However, there is an optimum level of hydrophobicity. Substituting both sets of dimethyl for diethyl (Figure 8D), affected the performance negatively. Results showed lower activity of



**Figure 6** Heteroarotinoide (A) with a 2-atom linker activates RAR and RXR receptors, leading to side effects. Flex-Hets with a 3-atom (B) or 4-atom (C) linker activate these receptors.



**Figure 7** Molecular docking SHetA2 with the substrate-binding domain of mortalin (PDB ID: 3N8E). The protein is shown with its secondary structure motifs in gray, and hydrophobic amino acids in the substrate binding channel in surface style (orange). SHetA2 is shown in sticks, with oxygen in red, carbon in cyan, hydrogen in white, and sulfur in yellow. Residues R513 and S473 are shown in ball and stick style. The hydrogen bonds are marked in dotted lines (green).

compounds containing two diethyl groups, compared to compounds with two dimethyl groups. Also apart from the size of hydrophobic substituents, their position is also an important factor in determining the activity. Result showed improved activity of (1) urea linker-containing compounds over thiourea counterparts, (2) compounds with higher degree of hydrophobicity over the ones with lower hydrophobicity, (3) geminal diethyl-containing compounds at C<sub>2</sub> position of ring A over compounds with geminal diethyl at C<sub>4</sub> or compounds with geminal diethyl at both C<sub>4</sub> and C<sub>2</sub> position of the double ring, and (4) NO<sub>2</sub>-containing compounds over CF<sub>3</sub> or other ring B substituents. Among the tested compounds, the group of compounds with NO<sub>2</sub> substituent and urea linker, had efficacy values of 91-94% and IC<sub>50</sub> values (half-maximal inhibitory concentration) of 2.0-2.4 μM which are significantly better than the corresponding values for SHetA2 being 84% and 3.2 μM respectively. Compounds with smaller IC<sub>50</sub> value are expected to have stronger affinity for the receptor protein, mortalin, thus be more effective in competing for the receptor versus other partner proteins.

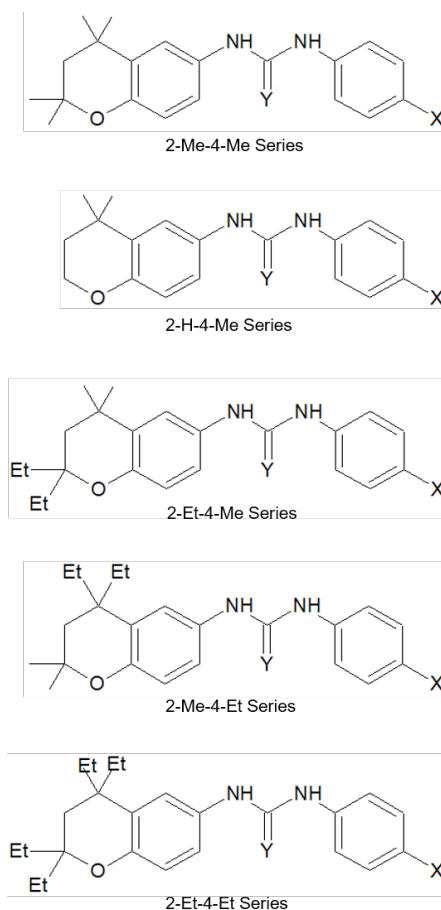
## PHARMOCOKINETICS STUDIES

SHetA2 absorption can be affected by factors like dose, species (thus different dietary habits, etc.), and formulation<sup>[98]</sup>. Effect of SHetA2 is time dependent and dose dependent. For example in ovarian cancer cell lines, cell cycle arrest contributes to SHetA2 growth inhibition, time-dependently and dose-responsively<sup>[99]</sup>. At lower concentrations, differentiation is observed. However in higher concentrations apoptosis dominates<sup>[34,35]</sup>. Thus, it is important to determine concentrations, bioavailability, and stability of SHetA2 in order to ensure sufficient dosing of SHetA2 in clinical trials to target apoptosis inducing concentrations and maximize the activity of the drug.

