Bioorganic & Medicinal Chemistry 22 (2014) 6980–6988

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of 21-alkynylaryl pregnenolone derivatives and evaluation of their anticancer activity

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ARTICLE INFO

Article history:
Received 22 June 2014
Revised 4 October 2014
Accepted 9 October 2014
Available online 25 October 2014

Keywords:
Alkynylaryl derivatives
Pregnenolone
Anticancer activity

ABSTRACT

A series of novel C21-alkynylaryl derivatives of pregnenolone were synthesized and screened for anticancer activity against a panel of seven human cancer cell lines (LNCaP, A549, MCF7, HeLa, A431, HepG2, HT29). The data revealed that these compounds can be potential antitumour agents against the specific cell models. Compound **6f** bearing a 2-trifluoromethylphenyl group displayed improved cytotoxicity towards all cancer cell lines used. A431 cells were the most sensitive with derivatives **6e**–**6h** bearing electron withdrawing substituents exhibiting high potency with IC_{50} values ranging between 2.18 and 0.54 μM and drastic inhibition of the prosurvival PI3K-Akt/PKB pathway.

1. Introduction

Cancer is one of the leading causes of death affecting millions of people worldwide. Even though there is an arsenal of available drugs there is an urgent need to develop new agents to overcome the limitations of the current therapies.

Steroids represent an important class of naturally occurring biomolecules which exhibit an astonishing range of biological activities. The rigid tetracyclic skeleton, the presence of several functional groups and the easy modification of the steroids have made them attractive substrates for many different targets. An extensive focus of research activities have been directed towards the rational modification of steroids. In particular, a number of steroid derivatives have been reported to possess anticancer activity while, their excellent ability to penetrate cell membranes is beneficial to their bioavailability.[1,2] The majority of structural studies towards new anticancer steroids involved A and D rings of the steroid skeleton and to a lesser extent B ring modifications.[3–5] In particular, the presence of heterocyclic moieties in D-ring modified steroids has been reported to enhance their biological activities.

Pregnenolone, a naturally occurring steroid has been utilized as a template for the synthesis of steroid derivatives with improved anticancer profile. Thus, the nitrochlorambucil ester of pregnenolone exhibited a significant in vitro cytotoxic activity in brain posterior fossa, medulloblastoma (Daoy), and lung large cell carcinoma (H460) cell lines.[6] In addition, pregnenolone derivatives bearing heterocyclic moieties were found to exhibit anticancer activity. More specifically, 17-pyrazolinyl derivatives of pregnenolone possess interesting activity in vitro against a panel of five human cancer cell lines and especially against HT-29, HCT-15 and 502713 cell lines.[7] In addition, 21-triazolyl derivatives of pregnenolone were found to possess anticancer activity.[8] A more detailed study of heterocylic analogues of pregnenolone, involved pyrazolines, hydrazones, pyrazoles and oximes which were screened against HepG2 and MDA-MB-231 cancer cell lines.[9] 17β-Chalconyl derivatives of pregnenolone and their corresponding pyrazoline epimers were evaluated against a panel of four human cancer cell lines.[10]

Recently, benzylidine pregnenolones and their oxime derivatives were tested for their cytotoxic activities against a panel of six human cancer cell lines and three of them were very potent especially against HCT-15 and MCF-7 cell lines.[11]

We have been involved in the area of steroid research for the past few years and we have successfully synthesized various congeners with neuroprotective,[12] $GABA_A$ modulating[13] and anticancer activities.[14] Thus, in continuation of our research on steroid based medicinal chemistry we set out to explore the anticancer properties of alkynylaryl pregnenolones. We were inspired by the promising results of benzylidine pregnenolones[11] and we incorporated in our design the substitution of the double bond spacer between the aromatic moiety and the C17-carbonyl by an alkyne functionality. Thus, we maintained the same number of carbons (two) however, we introduced a nonflexible spacer of high electron density

