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REVIEW ARTICLE

p53 Protein: Master Regulator of Apoptosis and its Application in Cancer Therapy

Chiragkumar J. Gohil[1]*, Malleshappa N. Noolvi[2]

[1]Discipline of Pharmacy, Gujarat Technological University, Ahmedabad 382424, Gujarat, India, [2]Department of Pharmaceutical Chemistry, Shree Dhanvantary Pharmacy College, Kim (E), Dist.: Surat 394 110, Gujarat, India

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ABSTRACT

Cancer is characterized by uncontrolled and abnormal cells growth. In the body, the cell growth and cell division are governing by apoptosis. The process of apoptosis is mainly regulated by the p53 protein. p53 is a tumor suppressor protein. The amount of p53 in a cell is mainly controlled by the negative regulator murine double minute 2 (MDM2), which on complex formation with p53 leads to an overall reduction of the p53 level. Inhibition of p53 function will inhibit the apoptosis and leads to cancer. Consequently, inhibition of the MDM2/p53 interaction using the small molecules activates the p53 function and apoptosis in the cells which contain the wild-type p53. It is a promising new therapeutic strategy for the treatment of cancers retaining wild-type p53. However, the safety window of this class of compounds must be evaluated. Moreover, it has to require the development of compounds, which can also be able to target the cells which contain the mutated or deleted p53.

Keywords: MDM2 protein, p53 protein, p53/MDM 2 interaction, small molecule p53/MDM 2 interaction inhibitors

INTRODUCTION

Cancer is a major challenge for medical science. Uncontrolled cell growth causes cancer.[1] Cancer is characterized by the appearance of the tumor; the tumor is an abnormal mass of the cells. Activation of the oncogenes in the body is responsible for cancer. Mainly there are two types of tumors, malignant tumor and benign tumor. Tumors can invade the vital organs of the body and can modulate the normal physiology of the body.[2]

In our body, cell division and cell growth are regulated by the various biological processes such as apoptosis and necrosis. Among these two, apoptosis is main mechanism which regulates the cell division and cell growth. Hence, cancer cannot be arising, if apoptosis is occurring properly. Every cancer tumors have an inhibited apoptosis process. Hence, the normal apoptosis process is mandatory for the inhibition of cancer genesis.

***Corresponding Author:**
Chiragkumar J. Gohil,
E-mail: gohil2711@gmail.com

APOPTOSIS AND NECROSIS

Programmed death of the cells is known as apoptosis. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. However, there are one more phenomena, which involves cell death and that is necrosis.[3]

Apoptosis and necrosis are a totally different process. Apoptosis is generally initiated by a normal homeostasis process of the body. During normal healing process, it can be occur as a defense mechanism. Apoptosis is a normal and routine process of the body, whereas necrosis is a premature death of cells and living tissue. External factors/disease (trauma or infection) can initiate the necrosis and it is always abnormal and harmful. Apoptosis involves a wide variety of physiological and pathological stimuli.[4] At low doses, a variety

of injurious stimuli such as heat, radiation, hypoxia, and cytotoxic anticancer drugs can induce apoptosis, but these same stimuli can result in necrosis at higher doses.

Apoptotic cell death occurs through a p53-dependent pathway. When this process of apoptosis is altered or override, then it leads to cancer. Hence, to maintain the normal apoptosis processor to trigger the apoptosis in cancer cells is an essential part of the treatment of cancer [Figure 1].[5]

p53 PROTEIN

In 1979, p53 was originally identified as a 53 kD protein bound by the large T-antigen of the sarcoma-associated virus,[6] assuming that it is a cell-cycle accelerator. Later on, it was characterized as an oncogene and tumor suppressor protein.

p53 in humans is encoded by the TP53 gene (located on the short arm of the chromosome).[7] p53 protein regulates the apoptosis. It also regulates the DNA damage response, cell cycle arrest, and autophagy. It is a powerful anti-proliferative protein. p53 induces the apoptosis, cell cycle arrest, and senescence depending on the cellular stress (DNA damage, oncogenic activity, ribosomal stress, hypoxia, and metabolic stress).

In normal or non-stressed condition, p53 is having a short half-life and low cellular level. It has been achieved by the inhibition of the p53 accumulation and p53 mediated pathways.

