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Original article

5-Benzylidene-hydantoins: Synthesis and antiproliferative activity on A549 lung cancer cell line

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ABSTRACT

Benzylidene hydantoins have been recently reported as a new class of EGFR inhibitors. We describe here a simple and efficient methodology for the parallel solution-phase synthesis of a library of 5-benzylidene hydantoins, which were evaluated for antiproliferative activity on the human lung adenocarcinoma A549 cell line. Various substituents at positions 1, 3 and 5 on the hydantoin nucleus were examined. In the presence of a 5-benzylidene group and of a lipophilic substituent at position 1, most of the tested compounds inhibited cell proliferation at a concentration of 20 μM. Compound **7** (UPR1024), bearing 1-phenethyl and (*E*)-5-*p*-OH-benzylidene substituents, was found to be the most active derivative of the series. It inhibited EGFR autophosphorylation and induced DNA damage in A549 cells. Compound **7** and other synthesized 5-benzylidene hydantoin derivatives increased p53 levels, suggesting that the dual mechanism of action was a common feature shared by compound **7** and other member of the series.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most frequent lung cancer in humans and is usually associated with poor prognosis [1]. Since only a minority of NSCLC patients is suitable for radical treatment with curative intent, the availability of new cytotoxic drugs and novel therapeutic strategies to improve the prognosis of lung cancer are urgently needed.

Epidermal growth factor receptor (EGFR) plays a central role in signal transduction pathways, regulating cell division and differentiation, and it is aberrantly activated in several epithelial solid tumors, including NSCLC. Small molecule EGFR tyrosine kinase inhibitors, such as gefitinib (Iressa™) and erlotinib (Tarceva™), are currently in clinical use or under development for the treatment of NSCLC (Fig. 1).

We have recently reported preliminary results on the antiproliferative action and the inhibition of EGFR kinase activity by a series of 1,5-disubstituted hydantoins [2]. These compounds were designed in view of the known interactions between 4-anilinoquinazolines, potent EGFR inhibitors, and the adenine-binding portion of the ATP-binding site of the receptor [3–5]. Molecular modeling showed that hydantoin could mimic the interactions of the quinazoline or 3-cyanoquinoline scaffolds with the hinge region of the EGFR ATP-binding site, accommodating an aromatic group at position C5 within the lipophilic pocket occupied by the 4-anilino one in the EGFR-erlotinib co-crystal. To improve the superposition on the 4-anilinoquinazoline ring, a rigid structural element, conferring planarity to the substituted hydantoin nucleus, was introduced. The resulting 5-benzylidene hydantoins both inhibited the EGFR kinase and exhibited an antiproliferative action on A431 human epidermoid carcinoma cells. The conjugated *exo*-cyclic double bond at the C5 position appeared essential for both EGFR inhibition and cell growth inhibition, indicating that the 5-benzylidene hydantoin core would be a suitable scaffold to generate new antiproliferative compounds [2]. Pharmacological characterization of the most active compound of the previous series (*E*)-5-(*p*-OH-benzylidene)-1-phenethyl hydantoin (**7**, UPR1024, Fig. 1), showed that this derivative also induced DNA damage, with up-regulation of p53 and S phase cell cycle arrest in the A549 cell line [6]. These observations contrast with the action of gefitinib in the same cell line, which is known to induce G_1 arrest without any changes in p53 expression (Table 1) [7,8].

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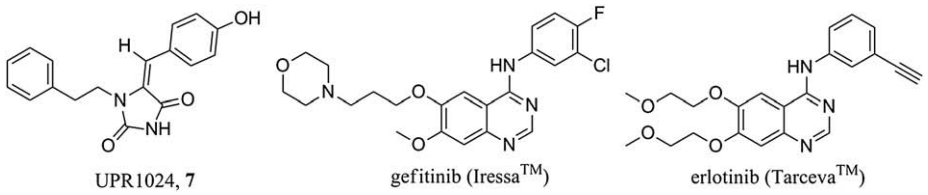
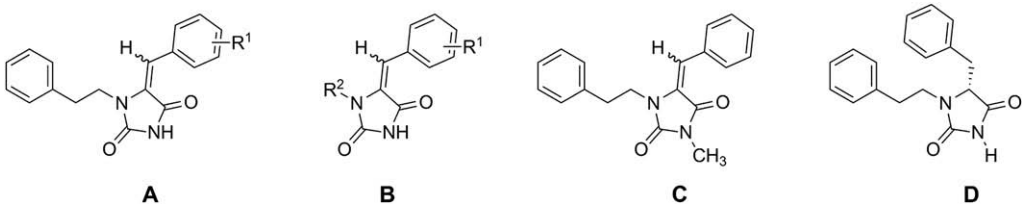


Fig. 1. EGFR TK inhibitors: UPR1024 (compound **7**), gefitinib (Iressa™) and erlotinib (Tarceva™).

These findings suggested that compound **7** exerted its antiproliferative activity by a dual mechanism of action potentially dependent on the presence of the conjugated *exo*-cyclic double bond, conferring on the hydantoin nucleus the ability to both interact with the EGFR active site and to alkylate bionucleophiles. This hypothesis was supported by the known cytotoxic antitumor activity of various natural products having an α,β-unsaturated carbonyl system substructure (e.g. helenalin, tenulin, and acronycine derivatives) [9–11].

In the present study, in a search for more potent antiproliferative derivatives and to find out whether the dual action of compound **7** (e.g. DNA damage induction and EGFR inhibition) was a unique characteristic of this compound or whether it was a common feature shared by other members of the series, we extended our exploration of the substituent space on the hydantoin ring. We devised a versatile route to the substituted 5-*exo*-methylene hydantoins, introducing different substituents at N1, N3 and C5 positions. Combining the advantages of solution-phase parallel synthesis and microwave irradiation, a simple and efficient methodology was developed and a small library of benzylidene hydantoin derivatives was synthesized in good yield and purity.

Table 1
Biological activity for compounds **1**–**30**.