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http://dx.doi.org/10.1016/j.bmc.2014.10.012
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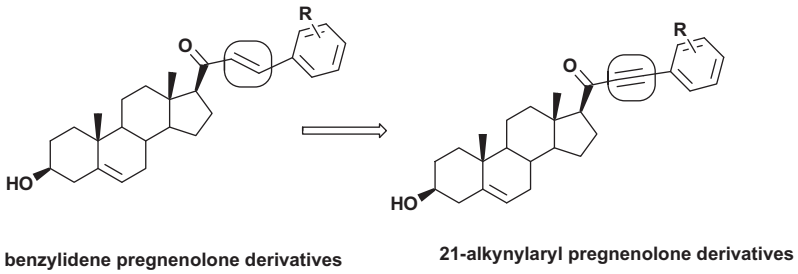


Figure 1. Design of new derivatives.

(Fig. 1). Furthermore, to the best of our knowledge this class of compounds has not been previously investigated.

Therefore, in this study, we describe the synthesis and SAR of C21-alkynylaryl pregnenolone derivatives and their cytotoxic activity against a panel of seven human cancer cell lines.

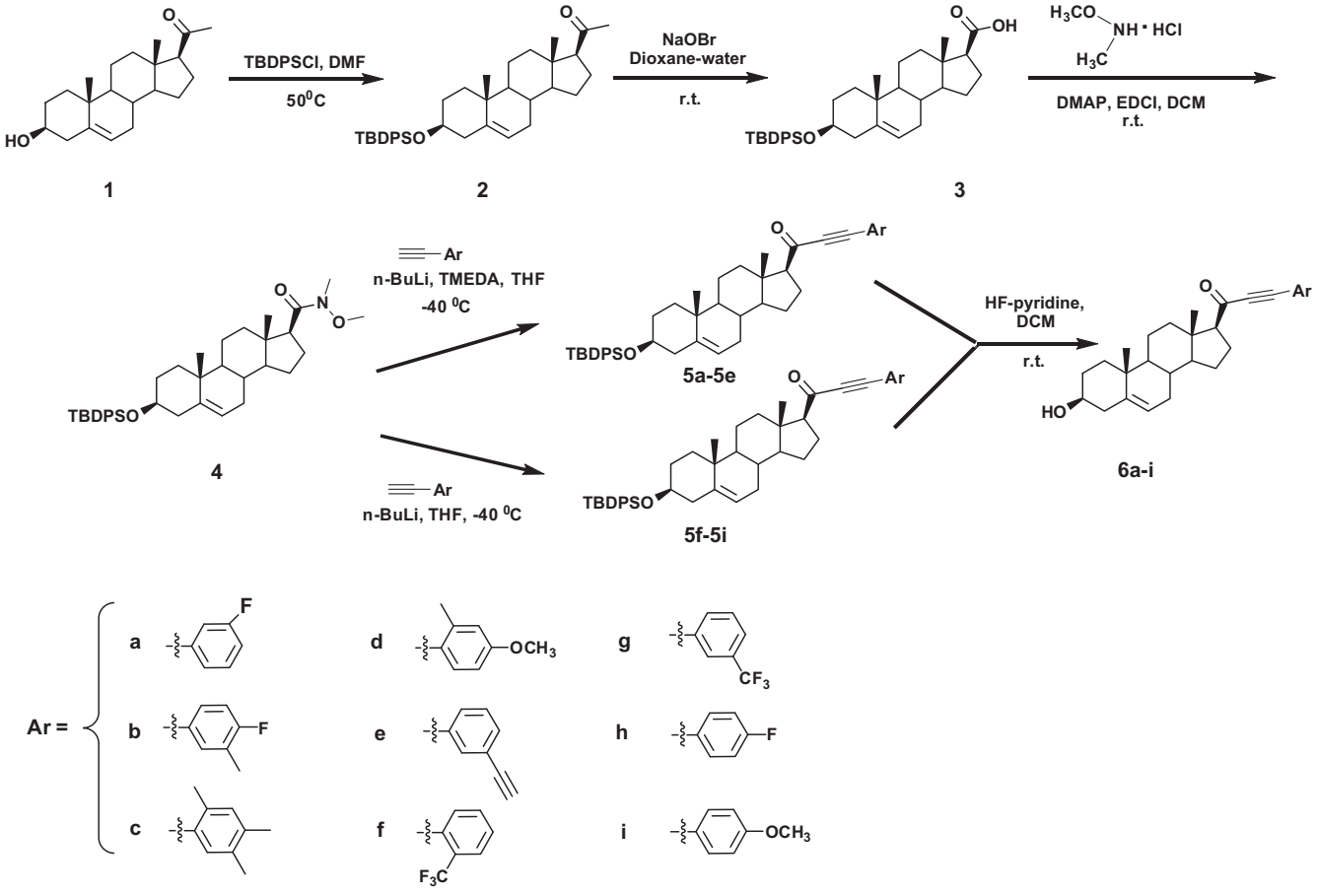
2. Results and discussion

2.1. Chemistry

The synthesis of the compounds of the present study **6a–i** is depicted in Scheme 1. Thus, protection of the 3-hydroxyl group of pregnenolone (**1**) as the *tert*-butyldiphenylsilyl ether afforded compound **2**, which was treated with sodium hypobromide in dioxane/water to give the corresponding 17β-carboxylic acid **3**, which in turn was transformed to the Weinreb amide **4** by treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and *N*,*O*-dimethylhydroxyl amine hydrochloride in the presence of DMAP. Subsequent reaction of compound **4** with the *n*-BuLi and the appropriate arylalkyne in the presence or absence of TMEDA at −40 °C afforded compounds **5a–5e** and **5f–5i**, respectively. Finally, deprotection of the 3β-hydroxyl group using HF·pyridine complex afforded the final compounds **6a–6i**.

2.2. Evaluation of cytotoxic activity