Domains of p53

As a potent transcription factor, p53 regulates the major pathway and protects the cells from malignant transformation.[8] It can act as a transcription factor to activate targets genes (e.g. PUMA, NOXA, and BAX) to induce apoptosis.[9] Post-translational modifications (phosphorylation, acetylation, and sumoylation) occur on multiple sites of p53. These modifications and phosphorylation on its N-terminus play an important role in the dissociation of p53 from murine double minute 2 (MDM2) and in the activation of p53 as a transcription factor too.[10,11]

Transcriptional activity of p53 has been controlled by four functional domains of p53 which are as follow: (1) N-terminal transactivation domain (required for the interaction with transcriptional protein factors as well as a proline-rich domain), (2) central conserved DNA-binding core domain, (3) tetramerization domain (assists in sequence-specific DNA binding), and (4) C-terminal negative regulatory domain (used for the sequence-specific DNA-binding when getting phosphorylated).[12]

Most of the negative regulators of a p53 bind with N-terminal transactivation domain, including MDM2.[13]

In the situation of cellular stress, upstream signaling in the p53 pathway cause the p53 stabilization and activation of p53 in response to stress, while downstream p53 signaling executes the appropriate

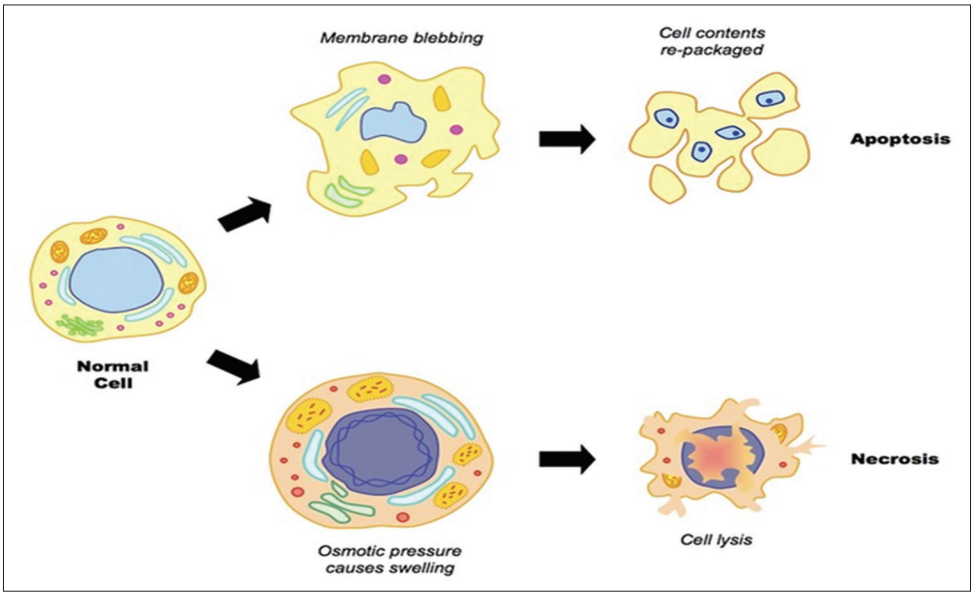

Figure 1: Apoptosis and necrosis[2]

cellular response. This upstream or downstream regulation has been modulated or disables in the cancer cells.[14]

In a stressed condition, p53 can be stabilized (cellular level of p53 increase) by the post-translational mechanism. Hence, it will be accumulated in the cell nuclei and activates or inhibits its target genes to induce cell-cycle arrest or apoptosis.[12] In case of activation of p53 target genes, various cyclin dependent kinase inhibitors such as p21, WAF1/CIP1 getting blocked the cell cycle in G1 and G2 phases.

Various stimulation stabilized and activated the p53, the various stimulation are as follow: (a) Aberrant growth signals (p14ARF and oncogenes Ras, Myc or Fas involved), (b) DNA damage (ATM and Chk2 involved), and (c) a number of chemotherapeutic drugs, ultraviolet light, and protein-kinase inhibitors (ATR and CKII involved).[15]

In response to circumstances, p53 protein may induce cell death (apoptosis) or cell cycle arrest.

Cell cycle arrest: It activates DNA repair proteins when DNA has sustained damage. Moreover, in this situation, it induces growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition.

Cell death/apoptosis: It can initiate apoptosis if DNA damage proves to be irreparable.

