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Identification and structure–activity relationship studies of 3-methylene-2-norbornanone as potent anti-proliferative agents presumably working through p53 mediated apoptosis

N. Laxma Reddy,* Jeanette Hill, Long Ye, Prabhavathi B. Fernandes† and David M. Stout

Ricerca Biosciences, 7528 Auburn Road, Concord, OH 44077, USA

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Abstract—We have identified a novel series of α-methylene carbonyl compounds through structure–activity relationship (SAR) studies with high levels of anti-proliferative activities. The lead molecule, 3-methylene-2-norbornanone (**3**) showed potent activity (LC_{50} = 3–8 μM) against mutant p53 cell types and many fold selectivity (>13–29) over wild-type p53 cells. Further, compound **3** and its analogs showed refolding of mutant p53 protein comparable to their anti-proliferative activities suggesting possible interaction with mutant p53 protein.

1. Introduction

One of the most promising, but challenging approaches for the treatment of cancer involves the selective induction of apoptosis in tumor cells. It is well recognized that one of the causes of cancer is the perturbation of the intricate balance (homeostasis) between cell growth and cell death and that the tumor suppressor gene, TP53, is intimately connected with this process. The mutation of the TP53 gene is linked to the proliferation of various types of tumors, such as colon, glioblastoma, breast, lung, and osteosarcoma. The gene product of TP53 is the tumor suppressor protein p53 (also referred to as 'wild-type' p53), which is directly involved in inducing apoptotic cell death in response to various stress signals encountered during the development and progression of malignant tumors.[1] In addition to having tumor suppression properties, p53 is also implicated in neurodegenerative disorders.[2]

The activity of p53 protein is dependent not only on its concentration and stability, but also on the maintenance of its intact native conformation. The half-life of p53 is very short thus its concentration must be strictly regulated because too little p53 will cause tumor proliferation and too much p53 will result in death of normal cells. Furthermore, even a single point mutation in TP53 causes considerable conformational change in the resulting protein, thereby rendering this 'mutant p53' protein inactive toward the induction of apoptosis. Indeed, more than half of all human cancer tumor cells are known to express high levels of mutant p53. The mutant p53 acts as a dominant negative inhibitor of wild-type p53, which functions as a tetramer.[3] In addition, some of the tumor-associated mutant p53 also acquires new transforming functions that contribute to tumor development.[4] The activity of wild-type p53 is also dependent on the level of MDM2 protein that destroys p53.[5] MDM2 is essential for normal cell proliferation, otherwise an uncontrolled level of wild-type p53 causes apoptosis of normal cells.

There has been considerable effort to develop small molecules that either enhance the stability of wild-type p53 or restore the function of mutant p53. Recently, it was reported (Fig. 1) that an amino pyrimidine derivative (**1**) stabilizes wild-type p53 and restores the active conformation to mutant p53.[6] More recently, it was shown that a quinuclidinone derivative (**2**) and certain maleimide derivatives, identified in screening a library of low-molecular weight compounds, restores the DNA binding activity of mutant p53 in tumor cells, reportedly through restoration of the active conformation to

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* Corresponding author. Tel.: +1 440 357 3369; fax: +1 440 354 6276; e-mail: reddy_l@ricerca.com
† Present address: DarPharma, Inc., 215 Cloister Ct., Chapel Hill, NC 27514, USA.

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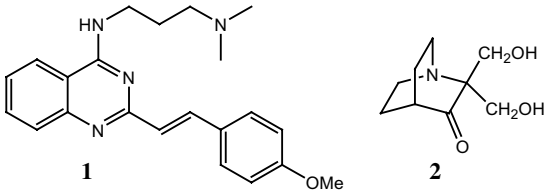

Figure 1.

mutant p53.[7] Compound **2** also induced apoptosis in a range of cell lines containing mutations in the p53 DNA binding domain, but was less toxic to cells containing wild-type p53. It has also been shown that 2-methoxy estradiol facilitates wild-type p53-mediated apoptosis.[8] The effectiveness of known compounds that target mutant p53 is sub-optimal, as compound **1** stabilizes only the newly synthesized p53 protein, while compound **2** does not reactivate all the known mutant p53 proteins. Thus, there continues to exist the need to develop novel small molecules that increase the concentration of wild-type p53 in tumors or restores the activity of mutant p53 or prevents the degradation of wild-type p53, or any combination of these processes. Therefore, we have undertaken a study to screen small-molecule compound libraries against mutant and wild-type p53 cell based anti-proliferative assays to identity novel chemical entities.