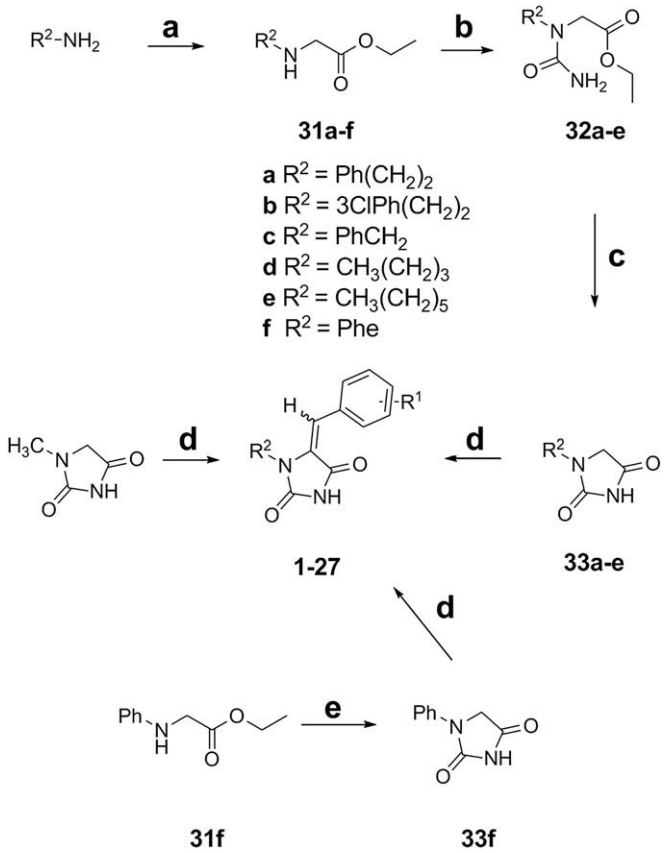
no.	Type	R^1	R^2	Isomer	A549 cells proliferation, % Inhibition (20 μM)[a]	A549 cells, p53 fold increased (20 μM, 48 h)[b]
1	A	H		*E*	9.4 ± 5.2	1.2 ± 0.2
2	A	H		*Z*	42 ± 6	1.3 ± 0.3
3	A	3-Cl		*E*	35 ± 7	n.d.
4	A	3-Cl		*Z*	43 ± 7	1.2 ± 0.2
5	A	3-OH		*E*	28 ± 8	0.9 ± 0.1
6	A	3-OH		*Z*	40 ± 5	1.1 ± 0.3
7	A	4-OH		*E*	53 ± 7	2.3 ± 0.2**
8	A	4-OH		*Z*	44 ± 5	1.5 ± 0.1
9	A	4-OCH_3		*E*	35 ± 6	1.0 ± 0.1
10	A	4-$NHCOCH_3$		*E*	39 ± 7	1.7 ± 0.2*
11	A	4-$NHCOCH_3$		*Z*	32 ± 4	n.d.
12	B	H	3-Cl$Ph(CH_2)_2$	*E*	10 ± 7	n.d.
13	B	H	3-Cl$Ph(CH_2)_2$	*Z*	49 ± 3	1.5 ± 0.2
14	B	H	$PhCH_2$	*E*	2.9 ± 3.4	n.d.
15	B	H	$PhCH_2$	*Z*	42 ± 9	1.7 ± 0.2*
16	B	4-OH	$PhCH_2$	*E*	41 ± 14	1.8 ± 0.1**
17	B	4-OH	$PhCH_2$	*Z*	32 ± 7	2.0 ± 0.2**
18	B	H	$CH_3(CH_2)_3$	*E*	6.8 ± 4.4	n.d.
19	B	H	$CH_3(CH_2)_3$	*Z*	18 ± 3	n.d.
20	B	4-Cl	$CH_3(CH_2)_3$	*E*	29 ± 4	n.d.
21	B	4-Cl	$CH_3(CH_2)_3$	*Z*	26 ± 6	n.d.
22	B	H	Phe	*E*	18 ± 5	n.d.
23	B	H	Phe	*Z*	26 ± 8	n.d.
24	B	H	$CH_3(CH_2)_5$	*E*	14 ± 3	n.d.
25	B	H	$CH_3(CH_2)_5$	*Z*	0.0 ± 1.0	n.d.
26	B	H	CH_3	*E*	5.7 ± 8.4	1.1 ± 0.1
27	B	H	CH_3	*Z*	16 ± 9	1.0 ± 0.1
28	C			*E*	31 ± 6	1.0 ± 0.1
29	C			*Z*	45 ± 13	1.0 ± 0.2
30	D				7.2 ± 5.6	1.0 ± 0.1
Gefitinib					63 ± 6[c]	1.0 ± 0.0

*$P < 0.05$; **$P < 0.01$ vs. 1; $n = 3$.
[a] Percent inhibition of cell proliferation at 20 μM; mean values of three independent experiments ± SD are reported.
[b] p53 protein level expressed as fold increase respect to the control; mean values of three independent experiments ± SD are reported. One-tail Student's *t*-test.
[c] gefitinib showed IC_{50} 7.0 μM when tested on A549 cell proliferation.

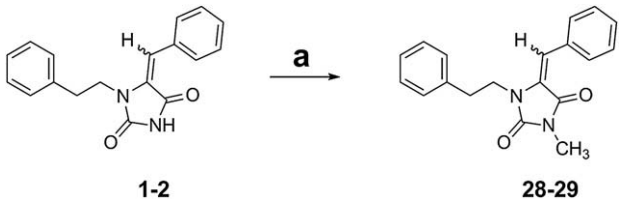
The antiproliferative activity of the synthesized compounds was assayed on the human NSCLC cell line A549, which responds to EGFR tyrosine kinase inhibitors such as erlotinib. Increased levels of the p53 protein have been reported as biological markers of DNA damage, and are known to lead to cell cycle arrest and apoptosis [12,13]. Therefore, to identify additional antiproliferative mechanisms of action, the effect of the newly synthesized compounds on the expression of the transcription factor p53 was also evaluated in A549 cells.

2. Chemistry

The small library of 5-*exo*-methylene hydantoin derivatives **1**–**27** was synthesized in parallel, according to the pathway described in Scheme 1. The four-step procedure proved efficient for hydantoin derivatives with different N1 and C5 substituents. The *N*-substituted glycine ethylesters **31** were submitted to acid-catalyzed cyclization to hydantoins **33**, *via* the urea intermediates **32**. Knoevenagel condensation between 1-substituted hydantoins **33** and benzaldehydes in piperidine was conducted under microwave irradiation at 130 °C for 5 min. Products **1**–**27** were produced as mixtures of *E*/*Z* isomers in 4:1 to 1:1 proportions. After purification of the isomers by silica gel chromatography, the geometry of the *exo*-cyclic double bond was determined by ^{1}H NMR spectral analysis (see Experimental section). Finally, alkylation of hydantoins **1** and **2** with methyl iodide gave compounds **28**, **29** (Scheme 2).



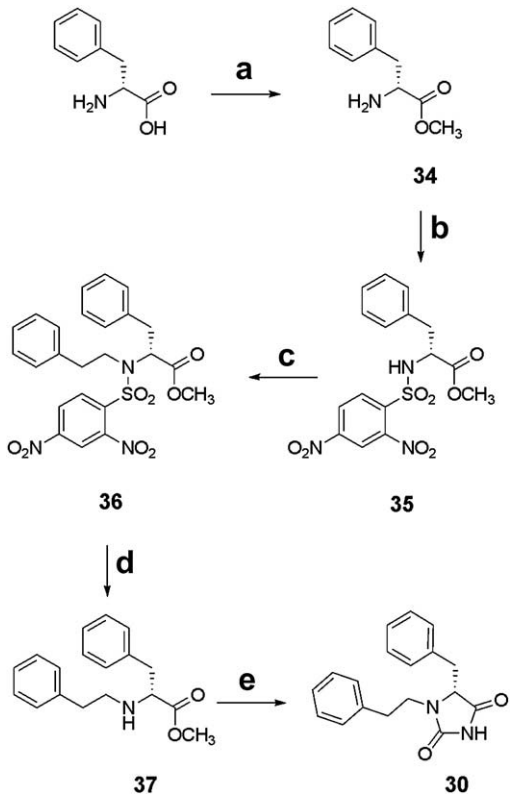
Scheme 1. Reagents and conditions: (a) R^2NH_2 (2 eq), $BrCH_2COOEt$ (1 eq), anhydrous $CHCl_3$, rt, 2 h, 94–97%; (b) HCl (1.5 eq), KCNO (1.5 eq), water, rt, 20 h, 84–89%; (c) HCl 25%, reflux, 4 h, 99%; (d) substituted benzaldehyde (1 eq), dry piperidine, 130 °C, μW, 5 min, 40–80%; (e) i. 0.1% CH_3COOH, 60 °C, 30 min, KCNO (1 eq), water, reflux, 1.5 h; ii. conc. HCl, 90 °C, 16 h, 40%.



Scheme 2. Reagents and conditions: (a) DMF, K_2CO_3 (1 eq), CH_3I (1 eq), rt, 2 h, 64–78%.

The rate of *E*/*Z* isomerization was investigated by NMR in DMSO, in the presence or absence of light. When the solutions were protected from light, no isomerization was detected after 7 days for compounds **7** (*E*-isomer) and **8** (*Z*-isomer). When the DMSO solutions were kept in the presence of light, the *E*-isomer gradually isomerized to the *Z*-one, reaching after 3 days a constant ratio of compound **7**/compound **8** close to 1:1. Isomerization of compounds **7** and **8** was also measured in the cell culture medium D-MEM by HPLC–UV analysis. When compounds **7** and **8** were incubated in the cell culture medium and protected from the light, no isomerization was detected after 3 days.

The (*R*)-5-benzyl-1-phenethylhydantoin **30** was synthesized from the aminoacid D-phenylalanine as previously reported (Scheme 3) [2]. Briefly, the 2,4-dinitrobenzensulfonamide **35**, readily prepared from 2,4-dinitrobenzensulfonyl chloride and the D-phenylalanine methyl ester **34**, was alkylated under Mitsunobu conditions and deprotected to **37** by treatment with thioglycolic acid. Cyclization of **37** with potassium cyanate afforded the target hydantoin **30** in good yield. Chemical and physical data of the new compounds are listed in Table 3.