In response to DNA damage, the various kinases including ataxia telangiectasia mutated (ATM) kinase activates the p53 protein. In mechanism point of view, ATM kinase does the phosphorylation of MDM2 at S395 (present in the RING domain) which, in turn, activate the p53.[16,17]

Hence, apoptosis is mainly governed by the p53 protein. During apoptosis process, Bcl-2 family of proteins controls the release of cytochrome-c from mitochondria, by altering the permeability of the mitochondria. This Bcl-2 family of proteins is regulated by the p53 protein.[18]

p53 can also play a pro-apoptotic function, by mediating a large number of factors and involves multiple pathways.

p53 is the most frequently mutated protein in human cancers. Its mutation and inhibition causes its inactivation and leads to malignancy. Approximately half of all cancers have inactivated mutation of p53 protein, while rests of have adopted the ways to override its function.

Nearly 50% of all cancer cells which contain mutated p53 are having the mutations within the DNA-binding domain of p53[19] and makes the p53 unable to act as a sequence-specific DNA binding transcription factor.

Apart from the inhibition of p53 by MDM2, some other mechanisms can also alter the p53 pathway or its regulation. Conditions which alter the p53 function, without its mutation: (i) Although half of the all cancer cells bearing the wild-type p53, its function will be inadequate due to abnormal regulation of p53 and defective signaling in pathways of p53. Other situation incorporates the overexpression of MDM2 without gene amplification.[20] (ii) Many human tumors are having an MDM2 which is overproduction due to an amplification of a chromosome segment, including the MDM2 gene.[21] In such situations such as (i) and (ii), p53 function is effectively suppressed without the need for mutation.

In addition to the above conditions, other causes of p53 inactivation are as follow: (a) Gene mutation and (b) interaction of p53 with viral proteins.[22,23]

Therefore, activation of p53 protein is a promising approach for cancer therapy [Figure 2].[24]

MDM2 PROTEIN

MDM2 was cloned from transformed mouse cell line 3T3-DM in 1987.[25] It is a 491 amino-acid protein. HDM2 or human MDM2 gene is present on chromosome 12q14.3–q15. The MDM genes are located on small, centromeric extrachromosomal nuclear bodies, called double minutes.[26]

It is an oncoprotein. Moreover, the expression and function are mainly governed at the transcriptional level. It controls the activity and protein levels of p53 in normal cells. Hence, p53 protein is mainly inhibited by MDM2.

p53 is a potent antiproliferative and pro-apoptotic protein so it can harm normal cells. That is why the cellular level of p53 is preciously controlled in normal/unstressed cells by MDM2.

MDM2 is a p53 specific E3 ubiquitin ligase, and the antagonist of p53 protein. It also limits the p53 growth suppressive function in unstressed cells.

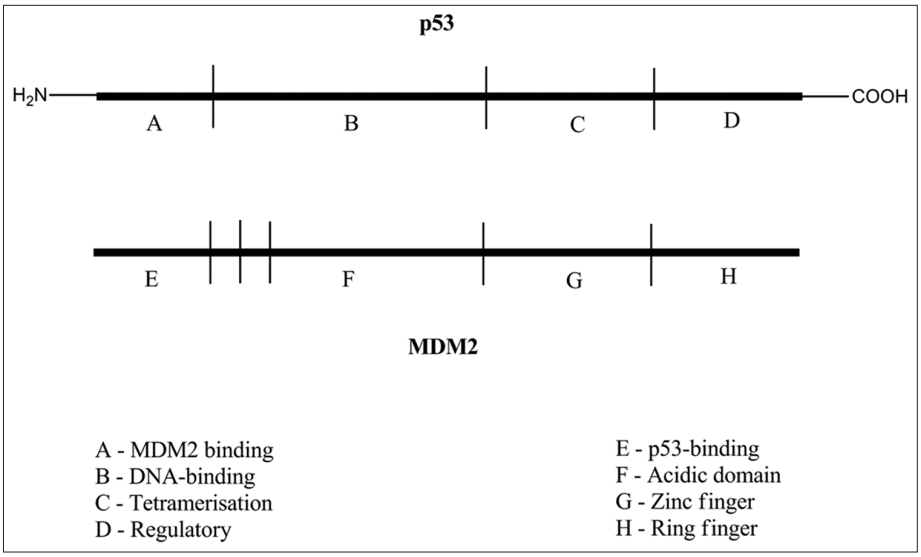

Figure 2: Domain composition of p53 and murine double minute 2 protein[12,13]

Protein-protein interaction (p53-MDM2) maintains the basal level of p53. In many of the cancers, MDM2 is overexpressed (by means of gene amplification).[27]

Domains of MDM2

There are five structural domains present on MDM2. (1) p53 binding domain, (2) p300-binding domain, (3) acidic domain, (4) zinc finger domain, and (5) RING domain.

p53 binding domain of the MDM2 protein can bind with the transactivation domain of p53.[28] MDM2 binds with the alpha-helical transactivation domain near N-terminus of p53 protein. Hence, it is responsible for p53-MDM2 binding.