Compounds **6a–6i** were assayed for in vitro cytotoxicity against a panel of human cancer cell lines namely, LNCaP (prostate carcinoma), A549 (lung adenocarcinoma), MCF7 (breast adenocarcinoma), HeLa (cervix adenocarcinoma), A431 (epithelial carcinoma), HepG2 (hepatocellular carcinoma) and HT29 (colon carcinoma) using the MTT assay. The IC_{50} values (μM) for 24 h

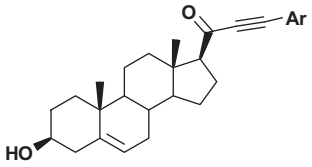


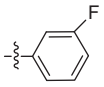
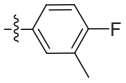
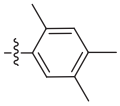
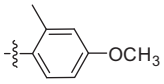
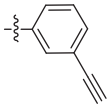
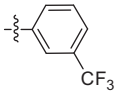
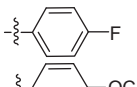
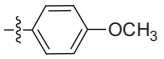
Scheme 1. Synthesis of compounds **6a–i**.

and 48 h incubation are reported in Table 1. Our design involves the variation of the substitution pattern of the aromatic ring of the 21-alkynylaryl moiety in order to obtain information on the structure activity relations of this class of compounds. Thus, we introduced electron donating groups (methoxy, methyl) or electron withdrawing groups (fluorine, trifluoromethyl, ethynyl) or combinations thereof. A number of correlations can be drawn by reviewing the data in Table 1. In general, all the compounds exhibit increased in vitro cytotoxicity after 48 h incubation when compared with the 24 h experiment. Therefore, we opted to target the discussion of our results using the IC_{50} values of the longer experiment.