Scheme 3. Reagents and conditions: (a) CH_3OH, HCl gas, reflux, 10 min, 88%; (b) 2,4-dinitrobenzensulfonyl chloride (1 eq), pyridine (3 eq), CH_2Cl_2, rt, 16 h, 78%; (c) $PhCH_2CH_2OH$ (2 eq), DIAD (2 eq), PPh_3 (2 eq), benzene, rt, 20 min, 94%; (d) $HSCH_2CO_2H$ (1.3 eq), Et_3N (2 eq), CH_2Cl_2, rt, 1 h, 97%; (e) KCNO (2 eq), CH_3CO_2H, rt, 4 h, 75%.

3. Pharmacology

Compounds **1**–**30** were evaluated for their antiproliferative properties against the human NSCLC cell line A549 and activities are expressed as percentage inhibition of cell proliferation at 20 μM concentration. Results are listed in Table 1. Moreover, modulation of p53 protein expression during treatment with compounds **1**, **2**, **4**–**10**, **13**, **15**–**17**, and **26**–**30** was evaluated by Western blot analysis (Table 1, Table 2 and Fig. 2). In particular, the effect of 48 h treatment at 20 μM concentration is reported in Table 1, while results obtained for compounds **4**, **7**, **8**, **13**, and **15** at 10 and 20 μM concentrations after 48 and 72 h treatment are reported in Table 2 and Fig. 2.

4. Results and discussion

The series of 5-benzylidene hydantoin derivatives **1**–**29** was synthesized via a simple and efficient solution-phase parallel process expanding our library of the initial series of compounds. Microwave irradiation reduced the reaction time and simplified the work-up providing good overall yields, and only final compounds were submitted to chromatography to separate the two geometric isomers.

Percentages of inhibition of the human lung adenocarcinoma cell line A549 proliferation at 20 μM are shown in Table 1. Compounds carrying a phenethyl side chain at position 1 (general formula A in Table 1, **1**–**11**) showed percentage inhibition of cell proliferation ranging from 9.4 to 53 at 20 μM concentration. With the exception of unsubstituted compounds **1** and **2**, no significant difference in cell growth inhibition was observed between *E* and *Z* isomers in the type A derivatives (**3**–**11**). The introduction of a substituent on the 5-benzylidene ring led to an improvement in the antiproliferative activity only in the case of the *E*-isomers (**3**, **5**, **7**, **9** and **10** vs. **1**). Different lipophilic side chains at the N1 position were also considered (type B compounds). *N*1-arylalkyl derivatives (**12**–**15**) paralleled the selectivity profile observed for compounds **1** and **2**, the *Z*-isomers being more potent then the *E*-ones. Also in the case of *N*1-benzyl hydantoin, the introduction of a substituent on the 5-benzylidene ring increased the activity of the *E*-isomer (**16**) with respect to the corresponding *Z*-one (**17**). Compounds **18**–**27**, carrying *N*1-phenyl and *N*1-alkyl side chains exhibited weak antiproliferative action, suggesting that a phenethyl or benzyl group at 1 position is important for cell growth inhibition. Methylation at the N3 position improved activity of the *E*-isomer (**28** vs. **1**) without affecting the activity of the *Z*-one (**29** vs. **2**). Finally, compound **30** had no significant antiproliferative effect, indicating that the *exo*-cyclic double bond at the 5 position on the hydantoin nucleus is essential not only for EGFR kinase inhibition, as previously reported [2], but also for growth inhibition in A549 cells.

Considering the possibility of interconversion between the *E* and *Z* geometric isomers, a stability study was performed on compounds **7** (*E*) and **8** (*Z*), in order to assess if the activity of an isomer could be affected by its isomerization to the other one in solution. The results obtained by ^{1}H NMR stability studies conducted in DMSO and HPLC–UV analysis in the D-MEM cell culture medium (see Experimental section) indicated that isomerization does not occur in the absence of light after 72 h of incubation. In the conditions applied to the cell tests here described, significant isomerization is ruled out.

Table 2
Evaluation of time- and dose-dependent p53 protein expression.

no.	p53 fold increase[a]			
	10 μM		20 μM	
	48 h	72 h	48 h	72 h
4	0.9 ± 0.1	1.4 ± 0.1*	1.2 ± 0.2	1.9 ± 0.3**
7	1.0 ± 0.1	1.4 ± 0.1**	2.3 ± 0.2**	3.2 ± 0.1**
8	0.9 ± 0.1	1.7 ± 0.1**	1.5 ± 0.1	2.7 ± 0.3**
13	0.7 ± 0.3	1.1 ± 0.1	1.5 ± 0.2	2.2 ± 0.2**
15	1.1 ± 0.1	1.8 ± 0.1**	1.7 ± 0.2*	2.6 ± 0.2**

*$P < 0.05$; **$P < 0.01$ vs. 1; $n = 3$.
[a] p53 protein expression, fold increase respect to the control; mean values of three independent experiments ± SD are reported. One-tail Student's *t*-test.

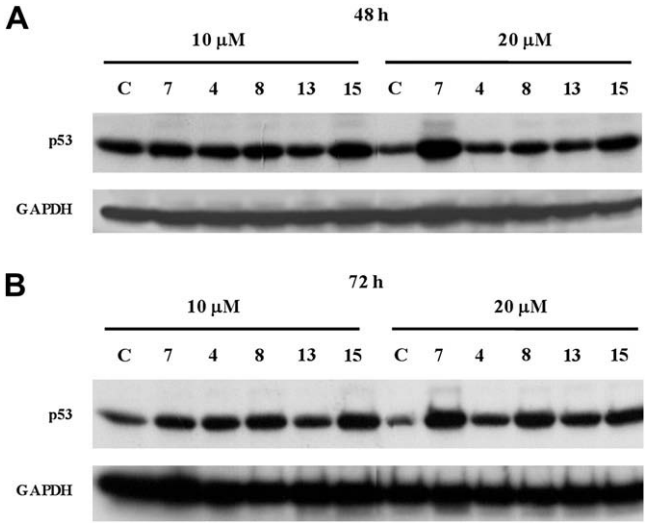


Fig. 2. Modulation of p53 protein expression during treatment with compounds **4**, **7**–**8**, **13** and **15**. A549 cells were incubated with each compound at 10 and 20 μM concentrations for 48 (A) and 72 h (B). Cells lysates were analyzed by Western blotting to assess the expression of p53 and GAPDH proteins. Representative blot of three independent experiments is reported.

With 53% growth inhibition at 20 μM, compound **7** (UPR1024) was the most active antiproliferative agent of the series. Further investigation of the cellular and molecular mechanisms of compound **7** indicated that it inhibited EGFR autophosphorylation in A549 cells in a dose-dependent manner with an IC_{50} of 19 μM [6]. It also induced S phase cell cycle arrest and DNA damage in human A549 cancer cells, with up-regulation of p53 [6].

p53 is a critical transcription factor that responds to signals from a wide range of cellular stresses and mediates the cellular apoptotic response to DNA damaging drugs in several tumor cell lines. Increased levels of p53 protein after treatment with anticancer drugs are considered as biological marker of DNA damage [12,13].

In order to find out whether the dual mechanism was a particular feature of compound **7** or whether it was shared with other hydantoin derivatives of the series, we tested the effect on p53 expression in A549 cells for compounds **1**, **2**, **4**–**10**, **13**, **15**–**17** and **26**–**30**. Compound **7** resulted the most active p53 inductor, giving 2.3-fold increase in the p53 protein level with respect to the control. Compounds **8**, **10**, **13**, and **15**–**17** gave p53 fold increase between 1.5 and 2.0 in A549 cells, while p53 expression was not altered in cells treated with any of the other selected hydantoin derivatives. When tested in the same conditions, gefitinib did not modify p53 expression (Table 1). The four 4-hydroxy-benzylidene derivatives (**7**, **8**, **16**, **17**) showed higher degrees of p53 expression. This could be due to possible transformation of this portion into a quinone-methide, that could bring cell damages by additional mechanisms, i.e. acting as a Michael acceptor, and this hypothesis is supported by the lack of activity observed for the 4-methoxy derivative, **9**. However, ability to increase p53 expression was also observed for compounds **13** and **15**, where the hydroxy group is absent, pointing out that its presence, although important for this kind of activity, is not mandatory.