The MDM2 binding site of p53 partially overlaps with its transactivation domain, and this is why MDM2 effectively inhibits p53 transcriptional activity. MDM2 plays a dual role; it serves as an E3 ubiquitin ligase for p53 and also its binding facilitates p53 proteolysis.[29]

RING (Really Interesting New Gene) domain is important domain of MDM2 protein which is responsible for the E3 ligase activity of the MDM2. This RING domain founds in E3 ubiquitin ligases, and RING domain plays an important role in protein-protein interaction.

Acting as an E3 ubiquitin ligase, MDM2 attaches ubiquitin groups to the carboxy terminus of p53 and induces the ubiquitin-dependent degradation of p53 through 26S proteasome.[30,31] E4 ligase activity of MDM2 is due to its binding with p300/CBP through RING domain, rendering it to poly-ubiquitinate and target the p53 for proteasomal degradation. MDM2 can also be ubiquitinate itself.[32] This RING domain and Zink finger domain provide the ability to MDM2 to uniquitinate and cause proteasomal degradation of its substrates.[33]

In response to the cellular stress, the post-translational modification occurs in MDM2 protein and MDM2 stops the inhibition of p53 protein. Hence, p53 can respond to damage/stress. In the oncogenic stress condition, MDM2 associated with the ARF. In the state of ribosomal stress, MDM2 associated with the ribosomal proteins (L5, L11, and L23),[34-36] and kinases (released by DNA damage) initiates the post-translational modifications of MDM2 and p53.[37,38]

Hence, inhibition of the activity of MDM2 protein is a fruitful strategy to reactivate the p53 function.[39]

P53/MDM2 INTERACTION

p53-MDM2 interaction is responsible for the inhibition of the p53 function, the over-expression of the MDM2 protein also inactivates the p53 protein. p53/MDM2 proteins interact with each other through an auto-regulatory negative feedback loop. Auto-regulatory negative feedback loop maintains the level of both proteins, MDM2, and p53.[40]

p53 binds with the P2 promoter region of MDM2 protein, in an activated condition which induced the expression of MDM2 protein and this MDM2 protein expression inhibits the p53 protein.

The p53/MDM2 interaction involves hydrophobic interaction. Hence, it involves the Van der Waals forces and H-bonds.[41]

Biological and functional studies indicated that 19–102 residues of MDM2 act as the domain for p53 protein binding[42] and 15-amino acid residues peptide of p53 acts as a domain for binding with MDM2.

Binding of MDM2 protein to the transactivation domain of p53 prevents the transcription (activated by p53).

p53 is getting mono-ubiquited by MDM2 (act as an ubiquitin ligase), so nuclear export of p53 occurs and also inhibits the translational activity of p53.[43,44] Ubiquitin-dependent degradation of protein includes three major steps: (1) Ub-activating (E1 enzyme), (2) Ub-conjugating (E2 enzyme), and (3) Ub protein (E3 ligase).

Ubiquitination of p53 by MDM2 causes the export of the p53 to the cytoplasm, from the nucleus so that p53 will no longer be able to bind with DNA and act as a transcriptional factor. This export also causes the proteasomes depended on degradation of p53.[45] RING domain (MDM2) and p53 domain (N-terminal transactivation domain) interaction will result in the proteasome-mediated degradation of p53 [Figure 3].[46,47]