Kabirov *et al* performed preclinical pharmacokinetics to determine the toxicokinetics of SHetA2 in animals<sup>[100]</sup>. HPLC-UV studies have been applied and pharmacokinetic profile of SHetA2 has been obtained to quantify SHetA2 in plasma in mouse and human by Sharma *et al*<sup>[101]</sup>. The result of a study performed by Zhang *et al*<sup>[102]</sup> indicates a linear and predictive pharmacokinetics in mouse plasma. The terminal half-life, total body clearance value, oral absorption, and bioavailability were measured. This study also showed high tissue uptake. It measured the observed total body clearance of SHetA2 to be 1.81 l/h/kg and suggested that the clearance of SHetA2 is due to degradation and metabolism. Liu *et al*<sup>[103]</sup> applied liquid chromatography-ultraviolet/multi-stage mass spectrometry (LC-UV/MS<sup>n</sup>) to evaluate SHetA2 metabolites in rat liver microsomes *in vitro*, and in human, mice, and rats *in vivo*.

It appeared that once the absorption saturation is reached, further increase of doses will decrease bioavailability and absorption<sup>[58]</sup>. Absorption saturation in mice, rats and dogs is about 100 mg/kg body weight/day<sup>[100,102,103]</sup>.

Highly hydrophobic SHetA2 has low water solubility and low gastrointestinal (GI) absorption, and exhibited low oral bioavailability in rats (< 1%) and dogs<sup>[100]</sup>. In dogs, suspension of SHetA2 in 30% aqueous Kolliphor HS15 (aka Solutol HS15), which is an excellent emulsifying system for both injectable and oral formulation of compounds with low solubility<sup>[104,105]</sup>, enhances the bioavailability, which is the proportion of a drug that enters the systemic circulation. Optimal dose is a dose at which chemoprevention occurs without toxicity. The optimal dose should reduce cyclin D1 in neoplastic and not in normal cells. The AIN76A diet formulation of SHetA2, 187 mg/kg/day SHetA2 suspended in Kolliphor HS15 for bioavailability enhancement has been reported in mice and effective tissue drug



**Figure 8** Flex-Hets designed to test hydrophobicity dependence of the activity at the double ring [97]. Compounds with decreased hydrophobicity (with R=Me, R'=H or vice versa) have dramatically decreased inhibition against cancer cell growth, while compounds with increased hydrophobicity (R=Me, R'=Et; R=Et, R'=Me; or R=Et, R'=Et) perform better than SHetA2.

levels were observed at this dose with no toxicity<sup>[58]</sup>. SHetA2 showed very low toxicity, with a no-observed-adverse-effect level (NOAEL) of 500 mg/kg/day in rats and over 1500 mg/kg/day in dogs<sup>[100]</sup>. Based on bioavailability and due to limited oral absorption at doses higher than 100 mg/kg, the new NOAEL dose was chosen 100 mg/kg<sup>[98]</sup>. On the other hand, the oral administered dose of SHetA2 that reduces the colon and small intestinal polyps incidence and sizes is 30 mg/kg/day<sup>[38]</sup>. A dose of 10 to 60 mg/kg/day can inhibit xenograft tumor growth<sup>[1,37,100]</sup>. Such large therapeutic window makes SHetA2 an ideal chemopreventive drug.

Responses to SHetA2 doses in APC<sup>min/+</sup> mice were found to be gender related, which suggests that males and females to be dosed differently<sup>[38]</sup>. Some administration routes include oral gavage<sup>[1,98]</sup>, dietary formulation<sup>[58]</sup>, intravenous injection<sup>[58]</sup>, and vaginal application<sup>[106]</sup>.

## CONCLUSION

Flex-Hets, with SHetA2 being a prominent example, have promising anti-cancer activities. SHetA2 contributes to both intrinsic apoptosis by directly targeting mitochondria and extrinsic apoptosis by enhancing death receptor activation. Interaction of SHetA2 with its target protein mortalin can regulate Bcl-2 protein family and affect stability of mitochondria and apoptosis. Efforts have been made to fine tune the chemical structure to enhance the anticancer activity of Flex-Hets.



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