We further selected five hydantoin derivatives (**4**, **7**, **8**, **13** and **15**), with inhibition of cell proliferation over 40% at 20 μM concentration, to evaluate dose- and time-dependent effects on p53 expression. Compounds **4**, **7**, **8**, **13** and **15** were tested on p53 at 10 and 20 μM concentrations for 48 and 72 h incubation times (Table 2 and Fig. 2). When incubated for 48 h, the selected compounds did not show any effect on p53 expression at 10 μM concentration, while 72 h incubation at the same concentration gave 1.4–1.8 fold increase in p53 level for compounds **4** (1.4), **7** (1.4), **8** (1.7) and **15** (1.8). Data show a time-dependent effect that become more evident when compounds are tested at 20 μM concentration. The tested derivatives showed p53 expression fold increase between 1.9 and 3.2 after 72 h incubation at 20 μM concentration. Treatment with compound **7** led to a 3.2 fold increase in p53 protein in A549 cells, confirming the derivative as the most potent of the series in this biological assay.

5. Conclusions

Parallel solution-phase and microwave-assisted synthesis were employed to prepare 5-benzylidene hydantoins with different substituents at the N1, N3 and C5 positions. Although none of the newly synthesized compounds resulted more active then compound **7**, most of them inhibits human A549 cell proliferation at 20 μM concentration with percentage inhibitions over 20%. In order to find out whether the observed antiproliferative effect was a result of a dual anti-EGFR and DNA damaging mechanism of action, selected derivatives were tested for their ability to increase p53 protein levels, and some of them showed p53 fold increase over 1.5 after 48 h treatment at 20 μM concentration, with no clear-cut structure–activity relationship. A time-dependent effect on p53 expression was also demonstrated for the most active hydantoin derivatives **4**, **7**, **8**, **13** and **15**. In conclusion, compound **7** and other synthesized 5-benzylidene hydantoin derivatives increased p53 levels, suggesting that the dual antiproliferative mechanism (e.g. DNA damage induction and EGFR inhibition) was a common feature of the most active compounds of the series.

6. Experimental

6.1. Chemistry

Reagents were obtained from commercial suppliers and used without further purification. Solvents were purified and stored according to standard procedures. Anhydrous reactions were conducted under a positive pressure of dry N_2. Melting points were not corrected and were determined with a Büchi instrument (Tottoli) and with a Gallenkamp melting point apparatus. The final compounds were analyzed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyzer, for C, H and N. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. The ^{1}H NMR spectra were recorded on a Bruker 300 MHz spectrometer and on a Bruker 300 MHz Avance spectrometer; chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. ^{1}H NMR spectra are reported in the following order: multiplicity, approximate coupling constant (J value) in hertz (Hz) and number of protons; signals were characterized as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) br s (broad signal). Mass spectra were recorded using an API 150 EX instrument (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). Reactions were monitored by TLC, on Kieselgel 60 F 254 (DC-Alufolien, Merck). Final compounds and intermediates were purified by flash chromatography (SiO_2 60, 40–63 μM), and by chromatography on preparative Gilson MPLC, using a SiO_2 column (SiO_2 60, 25–40 μM). Buchi Syncore® polyvap was used for parallel synthesis, filtration and evaporation. Microwave reactions were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). Yields and characteristic data of the final compounds are listed in Table 3.

6.1.1. Synthesis: general procedure for N-substituted glycine ethylesters (*31a–f*)

To a stirred solution of the appropriate primary amine (4.98 mmol) in 5 mL $CHCl_3$, ethylbromoacetate (0.28 mL, 2.52 mmol) was added dropwise. After 2 h, the precipitate was filtered off and the solution was evaporated to dryness. The residue was extracted with diethyl ether, the precipitate was filtered off and the filtrate was concentrated under vacuum to give compound **31** as an oil, used directly in the next step without further purification. Spectroscopic data for compounds **31a**, **c–f** are in agreement with those reported in the literature [14–17].

6.1.1.1. N-*(3-Chlorophenethyl)glycine ethylester (**31b**).* Yield 82%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.27 (t, $J = 7.2$ Hz, 3H), 2.80 (m, 2H), 2.88 (t, 2H), 3.42 (s, 2H), 4.18 (q, $J = 7.2$ Hz, 2H), 7.10–7.28 (m, 4H).

6.1.2. General procedure for N-substituted N-carbethoxymethylureas (*32a–e*)

To an ice-cooled sample of the appropriate *N*-substituted glycine ethylester **31** (18.71 mmol) conc. HCl (2.40 mL, 28.10 mmol) was slowly added. With continued stirring and cooling, a solution of potassium cyanate (2.30 g, 28.1 mmol) in 3.24 mL of water was added dropwise. The mixture was stirred at room temperature (rt) for 20 h and then was taken up in a mixture of CH_2Cl_2 and 10% HCl. The organic layer was separated, washed with water, and dried over anhydrous Na_2SO_4. Filtration and removal of the solvent under reduced pressure gave compounds **32** as viscous oils.

*6.1.2.1. 1-Carbethoxymethyl-1-phenethylurea (**32a**).* Crystallization from benzene/pentane gave pure **32a** as white crystals. Yield 84%. Mp 80–81 °C. ^{1}H NMR ($CDCl_3$; 400 MHz) δ 1.28 (t, $J = 7.2$ Hz, 3H), 2.89 (t, $J = 7.2$ Hz, 2H), 3.52 (t, $J = 7.2$ Hz, 2H), 3.91 (s, 2H), 4.19 (q, $J = 7.2$ Hz, 2H), 4.40 (br s, 2H), 7.21–7.36 (m, 5H).

*6.1.2.2. 1-Carbethoxymethyl-1-(3-chlorophenethyl)urea (**32b**).* Crystallization from benzene/pentane gave pure **32b** as white crystals. Yield 68%. Mp 84–86 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 1.30 (t, $J = 7.2$ Hz, 3H), 2.99 (t, $J = 7.3$ Hz, 2H), 3.53 (t, $J = 7.3$ Hz, 2H), 3.92 (s, 2H), 4.21 (q, $J = 7.2$ Hz, 2H), 4.48 (br s, 2H), 7.12–7.28 (m, 4H).

*6.1.2.3. 1-Carbethoxymethyl-1-benzylurea (**32c**).* Silica gel chromatography (CH_2Cl_2/CH_3OH = 97:3) followed by crystallization from CH_2Cl_2/petroleum ether gave pure **32c** as a white solid. Yield 68%. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 1.24 (t, $J = 7.1$ Hz, 3H), 4.03 (s, 2H), 4.16 (t, $J = 7.1$ Hz, 2H), 4.52 (s, 2H), 4.71 (br s, 2H), 7.26–7.37 (m, 5H).

*6.1.2.4. 1-Carbethoxymethyl-1-(*n*-butyl)urea (**32d**).* The target urea **32d** was obtained as a colorless oil. Yield 74%. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 0.88 (t, $J = 7.2$ Hz, 3H), 1.20–1.39 (m, 5H), 1.46–1.56 (m, 3H), 3.19 (t, $J = 7.3$ Hz, 2H), 3.95 (s, 2H), 4.14 (q, $J = 7.2$ Hz, 2H), 4.93 (s, 2H).

*6.1.2.5. 1-Carbethoxymethyl-1-(*n*-hexyl)urea (**32e**).* The target urea **32e** was obtained as a colorless oil. Yield 85%. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 0.89 (t, $J = 6.2$ Hz, 3H), 1.29–1.31 (m, 9H), 1.58 (t, $J = 6.9$ Hz, 3H), 3.24 (t, $J = 7.5$ Hz, 2H), 4.01 (s, 2H), 4.17–4.27 (m, 2H), 4.62–4.65 (m, 2H).