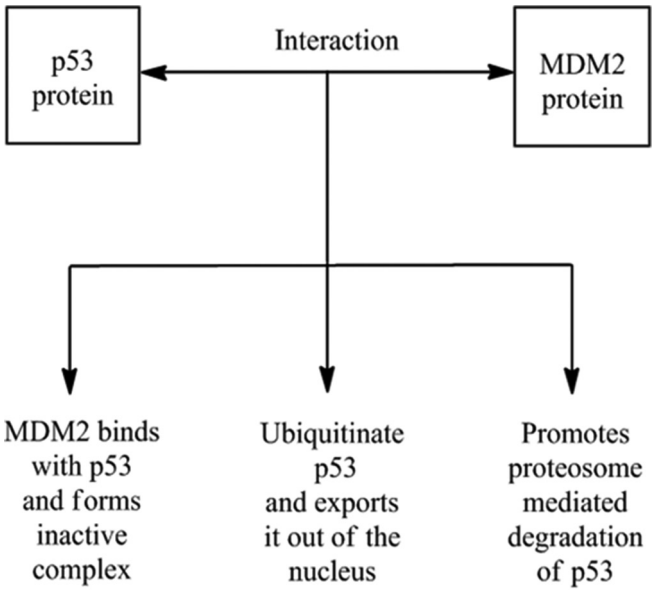

Figure 3: p53/murine double minute 2 interaction[41-45]

Along with the p53, MDM2 can also mono-ubiquitinate histones so that it inhibits transcription and transactivation of p53 targets.[48]

In certain situation like DNA damage, p53 will post-translationally modify to inhibit interactions with MDM2[49] and there are also several kinases present, which phosphorylate MDM2 and modulate its interactions with p53.

It has been found that MDM2 gene has been amplified/Overexpressed in many human cancers.[50] Hence, the suppression of MDM2 expression can activate p53 and inhibits the tumor growth.

The overexpression of MDM2 protein makes tumors less susceptible to natural and chemotherapeutic signals to undergo programmed cell death or apoptosis and will lead to the poor patient prognosis. Hence, disruption of this protein-protein interaction is, therefore, a therapeutic target for the treatment of cancer.

SMALL MOLECULE (NONPEPTIDIC) INHIBITORS OF THE P53-MDM2 INTERACTION

It is very much difficult to target protein–protein interactions by small molecules. Every protein–protein interactions incorporates the large surfaces, so they are very difficult to break by small molecules.[51] Hence, the inhibition of non-enzyme interaction using small molecules is difficult.

Crystal structure of MDM2 bound with the transactivation domain of p53 shows that MDM2 is having a deep hydrophobic pocket, which is filled with the three side chains of the helical region of p53. Hence, the presence of this kind of hydrophobic pocket on the MDM2 has raised the idea that this interaction can be inhibited using small molecules which mimics the three side chains of the p53 helix and can bind with the three pockets of MDM2 [Figure 4].

If we design small molecule (non-peptidic) that can bind with p53 binding pockets of MDM2 (3 pocket binding) will inhibit the p53-MDM2 interaction.[52] Hence, p53 will be liberated, and activation of p53 will occur. Ultimately triggers the apoptosis in cancer cells.

When it comes to p53–MDM2 interaction, fever amino acid residues are crucial for binding/

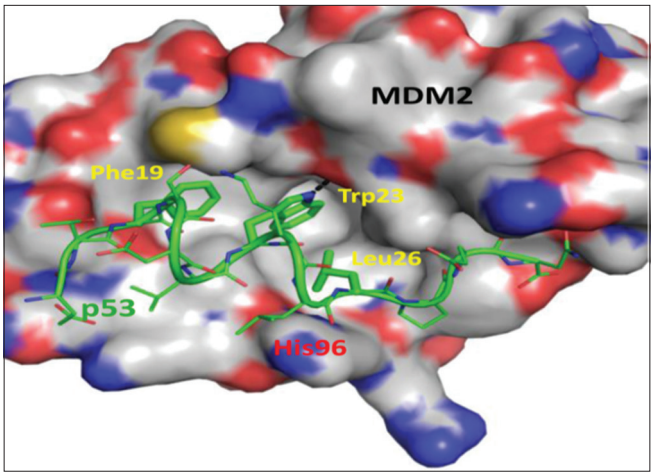

Figure 4: p53 bound with the murine double minute 2 (MDM2) protein (p53-MDM2 Complex)[54]

interaction. Three amino acids of p53 (Phe19, Trp23, and Leu26) are essential for the binding. All three amino acids bind/sits into the hydrophobic pocket of the MDM2 protein. The indole ring (Trp23) of p53 sits into the hydrophobic pocket of MDM2 protein and its NH group forms a hydrogen bond with the backbone carbonyl in MDM2. Therefore, Trp23 is most important for binding of p53 with the MDM2 protein.[53] Trp23 side chain of p53 forms the hydrogen bond as well as having the hydrophobic interactions with MDM2.