All compounds were tested for anti-proliferative activity (Tables 1 and 2) using two cell lines with single point mutations in the DNA binding region: C33A (human cervical cells with a mutation at Cys 273) and Ramos RA1 (human lymphocyte with a mutation at Asp 254). Caki-1, human renal carcinoma cells with wild-type p53, were used as a negative control. The cells were plated at sub-confluent levels (1×10^5 cells/mL) in RPMI media with 10% fbs and incubated with the compounds at concentrations between 0.001 and 100 μg/mL for 72 h at 37 °C, 5% CO_2. The number of viable cells was measured by mitochondrial reduction of Alamar Blue to a fluorescent dye. LC_{50} values for each compound were determined and the activity for mutant versus wild-type p53 was compared. Compound **2** was used as a positive control and vehicle as a negative control. Desirable results of a compound would have low LC_{50} concentration (sub-μM) and high selectivity for mutant p53 cells.

Several simple α-methylene carbonyl-containing compounds were identified in a primary-round of anti-proliferative assays. One of the molecules, 3-methylene-2-norbornanone (**3**) showed potent activity and selectivity for mutant p53 cell types over wild-type p53. It was reported earlier that natural products containing sesquiterpene lactones or compounds containing the α-methylene carbonyl group are cytotoxic and potential anti-tumor agents.[9] These reports supported our findings and led us to investigate further the structure–activity studies based on 3-methylene-2-norbornanone (**3**).

In a first attempt to understand the relative importance of each of the functional group of compound **3** for anti-proliferative activity, we have prepared several direct analogs (**4**, **5**, **6**) of compound **3** through simple chemical transformations and/or obtained compounds from commercial sources (Fig. 2).[10] For example, compounds **4** and **5** were obtained commercially, while compound **6** was prepared as mixture of *exo* and *endo* isomers from reduction of **3** with $NaBH_4$ in ethanol. Compound **7** was obtained as a mixture of *E* and *Z* isomers by direct condensation of **3** with hydroxylamine hydrochloride. Compounds **8**–**11** were prepared by reacting the corresponding tetralones and indanones with formalin/acetic acid followed by diethyl amine at 100 °C.[11] Similarly, compound **14** and camphor analogs **15** and **16** were prepared from commercially available tricyclodecane-8-one and D- and L-camphor, respectively. Compound **17** resulted during the introduction of an α-methylene group

Table 1. Structure–activity relationships of 3-methylene-2-norbornanone

	LC_{50} (μM)[a]			Selective activity against mutant p53 (fold increase)[e]	
Compound #	Caki-1[b]	C33A[c]	Ramos RA1[d]	Caki/C33A	Caki/Ramos RA1
1	9.3	13.4	14.3	0.69	0.65
2	162	25.3	17.2	6.40	9.42
3	109	3.67	8.28	29.70	13.16
4[f]	>618	>618	>618	1.0	1.0
5[f]	>924	>924	>924	1.0	1.0
6	805	206	805	3.90	1.0
7	>728	>728	>728	1.0	1.0
8	30.6	27.7	12.9	1.10	2.37
9	16.2	24.8	1.80	0.65	9.0
10	15.9	22.8	4.9	0.70	3.24
11	30.5	21.1	3.2	1.45	9.53
12[f]	55.4	49.2	39.3	1.13	1.41
13[f]	657	359	509	1.83	1.29

[a] Concentration required to kill 50% of the cells.
[b] Caki-1 cells are human renal carcinoma with wt p53 and are used as a negative control.
[c] C33A are human cervical cells with a mutation at Cys 273.
[d] Ramos RA1 are human lymphocyte with a mutation at Asp 254.
[e] Values are means of three experiments.
[f] Obtained compounds commerciallly.

Table 2. Structure–activity relationships of 3-methylene-2-norbornanone

	LC_{50} (μM)[a]			Selective activity against mutant p53 (fold increase)[e]	
Compound #	Caki-1[b]	C33A[c]	Ramos RA1[d]	Caki/C33A	Caki/Ramos RA1
3	109	3.67	8.28	29.7	13.16
14	271	26.7	35.0	10.15	7.74
15	609	185	609	3.29	1.0
16	609	609	609	1.0	1.0
17	15.9	2.5	2.1	6.36	7.57
18	515	447	353	1.15	1.46
19	36.0	23.4	26.4	1.54	1.36
20	171	21.2	21.5	8.07	7.95
21	108	15.2	14.3	7.11	7.55
22	120	16.4	15.9	7.31	7.55
23	334	171	157	1.95	2.13
24	114	13.4	12.7	8.51	8.98
25	190	98.2	69.9	1.93	2.72

[a] Concentration required to kill 50% of the cells.
[b] Caki-1 cells are human renal carcinoma with wt p53 and are used as a negative control.
[c] C33A are human cervical cells with a mutation at Cys 273.
[d] Ramos RA1 are human lymphocyte with a mutation at Asp 254.
[e] Values are means of three experiments.