6.1.3. General procedure for 1-substituted hydantoins (*33a–f*)

A mixture of the appropriate *N*-substituted *N*-carbethoxymethylureas **32a–e** (14.00 mmol) and 12.50 mL of 25% HCl was

Table 3
Chemical and physical data for the synthesized hydantoin derivatives **1**–**30**.

no.	SiO_2 chromatography[a]	*E*/*Z* ratio	Yield%[b]	Crystallization solvent	Mp (°C)	Formula
1	I	1:1	70	EtOH/H_2O	173–175	$C_{18}H_{16}N_2O_2$
2	I	1:1	70	EtOH/H_2O	156–158	$C_{18}H_{16}N_2O_2$
3	I	1:1	80	EtOH/H_2O	168–170	$C_{18}H_{15}N_2O_2Cl$
4	I	1:1	80	EtOH/H_2O	153–155	$C_{18}H_{15}N_2O_2Cl$
5	II	1:1	76	EtOH/H_2O	185–187	$C_{18}H_{15}N_2O_3$
6	II	1:1	76	EtOH/H_2O	192–195	$C_{18}H_{15}N_2O_3$
7	II	1.5:1	57	EtOH/H_2O	213–215	$C_{18}H_{15}N_2O_3$
8	II	1.5:1	57	EtOH/H_2O	210–212	$C_{18}H_{15}N_2O_3$
9	I	2.4:1	51	EtOH/H_2O	190–191	$C_{19}H_{18}N_2O_3$
10	I	4:1	80	EtOH/H_2O	255–256	$C_{20}H_{19}N_3O_3$
11	I	4:1	80	EtOH/H_2O	184–185	$C_{20}H_{19}N_3O_3$
12	I	1:1	76	EtOH/H_2O	147–149	$C_{18}H_{15}N_2O_2Cl$
13	I	1:1	76	EtOH/H_2O	153–155	$C_{18}H_{15}N_2O_2Cl$
14	I	1.9:1	69	EtOH	181–183	$C_{17}H_{14}N_2O_2$
15	I	1.9:1	69	EtOH/H_2O	132–133	$C_{17}H_{14}N_2O_2$
16	II	1:1	80	CH_2Cl_2	223–226	$C_{17}H_{14}N_2O_3$
17	II	1:1	80	EtOH/H_2O	233–235	$C_{17}H_{14}N_2O_3$
18	I	1:1	78	EtOH/H_2O	145–148	$C_{14}H_{16}N_2O_2$
19	I	1:1	78	EtOH/H_2O	86–87	$C_{14}H_{16}N_2O_2$
20	I	1:1	76	EtOH/H_2O	113–115	$C_{14}H_{15}N_2O_2Cl$
21	I	1:1	76	EtOH/H_2O	104–105	$C_{14}H_{15}N_2O_2Cl$
22	I	4:1	75	EtOH	235–240	$C_{16}H_{12}N_2O_2$
23	I	4:1	75	CH_2Cl_2	190–194	$C_{16}H_{12}N_2O_2$
24	I	1:1	72	EtOH/H_2O	105–108	$C_{16}H_{20}N_2O_2$
25	I	1:1	72	–	(oil)	$C_{16}H_{20}N_2O_2$
26	III	2.4:1	80	Et_2O/petrol. ether	188–190	$C_{11}H_{10}N_2O_2$
27	III	2.4:1	80	Et_2O/petrol. ether	135–136	$C_{11}H_{10}N_2O_2$
28	IV	1:0[c]	78	EtOH/H_2O	90–92	$C_{19}H_{18}N_2O_2$
29	IV	0:1[c]	64	EtOH/H_2O	110–111	$C_{19}H_{18}N_2O_2$
30	I	–	63[d]	benzene/petrol. ether	117–119	$C_{18}H_{17}N_2O_2$

[a] Silica gel chromatography eluents: I = DCM/MeOH(5% NH_3) 30:1. II = DCM/EtOAc 8:1 ≥ 5:1. III = DCM/EtOAc/MeOH(1% NH_3) 21:3:0.2. IV = DCM/MeOH 9:1.
[b] *E*/*Z* isomers mixture yield.
[c] Synthesized from the pure corresponding isomer.
[d] Overall yield.

heated under reflux for 4 h and then cooled in ice. The precipitate was collected by filtration, washed with cold water and dried to afford crude hydantoins that were purified by crystallization. Spectroscopic data and melting point for compound **33d** are in agreement with those reported in the literature [18]. 1-Phenylhydantoin **33f** was prepared according to a previously described procedure [19].

*6.1.3.1. 1-Phenethylhydantoin (**33a**).* Crystallization from ethanol 95% gave pure **33a** as a white solid. Yield 99%. Mp 180–181 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.79 (t, J = 7.3 Hz, 2H), 3.46 (t, J = 7.3 Hz, 2H), 3.86 (s, 2H), 7.18–7.32 (m, 5H).

*6.1.3.2. 1-(3-Chlorophenethy)hydantoin (**33b**).* Crystallization from ethanol gave pure **33b** as a white solid. Yield 89%. Mp 164–166 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.26 (t, J = 7.5 Hz, 2H), 3.64 (t, J = 7.6 Hz, 2H), 6.56 (s, 1H), 6.65 (d, 1H), 6.76 (s, 1H), 7.18–7.20 (m, 2H), 7.42–7.51 (m, 5H), 11.49 (br s, 1H).

*6.1.3.3. 1-Benzylhydantoin (**33c**).* Crystallization from ethanol gave pure **33c** as a pale yellow solid. Yield 52%. Mp 135–138 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 3.77 (s, 2H), 4.52 (s, 2H), 7.39–7.24 (m, 5H), 9.10 (br s, 1H).

*6.1.3.4. 1-*n*-Hexylhydantoin (**33e**).* Crystallization from ethanol gave pure **33e** as a white solid. Yield 56%. Mp 76–78 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.85 (t, J = 6.8 Hz, 3H), 1.19–1.28 (m, 6H), 1.45 (m, 2H), 3.19 (t, J = 7.0 Hz, 2H), 3.89 (s, 2H), 10.67 (br s, 1H).

*6.1.4. General procedure for 5-(substituted benzylidene)hydantoin analogs (**1**–**27**)*

In a microwave vessel, dry piperidine (2 mmol) was added to a mixture of benzaldehyde or its derivatives (1 mmol) and the proper 1-substituted hydantoin **33a**–**f** (1 mmol), under an inert atmosphere. The reaction mixture was then irradiated at 130 °C for 5 min under 200 W microwave power. The mixture was then cooled to rt, and water and diethyl ether were added. The organic phase was washed with water, 5% HCl and water to neutrality. The organic layer was dried over Na_2SO_4 and the solvent removed under reduced pressure. The *E*/*Z* mixtures were resolved by silica gel chromatography and each isomer was then crystallized. Compounds **26** and **27** were synthesized following the same procedure starting from commercially available 1-methylhydantoin. Silica gel chromatography eluents, *E*/*Z* ratios, yields, crystallization solvents, melting points, and formulas for the target hydantoin derivatives **1**–**27** are listed in Table 3.

*6.1.4.1. (E)-5-Benzylidene-1-phenethylhydantoin (**1**).* Mp 173–175 °C. ^{1}H NMR (DMSO-d_6; 400 MHz) δ 2.89 (t, J = 7.1 Hz, 2H), 3.87 (t, J = 7.1 Hz, 2H), 6.42 (s, 1H), 7.09–7.45 (m, 8H), 7.82 (d, J = 7.5 Hz, 2H), 11.27 (br s, 1H). MS-APCI *m*/*z*: 291.3 [M-H]$^-$. Anal. $C_{18}H_{16}N_2O_2$ (C, H, N).

*6.1.4.2. (Z)-5-Benzylidene-1-phenethylhydantoin (**2**).* Mp 156–158 °C. ^{1}H NMR (DMSO-d_6; 400 MHz) δ 2.25 (t, J = 7.8 Hz, 2H), 3.63 (t, J = 7.8 Hz, 2H), 6.62 (dd, J = 7.0, 2.1 Hz, 2H), 6.74 (s, 1H), 7.11–7.16 (m, 3H), 7.41–7.49 (m, 5H), 11.40 (s, 1H). MS-APCI *m*/*z*: 291.2 [M-H]$^-$. Anal. $C_{18}H_{16}N_2O_2$ (C, H, N).