In 2004, Vassilev *et al*. at Roche Pharmaceutical reported the compound which inhibits the p53/MDM2 interaction. Later on, that compound was termed as "Nutlin." Hence, the first potent compound, which can inhibit this interaction, was "Nutlins" (cis-imidazoline analogs). Nutlin can displaced p53 from MDM2 (IC_{50} = 100–300 nM).[55] Among the nutlin class of compounds, Nutlin-3 is most potent and again in nutlin-3, one of its enantiomer nutlin-3a is 150 times more active than its other enantiomer Nutlin-3b. The nutlin can mimic the alpha-helical structure of the p53 backbone and having the three pockets binding with MDM2 protein. Superimposition of the 3D structure of Nutlin on the cocrystal structure of MDM2 protein reveals, Trp23 pocket of MDM2 protein has been occupied by bromophenyl ring of the Nutlin. And second bromophenyl ring and ethyl ether side chains sits deeply into the Leu26 and Phe19 pocket of MDM2, respectively.[18] Hence, it is having the three pocket binding [Figure 5].

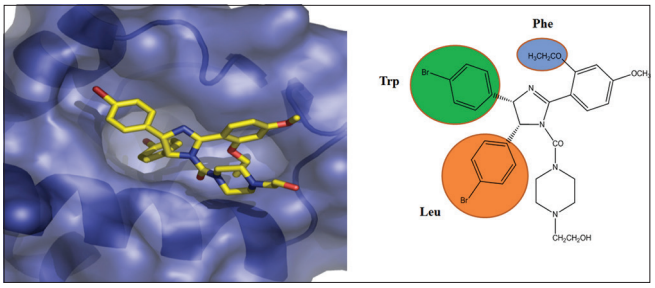

Figure 5: Crystal structure of the nutlin-2 bound murine double minute 2 protein[42]

This study provides the basis for the design of non-peptide, small molecule inhibitors of p53/MDM2 interaction. Recently, we are working on the development of this class of compounds.

Many of the MDM2 inhibitors contain the chlorophenyl substituent; this chlorophenyl substituent mimics the 6-Cl-Trp moiety.

To design potent p53-MDM2 interaction inhibitors, it should produce the following effects:

1. Stabilization and accumulation of p53 protein (By preventing its degradation and its export from the nucleus).
2. Activation of p53 pathway and various genes, which regulates p53 function (Induce cell cycle arrest in the G1and G2 phases and/or apoptosis).

Fortunately, data from various studies indicate that p53 gets accumulated in the nuclei of cancer cells after liberating from MDM2 and activates p53 target genes and the p53 pathways. Moreover, the proliferating cancer cells were effectively blocked in G1 and G2 phases (G2/M phase fraction and nearly complete depletion of the S-phase),[55] which subsequently undergoes to the apoptosis/cell cycle arrest (only cells which contain wild type p53).

However, clinical data evident that cell cycle arrest and/or apoptosis by MDM2 inhibitor have only occurred in cells with wild-type p53 and not in mutant or deleted p53.[56] With contrary to this, the low cytotoxicity of MDM2 inhibitors results in the cells, which contain the mutant p53 protein.

The plus point of non-peptide small molecule MDM2 inhibitors over peptide-based inhibitors is that they have greater cell permeability.

Although many of the MDM2 inhibitors are at the upper limits of a molecular weight cut-off (Lipinski's rule of five), they proved to have drug-like properties.[57]

Since from the discovery of Nutlin, a number of p53-MDM2 interaction inhibitors have been reported by various researchers and companies, which includes the compounds having several different chemical classes including benzodiazepinediones, cis-imidazolines, oxindoles, spirooxindoles, chalcones, chlorofusin (natural product), tryptamine, and other numerous miscellaneous groups.

In this review, we have covers the details of the non-peptide small molecule p53-MDM2 interaction inhibitors which are under clinical trials and we have also included the structures of respective compounds [Table 1 and Figure 6].