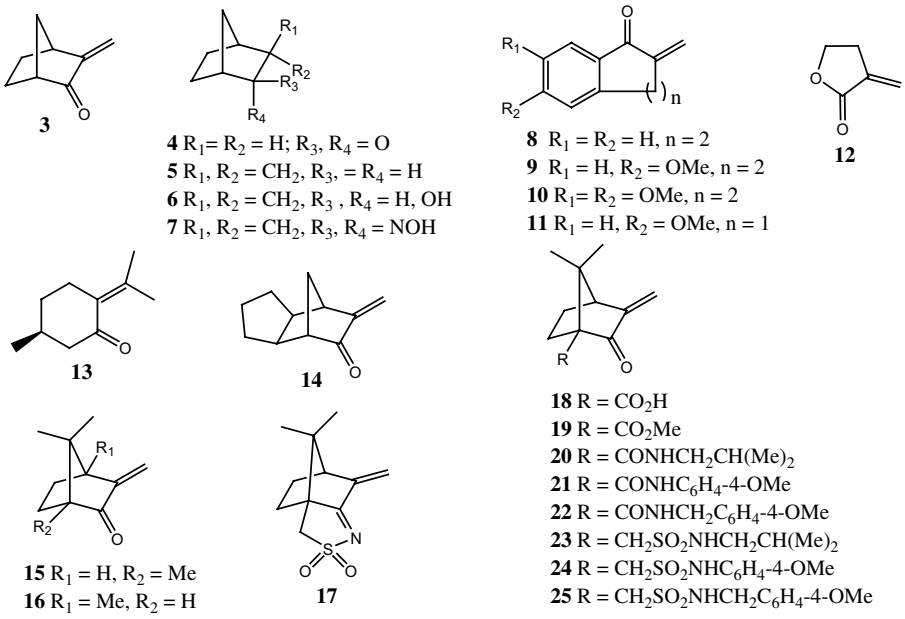

Figure 2.

on camphor sulfonamide. Several compounds analogous to **3** (**18**–**25**) were prepared using either ketopinic acid or camphor sulfonyl chloride as starting materials. Ketopinic acid was first converted to the acid chloride ($SOCl_2$) before condensation with the appropriate amines. The resulting amides/sulfonamides were finally converted to the α-methylene analogs **18**–**25** using the above reaction method.

Initially we focused on the relative importance of the methylene and ketone functionalities of compound **3** for anti-proliferative activity (Table 1). Simple norbornanone (**4**) or 2-methylene norbornane (**5**) were found to be far less active and selective compared to **3**, indicating that both functional groups are required for retaining biological activity. This observation was further supported when the alcohol (**6**) and oxime (**7**) were found to be less active and selective compared to compound **3**. The loss of activity and selectivity of these compounds for mutant and wild-type p53 indicate the importance of the α-methylene carbonyl functionality for retaining biological activity.

Next we focused on modifying the carbocyclic part of compound **3** while keeping the α-methylene carbonyl moiety intact. First we investigated the extension of the carbocyclic frame with an aromatic ring and also eliminating the methylene bridge. The addition of an aromatic ring would allow us to introduce additional groups on to it for optimization studies and further it

may improve the physico-chemical properties of the compounds as 3-methylene-2-norbornanone (**3**) is a volatile liquid and difficult to handle in the biological assays. The first compound of this series, an α-methylene-1-tetralone (**8**), showed moderate activity and selectivity against mutant p53 cell types over wild-type p53 (Table 1). The 6-methoxy analog (**9**) showed improved activity and selectivity for mutant p53 cell types and more importantly showed a >10-fold improvement in activity for mutant p53 (Ramos-RA-1) cell types compared to parent tetralone (**8**). However, the 6,7-dimethoxy analog (**10**) showed decreased selectivity in spite of retaining activity for mutant p53 cell types. In order to explore the effect of the ring size on activity and selectivity for mutant p53 cell types, we have tested the 5-methoxy-α-methylene-1-indanone (**11**). Its activity and selectivity for mutant p53 cell types was found to be comparable to the homologue, compound **9**. As earlier reports indicate that α-methylene carbocyclic ketones and lactones are cytotoxic and potential anti-cancer agents, we tested compounds **12** and **13** to see whether they showed any anti-proliferative activity.[9] Biological data for these compounds show reduced activity and selectivity for mutant p53 cell lines compared to compound **3**.