*6.1.4.3. (E)-5-(3-Chlorobenzylidene)-1-phenethylhydantoin (**3**).* Mp 168–170 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.89 (t, 2H), 3.87 (t, 2H), 6.41 (s, 1H), 7.18–7.38 (m, 7H), 7.44–7.77 (m, 1H), 7.99 (d, 1H), 11.33 (br s, 1H). MS-APCI *m*/*z*: 327.2, 325.3 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.4. (Z)-5-(3-Chlorobenzylidene)-1-phenethylhydantoin (**4**).* Mp 153–155 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.30 (t, 2H), 3.62 (t, 2H), 6.67–6.70 (m, 3H), 7.14–7.19 (m, 3H), 7.39–7.44 (m, 1H), 7.48–7.50 (m, 2H), 7.55 (d, 1H), 11.30 (s, 1H). MS-APCI *m*/*z*: 327.3, 325.3 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.5. (E)-5-(3-Hydroxybenzylidene)-1-phenethylhydantoin (**5**).* Mp 185–187 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.88 (t, 2H), 3.87 (t, 2H), 6.33 (s, 1H), 6.70–6.73 (m, 1H), 7.10–7.34 (m, 8H), 9.36 (s, 1H), 11.23 (s, 1H). MS-APCI *m*/*z*: 307.2 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_3$ (C, H, N).

*6.1.4.6. (Z)-5-(3-Hydroxybenzylidene)-1-phenethylhydantoin (**6**).* Mp 192–195 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.31 (t, 2H), 3.66 (t, 2H), 6.66 (s, 1H), 6.69–6.86 (m, 1H), 7.14–7.29 (m, 8H), 9.62 (s, 1H), 11.36 (br s, 1H). MS-APCI *m*/*z*: 307.3 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_3$ (C, H, N).

*6.1.4.7. (E)-5-(4-Hydroxybenzylidene)-1-phenethylhydantoin (**7**).* Mp 213–215 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.86 (t, J = 7.2 Hz, 2H), 3.86 (t, J = 7.3, 2H), 6.37 (s, 1H), 6.75 (d, J = 9.0 Hz, 1H), 7.18–7.33 (m, 5H), 7.85 (d, J = 8.7 Hz, 2H), 9.84 (br s, 1H), 11.19 (br s, 1H). MS-CI *m*/*z*: 308 [M + H]$^+$. Anal. $C_{18}H_{15}N_2O_3$ (C, H, N).

*6.1.4.8. (Z)-5-(4-Hydroxybenzylidene)-1-phenethylhydantoin (**8**).* Mp 210–212 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.29 (t, J = 7.8 Hz, 2H), 3.70 (t, J = 7.8, 2H), 6.67 (s, 1H), 6.70–6.72 (m, 2H), 6.85 (d, J = 8.1 Hz, 2H), 7.14–7.19 (m, 3H), 7.28 (d, J = 8.7 Hz, 2H), 9.83 (br s, 1H), 11.31 (br s, 1H). MS-APCI *m*/*z*: 307.2 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_3$ (C, H, N).

*6.1.4.9. (E)-5-(4-Methoxybenzylidene)-1-phenethylhydantoin (**9**).* Mp 190–191 °C.; ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.88 (t, J = 7.1 Hz, 2H), 3.87 (t, J = 7.3 Hz, 2H), 3.91 (s, 3H), 6.41 (s, 1H), 6.94 (d, J = 9.0 Hz, 2H), 7.20–7.33 (m, 5H), 7.92 (d, J = 8.9 Hz, 2H), 11.25 (br s, 1H). MS-CI *m*/*z*: 323.1 [M + H]$^+$. Anal. $C_{19}H_{18}N_2O_3$ (C, H, N).

*6.1.4.10. (E)-N-(4-(3-Phenethylhydantoin-4-ylidene)methyl)phenylacetamide (**10**).* Mp 255–256 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.06 (S, 3H), 2.87 (t, J = 7.0 Hz, 2H), 3.87 (t, J = 7.0 Hz, 2H), 6.39 (s, 1H), 7.18–7.33 (m, 5H), 7.56 (d, J = 8.5 Hz, 2H) 7.86 (d, J = 8.5 Hz, 2H), 10.07 (s, 1H), 11.27 (s, 1H). MS-APCI *m*/*z*: 347.06 [M-H]$^-$. Anal. $C_{20}H_{19}N_3O_3$ (C, H, N).

*6.1.4.11. (Z)-N-(4-(3-Phenethylhydantoin-4-ylidene)methyl)phenylacetamide (**11**).* Mp 184–185 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.09 (S, 3H), 2.27 (t, J = 7.0 Hz, 2H), 3.70 (t, J = 7.0 Hz, 2H), 6.66–6.70 (m, 3H), 7.12–7.14 (m, 3H), 7.39 (d, J = 8.5 Hz, 2H) 7.67 (d, J = 8.5 Hz, 2H), 10.14 (s, 1H), 11.38 (s, 1H). MS-APCI *m*/*z*: 347.06 [M-H]$^-$. Anal. $C_{20}H_{19}N_3O_3$ (C, H, N).

*6.1.4.12. (E)-5-Benzylidene-1-(3-chlorophenethyl)hydantoin (**12**).* Mp 147–149 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.91 (t, J = 7.2 Hz, 2H), 3.90 (t, J = 7.2 Hz, 2H), 6.51 (s, 1H), 7.18–7.47 (m, 7H), 7.85 (d, J = 7.2 Hz, 2H), 11.33 (s, 1H). MS-APCI *m*/*z*: 327.3, 325.3 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.13. (Z)-5-Benzylidene-1-(3-chlorophenethyl)hydantoin (**13**).* Mp 153–155 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.26 (t, J = 7.5 Hz, 2H), 3.64 (t, J = 7.6 Hz, 2H), 6.56 (s, 1H), 6.65 (d, 1H), 6.76 (s, 1H), 7.18–7.20 (m, 2H), 7.42–7.51 (m, 5H), 11.49 (s, 1H). MS-APCI *m*/*z*: 327.2, 325.3 [M-H]$^-$. Anal. $C_{18}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.14. (E)-5-Benzylidene-1-benzylhydantoin (**14**).* Mp 181–183 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 4.92 (s, 2H), 6.22 (s, 1H), 7.25–7.40 (m, 8H), 7.74 (d, J = 7.8 Hz, 2H), 8.57 (br s, 1H). MS-APCI *m*/*z*: 277.1 [M-H]$^-$. Anal. $C_{17}H_{14}N_2O_2$ (C, H, N).

*6.1.4.15. (Z)-5-Benzylidene-1-benzylhydantoin (**15**).* Mp 132–133 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 4.66 (s, 2H), 6.55–6.59 (m, 2H), 6.64 (s, 1H), 7.10–7.18 (m, 5H), 7.26–7.33 (m, 3H), 11.67 (br s, 1H). MS-APCI *m*/*z*: 277.2 [M-H]$^-$. Anal. $C_{17}H_{14}N_2O_2$ (C, H, N).

*6.1.4.16. (E)-5-(4-Hydroxybenzylidene)-1-benzylhydantoin (**16**).* Mp 223–226 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 4.86 (s, 2H), 6.28 (s, 1H), 6.70 (d, J = 8.7 Hz, 2H), 7.23–7.38 (m, 5H), 7.79 (d, J = 8.7 Hz, 2H), 9.83 (br s, 1H), 11.43 (br s, 1H). MS-APCI *m*/*z*: 293.2 [M-H]$^-$. Anal. $C_{17}H_{14}N_2O_3$ (C, H, N).

*6.1.4.17. (Z)-5-(4-Hydroxybenzylidene)-1-benzylhydantoin (**17**).* Mp 233–235 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 4.71 (s, 2H), 6.57 (s, 1H), 6.65–6.72 (m, 4H), 7.05 (d, J = 8.4 Hz, 2H), 7.15–7.17 (m, 3H), 9.78 (s, 1H), 11.56 (s, 1H). MS-APCI *m*/*z*: 293.3 [M-H]$^-$. Anal. $C_{17}H_{14}N_2O_3$ (C, H, N).