Table 1: MDM2 inhibitors under clinical trials[58-62]

Compound	Phase of clinical trial	Status	Originator
RG7112 (RO5045337)	1	Active	Hoffmann-La Roche
RG7388 (RO5503781) (Idasanutlin)	1	Active	Hoffmann-La Roche
MI-77301 (SAR405838)	1	Active	University of Michigan, advanced into clinical trials by Sanofi in 2012
MK-8242 (SCH 900242)	1	Active	Merck
AMG232	1	Active	Amgen biopharma
DS-3032b	1	Active	Daiichi Sankyo
HDM201	1	Active	Novartis
CGM097	1	Active	Novartis
ALRN-6924 (stapled peptides)	1	Active	Aileron therapeutics and Roche

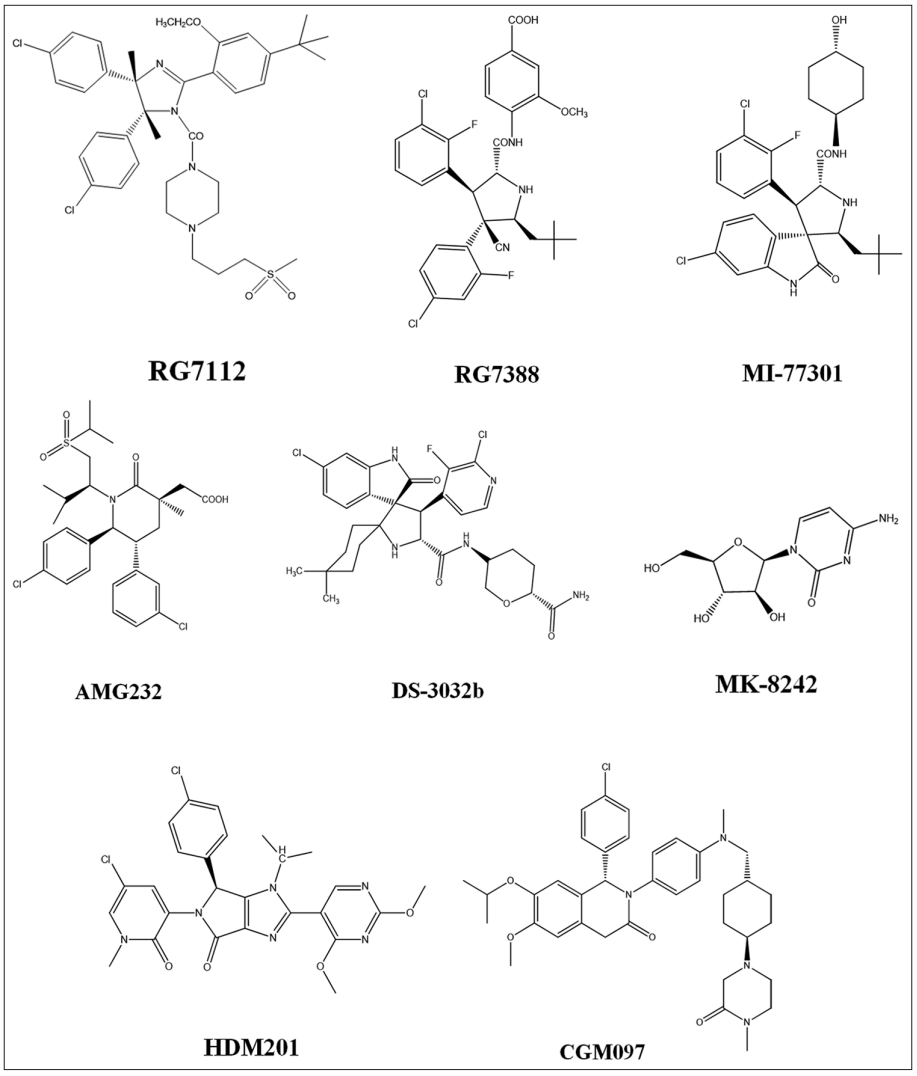

Figure 6: Structures of the murine double minute 2 inhibitors under Clinical trials[54,59-63]

The IC_{50} values of the MDM2 inhibitors which are under clinical trials are as follow:[54,63] (1) RG7112 (RO5045337) – 18 nM, (2) RG7388 (RO5503781) – 6 nM, (3) MI-77301 (SAR405838) – 100 to 200 nM, Cell lines – SJSA-1, RS411, LNCaP, HCT116, (4) MK-8242 – 20 nM, (5) AMG232 – 0.6 nM, (6) DS-3032b– 5.57 nM, (7) HDM201– 0.21 nM, and (8) CGM097– 1.7 nM.