Next we turned our focus to direct modifications to the norbornanone carbocyclic frame while keeping the α-methylene carbonyl moiety intact. Extension of the carbocyclic frame with the cyclopentane ring (**14**) did not significantly affect its anti-proliferative activity for mutant p53 cell types, while the α-methylene analogs of camphor (D and L; **15**, **16**) showed poor activity and selectivity for mutant p53 cells. However, the excellent activity and selectivity of the cyclic sulfonamide (**17**) for mutant p53 cell lines indicate that the carbocyclic frame will tolerate certain types of substitutions. This result led us to investigate further the SAR of this particular chemical series.

We have first tested the α-methylene analogs of simple acid, the ketopinic acid (**18**) and its methyl ester (**19**) (Table 2). The acid analog showed poor activity and selectivity, however the methyl ester showed about 8-fold and 23-fold improved activity for mutant p53 C33A and Ramos-RA-1 cell lines, respectively, compared to the parent camphor analog (**15**). We next investigated several amide (**20**, **21**, **22**) and sulfonamide (**23**, **24**, **25**) analogs of α-methylene camphor. In general these compounds showed good activity and selectivity against mutant p53 cell lines over wild-type p53. In particular, the 4-methoxyaniline analogs of ketopinic and camphor sulfonic acids (**21** and **24**) showed excellent activity and selectivity for the mutant p53 cell types. The first lead compound of this series, compound **3** was further investigated in biochemical, metabolic, and toxicological assays.

Refolding of mutant p53 into the active conformation and a corresponding loss of misfolded protein is the likely mechanism of compound **3** and derivatives. To measure the amount of misfolded p53 protein, Ramos cells were treated for 4 h with compounds at 100 μM and mutant p53 protein was measured by Mutant p53 ELISA (Oncogene, Boston, MA) according to the manufacturers instructions; this ELISA utilizes an antibody that is specific for the common conformation of misfolded p53 with mutations in the DNA binding region. The antibody does not recognize wild-type p53. Compound **2** was used as a positive control and vehicle and compound **19**, a partially active derivative, as a negative control. The results are expressed as percent loss of mutant p53 content/total protein content (Fig. 3) and show a correlation between loss of mutant p53 protein and anti-proliferation activity.

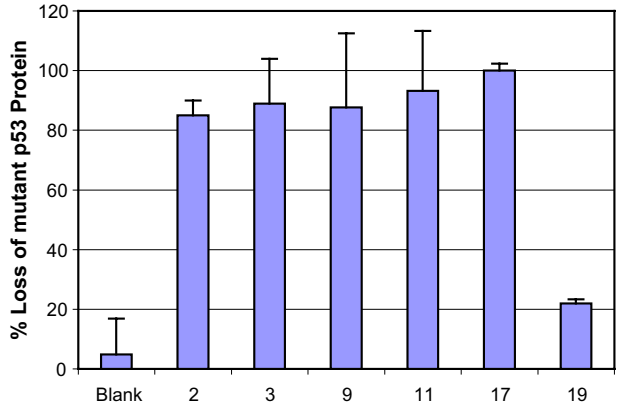

Figure 3.

As compounds containing α-methylene-γ-lactones or 2-cyclopentenone are known to react with thiol-rich proteins,[12] the whole cell glutathione content was measured by monochlorobimane.[9] Briefly, plated cells were incubated for 4 h with compounds or vehicle. The cells were washed with PBS and incubated at 37 °C for 30 min in the dark with 2 mM monochlorobimane (Sigma). The fluorescence resulting from reduced glutathione reaction with monochlorobimane was measured at ex 390 nm, em 520 nm. An IC_{50} value was calculated for compounds exhibiting a decrease in glutathione content. Compound **3** decreased the glutathione content in mutant p53 C33A cells at an IC_{50} concentration approximately 10 times higher than for anti-proliferation activity. For non-mutant p53 Caki-1 cells, it inhibited glutathione content at approximately the same concentration as anti-proliferation activity indicating that the anti-proliferative activity for mutant p53 cells observed for compound **3** is not due to interaction with glutathione. Compound **3** was incubated with WI-38 human fibroblast (non-cancer) cells under conditions similar to the anti-proliferation assay. The calculated LC_{50} for inhibition of WI-38 growth was approximately 17 and 12 times higher than for inhibition of mutant p53 cells Ramos and C33A, respectively, suggesting that toxicity is selective for cancer cells with mutated p53.

2. Conclusions

We have identified a novel series of small molecules based on a structure–activity relationship (SAR) study of 3-

methylene-2-norbornanone (**3**). This series showed potent activity and selectivity for mutant p53 cell types in anti-proliferation assays and specific interaction to mutant p53 protein refolding in an ELISA assay. Further, several compounds of this series showed desirable metabolic and tox profiles and are currently being investigated in additional biological studies including animal models.

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