*6.1.4.18. (E)-5-Benzylidene-1-n-butylhydantoin (**18**).* Mp 145–148 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.99 (t, J = 7.2 Hz, 3H), 1.39–1.46 (m, 2H), 1.61–1.71 (m, 2H), 3.70 (t, J = 7.2 Hz, 2H), 6.26 (s, 1H), 7.28–7.42 (m, 3H), 7.87 (d, J = 7.5 Hz, 2H). MS-CI *m*/*z*: 243.2 [M-H]$^-$. Anal. $C_{14}H_{16}N_2O_2$ (C, H, N).

*6.1.4.19. (Z)-5-Benzylidene-1-n-butylhydantoin (**19**).* Mp 86–87 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.27 (t, J = 7.4 Hz, 2H), 3.69 (t, J = 7.4 Hz, 2H), 3.82 (s, 3H), 6.64–6.69 (m, 2H), 6.71 (s, 1H), 7.04 (d, J = 8.2 Hz, 2H), 7.10–7.20 (m, 3H), 7.40 (d, J = 8.6 Hz, 2H), 11.25 (s, 1H). MS-APCI *m*/*z*: 243.0 [M-H]$^-$. Anal. $C_{14}H_{16}N_2O_2$ (C, H, N).

*6.1.4.20. (E)-5-(4-Chlorobenzylidene)-1-n-butylhydantoin (**20**).* Mp 113–115 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.91 (t, J = 7.2 Hz, 3H), 1.28–1.35 (m, 2H), 1.50–1.57 (m, 2H), 3.63 (t, J = 7.0 Hz, 2H), 6.47 (s, 1H), 7.42 (d, J = 8.5 Hz, 2H), 7.94 (d, J = 8.5 Hz, 2H), 11.39 (br s, 1H). MS-APCI *m*/*z*: 277.2 [M-H]$^-$, 279.1. Anal. $C_{14}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.21. (Z)-5-(4-Chlorobenzylidene)-1-n-butylhydantoin (**21**).* Mp 103–105 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.57 (t, J = 7.0 Hz, 3H), 0.79–0.86 (m, 2H), 0.89–1.02 (m, 2H), 3.42 (t, J = 7.1 Hz, 2H), 6.65 (s, 1H), 7.41 (d, J = 8.5 Hz, 2H), 7.49 (d, J = 8.5 Hz, 2H), 11.51 (br s, 1H). MS-APCI *m*/*z*: 277.1 [M-H]$^-$, 279.1. Anal. $C_{14}H_{15}N_2O_2Cl$ (C, H, N).

*6.1.4.22. (E)-5-Benzylidene-1-phenylhydantoin (**22**).* Mp 235–240 °C. ^{1}H NMR (CD_3OD; 300 MHz) δ 6.16 (s, 1H), 7.30–7.36 (m, 3H), 7.43–7.46 (m, 2H), 7.53–7.55 (m, 1H) 7.59–7.63 (m, 2H) 7.80–7.83 (m, 2H). MS-APCI *m*/*z*: 262.7 [M-H]$^-$. Anal. $C_{16}H_{12}N_2O_2$ (C, H, N).

*6.1.4.23. (Z)-5-Benzylidene-1-phenylhydantoin (**23**).* Mp 190–194 °C. ^{1}H NMR (CD_3OD; 300 MHz) δ 6.85–6.88 (m, 3H), 6.94 (t, J = 7.8 Hz, 2H), 7.04–7.09 (m, 3H), 7.14–7.16 (m, 3H). MS-APCI *m*/*z*: 262.9 [M-H]$^-$. Anal. $C_{16}H_{12}N_2O_2$ (C, H, N).

*6.1.4.24. (E)-5-Benzylidene-1-n-hexylhydantoin (**24**).* Mp 105–108 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.86 (t, J = 6.7 Hz, 3H), 1.29 (m, 6H), 1.54 (m, 2H), 3.63 (t, J = 7.2 Hz, 2H), 6.48 (s, 1H), 7.30–7.40 (m, 3H), 7.91 (d, J = 7.0 Hz, 2H), 11.33 (br s, 1H). MS-APCI *m*/*z*: 271.2 [M-H]$^-$. Anal. $C_{16}H_{20}N_2O_2$ (C, H, N).

*6.1.4.25. (Z)-5-Benzylidene-1-n-hexylhydantoin (**25**).* ^{1}H NMR (DMSO-d_6; 300 MHz) δ 0.70 (t, J = 7.0 Hz, 3H), 0.72–1.09 (m, 8H), 3.40 (t, J = 7.3 Hz, 2H), 6.69 (s, 1H), 7.32–7.43 (m, 5H), 11.46 (br s, 1H). MS-APCI *m*/*z*: 271.2 [M-H]$^-$. Anal. $C_{16}H_{20}N_2O_2$ (C, H, N).

*6.1.4.26. (E)-5-Benzylidene-1-methylhydantoin (**26**).* Mp 188–190 °C. ^{1}H NMR (CD_3OD; 300 MHz) δ 3.18 (s, 3H), 6.41 (s, 1H), 7.32–

7.36 (m, 3H), 7.93 (d, $J = 7.7$ Hz, 2H). MS-APCI m/z: 201.1 $[M + H]^+$. Anal. $C_{11}H_{10}N_2O_2$ (C, H, N).

*6.1.4.27. (Z)-5-Benzylidene-1-methylhydantoin (**27**).* Mp 135–136 °C. ^{1}H NMR (DMSO-d_6; 300 MHz) δ 2.81 (s, 3H), 6.66 (s, 1H), 7.33–7.41 (m, 5H), 11.43 (br s, 1H). MS-APCI m/z: 201 $[M-H]^-$. Anal. $C_{11}H_{10}N_2O_2$ (C, H, N).

*6.1.5. Synthesis of (*E*)-5-benzylidene-3-methyl-1-phenethylhydantoin (**28**)*

To a solution of (*E*)-5-benzylidene-1-phenethylhydantoin **1** (0.05 g, 0.17 mmol) in dry DMF (0.5 mL), K_2CO_3 (0.023 g, 0.17 mmol) and CH_3I (11 μL, 0.17 mmol) were added at rt under an inert atmosphere. The reaction mixture was stirred for 2 h at rt until complete consumption of the starting material assessed by TLC (SiO_2, $CH_2Cl_2/CH_3OH = 9:1$). A mixture of EtOAc/water was then added, the organic layer was separated and washed first with 0.1 N HCl, then with water. After drying over Na_2SO_4, the solvent was removed under reduced pressure. The pure product was collected after crystallization from ethanol/water as white crystals (78% yield). Mp 90–92 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 3.00 (t, $J = 7.5$ Hz, 2H), 3.11 (s, 3H), 3.97 (t, $J = 7.5$ Hz, 2H), 6.16 (s, 1H), 7.26–7.42 (m, 8H), 7.75–7.78 (m, 2H). MS-APCI m/z: 307.4 $[M + H]^+$. Anal. $C_{19}H_{18}N_2O_2$ (C, H, N).

*6.1.6. (Z)-5-Benzylidene-3-methyl-1-phenethylhydantoin (**29**)*

Starting from (*Z*)-5-benzylidene-1-phenethylhydantoin **2**, and following the procedure described above for the *E*-isomer, compound **29** was obtained after crystallization from ethanol/water as white crystals (64% yield). Mp 110–111 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 7.49–7.45 (m, 5H), 7.15–7.12 (m, 3H), 6.87 (s, 1H), 6.61–6.58 (m, 2H), 3.67 (t, $J = 7.8$ Hz, 2H), 2.95 (s3H), 2.29 (t, $J = 7.8$ Hz, 2H). MS-APCI m/z: 307.3 $[M + H]^+$. Anal. $C_{19}H_{18}N_2O_2$ (C, H, N).