Since last many years, the substantial efforts have been invested for this approach. Which yield some of the promising compounds of this class and some of the compounds have been successfully entered into the clinical trials.

APPLICATION OF p53/MDM2 INTERACTION INHIBITORS (MDM2 INHIBITORS) IN CANCER THERAPY

The conventional anti-neoplastic drugs are genotoxic in nature. Hence, it has activated the p53 and induced the apoptosis by phosphorylation of the protein on specific serine residues (Ser15 is phosphorylated most frequently) near the MDM2 binding domain. While MDM2 inhibitors can activate the p53/apoptosis and it has not been required the phosphorylation of the protein.[56] Hence p53/MDM2 interaction inhibitors are nongenotoxic.

p53 is a tumor suppressor protein so activation of p53 in cancer cells would induce the apoptosis in cancer cells.

Its application in cancer therapy can be achieved through various strategies such as (i) inhibiting MDM2 expression, (ii) inhibiting the p53-MDM2 interaction, (iii) modulating the E3 ubiquitin ligase activity of MDM2, and (iv) targeting the MDM2-p53 complex.

Among these strategies, the p53/MDM2 interaction inhibition is most promising and most studied.

Clinical experiments show that in the cancer cell lines, activation of p53 by MDM2 inhibitors induces p53 dependent cell cycle arrest, and cell death. (In cells with wild-type p53, but not in cells with deleted or mutated p53). Whereas in normal cells, p53 activation by MDM2 inhibitors leads to cell cycle arrest, but not cell death. Hence, p53 activation by MDM2 inhibitors might be selectively target the cancer cells only.

Other approach to minimize the side effects to normal cells is cyclotherapy. Cyclotherapy is a conditioning treatment with low doses of p53 activators to induce cell cycle arrest in normal cells with a cytostatic effect that will be protected from the toxicity of conventional drugs targeting the S/M phases of the cell cycle.

MDM2 inhibitors have a good anti-tumor activity to *in vitro* and *in vivo* too. However, the mutated p53 cannot be targeted and affected by this because mutated p53 protein cannot activate its negative regulator (MDM2 protein) and disruption of p53–MDM2 binding.

TP53 gene is either mutated or deleted in the cancer cells. Hence, it will be non-effective as a transcription factor.[64] Clinically, with the help of DNA microarray-based GeneChip, we can determine the mutant p53 protein.[65]

Clinical development of p53-MDM2 inhibitors

The first compound to enter into the clinical trials was RG7112 (R7112, RO5045337) RG7112 (R7112, RO5045337).

It was discovered and developed by the Hoffmann-La Roche pharmaceuticals and it is a nutlins (cis-imidazoline) class of compound.[58] It was first p53-MDM2 interaction inhibitors, which enter into the clinical trials. It is an orally administered agent. It inhibits p53/MDM2 interaction and inhibits the proteasomal degradation of p53. Hence, p53 getting stabilized and increased its amount.

Apart from MDM2 inhibitors, other applications of small molecules are as follow.

Discovery of ubiquitin ligase inhibitors

According to the latest studies, small molecules containing benzenesulfonamides, ureas, and imidazolone moieties, inhibit MDM2 depended on ubiquitylation of p53.[66] Apart from this, 5-deazaflavin compound can inhibit the MDM2 auto-ubiquitylation and induces the p53 activation.[42]

Small molecule activators of mutant p53

Some of the small molecules have been come across during the chemical library screening, which is having the anti-neoplastic activity by restoring the DNA-binding activity of a mutant p53 protein.[67]

DISCUSSION

MDM2 inhibitors cause the activation of p53 and leads to trigger the apoptosis in the cancer cells. It is a promising approach for the treatment of the cancers which are having low levels of TP53 mutations. From various studies and experiments, it is known that activation p53 function might be offered an effective therapeutic strategy for tumors that retain the wild-type p53 and in which the downstream p53 dependent apoptotic signaling is preserved. The major limitation of this class of compounds is that they cannot able to reactivate the p53 function in cancer cells which contains the mutated or deleted p53, so a strategy for reactivation of the p53 pathway in this context is desirable. Various studies and entry of some of the small molecule MDM2 inhibitors into clinical trials represent a significant advancement for the target. Alongside, improve bioavailability and optimized PK profile of small molecule MDM2 inhibitors have required further investigation.

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