*6.1.7. 2-(2,4-Dinitro-benzensulfonamido)-3-phenylpropionic acid methyl ester (**35**)*

D-Phenylalanine methyl ester hydrochloride **34** (6.50 g, 30.14 mmol) [20] and 2,4-dinitrobenzenesulfonyl chloride (8.04 g, 30.14 mmol) were suspended in 290 mL of CH_2Cl_2. Pyridine (7.28 mL, 90.42 mmol) was added dropwise at rt. The resulting orange solution was stirred for 16 h at rt. The precipitate was filtered off, and the organic solution was washed with 2 N HCl (×3), 1 N $NaHCO_3$ (×3), and brine. The organic phase was dried over Na_2SO_4, the solvent removed under reduced pressure and the solid residue crystallized from CH_2Cl_2/petroleum ether to afford 2,4-dinitrobenzensulfonamide **35** as yellow needles (88%). Mp 116–119 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 3.01–3.09 (dd, $J = 13.9, 7.9$ Hz, 1H), 3.17–3.24 (dd, $J = 13.8, 5.1$ Hz, 1H), 3.65 (s, 3H), 4.50–4.54 (dd, $J = 4.8$ Hz, 1H z), 5.98 (br, 1H), 7.07–7.10 (m, 2H), 7.15–7.21 (m, 3H), 8.06 (d, $J = 8.7$ Hz, 1H), 8.40 (dd, $J = 8.8, 2.2$ Hz, 1H), 8.62 (d, $J = 2.1$ Hz, 1H).

*6.1.8. 2-[*N*-(2,4-Dinitrobenzensulfonyl),*N*-phenethylamino]-3-phenylpropionic acid methyl ester (**36**)*

To a solution of the 2,4-dinitrobenzensulfonamide **35** (1.38 g, 3.37 mmol), phenethyl alcohol (0.82 g, 3.37 mmol), and triphenylphosphine (1.77 g, 6.74 mmol) in 30 mL of dry benzene, DIAD (1.36 g, 6.74 mmol) in 20 mL of dry benzene was added dropwise under inert atmosphere. The resulting yellow solution was stirred for 20 min at rt. The solvent was removed under reduced pressure and the resulting oil was purified by chromatography (SiO_2, ethylacetate/petroleum ether = 1:1). Compound **37** was obtained as a red oil (94%). ^{1}H NMR ($CDCl_3$; 300 MHz) δ 2.84–3.03 (m, 3H), 3.05–3.13 (m, 2H), 3.44–3.55 (m, 4H), 4.96–5.02 (dd, 1H), 7.19–7.35 (m, 10H), 7.93 (d, $J = 8.7$ Hz, 1H), 8.28 (dd, $J = 8.7$, $J = 2.2$ Hz, 1H), 8.35 (d, $J = 2.2$ Hz, 1H).

*6.1.9. 2-Phenethylamino-3-phenylpropionic acid methyl ester (**37**)*

To a solution of **36** (1.94 g, 3.78 mmol) and thioglycolic acid (0.45 g, 4.88 mmol) in 21 mL of dry CH_2Cl_2, Et_3N (1.05 mL, 7.53 mmol) was added dropwise at rt, under an inert atmosphere. The reaction mixture was stirred for 1 h at rt before the addition of 1 N $NaHCO_3$. The organic phase was dried over Na_2SO_4, the solvent evaporated and the crude residue purified by MPLC (SiO_2, CH_2Cl_2:$CH_3OH = 50:1$) to afford **37** as a yellow oil (97%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.70–2.94 (m, 6H), 3.54 (t, 1H), 3.61 (s, 3H), 7.12–7.29 (m, 10H).

*6.1.10. (R)-5-Benzyl-1-phenethylimidazolidin-2,4-dione (**30**)*

Compound **37** (0.22 g, 0.78 mmol) was dissolved in 4.5 mL of acetic acid at rt. KCNO (0.13 g, 1.55 mmol) was then added and the reaction mixture was stirred for 3.5 h at rt. The mixture was evaporated under reduced pressure and the residue was extracted several times with diethyl ether. The organic solvent was evaporated and the product was purified by MPLC (SiO_2, CH_2Cl_2:CH_3OH (1% NH_3) = 30:1). After crystallization from anhydrous benzene/petroleum ether, hydantoin **30** was obtained as a white solid (79%). Mp 117–119 °C. ^{1}H NMR ($CDCl_3$; 300 MHz) δ 2.72–2.89 (m, 2H), 2.99–3.17 (m, 3H), 3.93–4.00 (m, 2H), 7.08 (dd, $J = 7.6$, $J = 1.5$ Hz, 2H), 7.14 (dd, $J = 7.4$, $J = 1.8$ Hz, 2H), 7.24–7.33 (m, 6H). MS-CI m/z: 295 $[M + H]^+$. Anal. $C_{18}H_{17}N_2O_2$(C, H, N).

6.1.11. Isomer identification by ^{1}H NMR spectroscopy

The ^{1}H NMRs of each pair of *E*/*Z* isomers showed significant differences in the chemical shifts of the vinyl proton, and of the phenyl *ortho* protons on the benzylidene moiety at the C5 position. In addition, the methylene protons of the substituents at the N1 position on the hydantoin ring exhibited different chemical shifts in the *E*- and *Z*-isomers. The anisotropic effect of the 4-carbonyl group deshields the vinyl proton in the *Z*-isomers (δ 6.62–6.87) relative to that in the *E*-isomers (δ 6.16–6.51). For the same reason, the phenyl *ortho* protons on the benzylidene moiety in the *E*-isomers are more deshielded than the corresponding ones in the *Z*-isomers, so that the multiplets representing phenyl protons are more widely separated in the spectra of the *E*- than in those of the *Z*-isomers. Assignments were confirmed for compounds **1**, **2**, **7** and **8** by 2D NOESY spectral analysis. In the *E*-isomers (**1** and **7**), NOEs between the vinyl proton and the methylene groups on the phenethyl side chain at N1 could be observed. On the other hand, the same NOEs were not present for *Z*-isomers (**2** and **8**), while NOEs between the phenyl *ortho* protons on the 5-benzylidene and the methylene protons at N1 were detected.

*6.1.12. Isomerization study of compounds **7** and **8** in DMSO*

Pure compounds **7** and **8** (3–5 mg) were dissolved separately in DMSO-d_6, directly in NMR tubes. The solutions were kept at 25 °C, either in the presence or in the absence of light. ^{1}H NMR spectra were obtained at 0, 0.5, 1, 1.5, 2, 3, 8 h and at 1, 3, 7 days. The extent of isomerization was measured by comparing the peak ratio of the vinyl protons of compound **7** (6.37 ppm) and compound **8** (6.67 ppm).

*6.1.13. Isomerization of compounds **7** and **8** in cell culture media*

Stock solutions of compounds **7** and **8** were prepared in DMSO and an aliquot of each was added separately to D-MEM medium. The final concentrations of **7** and **8** in tested samples were 20 μM, with a final DMSO percentage of 0.1% v/v. Samples were incubated at 37 °C in the dark and, at set time intervals (0, 1.5, 3.0, 4.5, 6.0, 8.0, 24 and 72 h), 20 μL aliquots were withdrawn and directly injected into a Shimadzu HPLC gradient system (Shimadzu Corp., Japan), equipped with two LC-10AD pumps, an UV–VIS SPD-10A detector and a RP-C18 column (Supelcosil LC-18-DB, 15 cm × 4.6 mm, 5 μm) to determine the degree of isomerization. An isocratic elution

employing acidified HPLC grade water (0.1% v/v HCOOH) and CH_3CN (65:35) was chosen, at a flow rate of 1 mL/min; UV detection was set at 254 nm.

6.2. Biology

6.2.1. Cellular proliferation assay

The human NSCLC cell line A549 was cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS Gibco, Life Technologies). The cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained under standard cell culture conditions at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. Proliferation rate was evaluated essentially as described elsewhere [21]. Briefly, proliferation rate was determined by cell counting: cells incubated for 72 h with the inhibitor, were detached from the plates by trypsinization and counted in a Brücher hemocytometer by trypan blue exclusion.

6.2.2. Western blot analysis

Procedures for protein extraction, solubilization, and protein analysis by one-dimensional PAGE are described elsewhere [6]. Briefly, proteins (50 μg) from lysates were resolved by 5–15% SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with a mouse anti-human p53 monoclonal antibody (DO1, Santa Cruz Biotechnology, CA, USA) and a mouse anti-human GAPDH monoclonal antibody (Sigma Aldrich), washed and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence system.

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