Thus, the 2-trifluoromethyl substituted derivative **6f** is the most potent against LNCaP cells (IC_{50} = 5.44 ± 0.40 μM), A549 (IC_{50} = 6.30 ± 0.84 μM), MCF7 cells (IC_{50} = 4.42 ± 0.76 μM), HeLa cells (IC_{50} = 3.85 ± 0.60 μM), HepG2 cells (IC_{50} = 12.64 ± 2.17 μM), and HT29 cells (IC_{50} = 9.87 ± 1.55 μM), while it exhibits excellent activity against A431 cells (IC_{50} = 1.78 ± 0.22 μM). Conversely, the 3-trifluoromethylphenyl substituted analogue **6g** is less active than the 2-substituted congener with IC_{50} values for LNCaP IC_{50} = 17.40 ± 2.81 μM, for A549 cells IC_{50} = 20.74 ± 6.70 μM, for MCF7 cells IC_{50} = 18.56 ± 2.11 μM, for HeLa cells IC_{50} = 8.34 ± 1.26 μM, for HepG2 IC_{50} = 16.45 ± 2.55 μM and for HT29 IC_{50} = 26.45 ± 2.3 μM. Surprisingly however, compound **6g** is the most active derivative against A431 (IC_{50} = 0.54 ± 0.08 μM). The fluoro substituted compounds **6a** and **6h** are both very active against LNCaP cells exhibiting IC_{50} = 9.48 ± 0.75 μM and IC_{50} = 10.78 ± 1.42 μM, respectively. However, there is a marked difference in activity of **6a** and **6h** with respect to A549 and HT29 cells which is even more pronounced against MCF7 and HeLa cells. Specifically, the 3-fluoro derivative **6a** possesses an IC_{50} = 24.95 ± 3.70 μM for A549 cells, an IC_{50} = 28.58 ± 3.20 μM for HT29 cells, an IC_{50} = 14.70 ± 2.48 μM for MCF7 cells and an IC_{50} = 6.44 ± 0.82 μM for HeLa cells. In contrast the 4-fluoro analogue **6h** is slightly active against A549 cells (IC_{50} = 40.57 ± 5.76 μM) and HT29 cells (IC_{50} = 44.70 ± 5.92 μM) and inactive against MCF7 and HepG2 cells (IC_{50} >50 μM). It is worth noting however, that this order is reversed for A431 cells with compound **6h** to possess an IC_{50} = 2.18 ± 0.34 μM while, **6a** an IC_{50} = 45.32 ± 3.15 μM. Furthermore, the combination of a 3-methyl and a 4-fluoro substituent on the phenyl ring, compound **6b**, leads to an increase in activity against MCF7 (IC_{50} = 11.30 ± 0.42 μM), HT29 cells (IC_{50} = 26.70 ± 4.18 μM) and A549 cells (IC_{50} = 28.2 ± 2.55 μM), while, it decreases activity against LNCaP cells (IC_{50} =

Table 1
Cytotoxic activity of compounds **6a**–**6i**



Compound	Ar	(h)	LNCaP IC_{50}[a] (μM)	A549 IC_{50}[a] (μM)	MCF7 IC_{50}[a] (μM)	HeLa IC_{50}[a] (μM)	A431 IC_{50}[a] (μM)	HepG2 IC_{50}[a] (μM)	HT29 IC_{50}[a] (μM)
6a		24	14.70 ± 1.38	>50	28.40 ± 2.27	21.38 ± 2.81	>50	>50	>50
		48	9.48 ± 0.75	24.95 ± 3.70	14.70 ± 2.48	6.44 ± 0.82	45.32 ± 3.15	>50	28.58 ± 3.20
6b		24	>50	40.05 ± 5.45	17.60 ± 1.98	>50	>50	>50	>50
		48	38.40 ± 2.80	28.2 ± 2.55	11.30 ± 0.42	>50	>50	>50	26.70 ± 4.18
6c		24	>50	>50	39.00 ± 2.00	>50	39.31 ± 3.20	>50	>50
		48	46.2 ± 4.80	>50	7.78 ± 1.72	32.4 ± 2.80	12.46 ± 1.34	48.56 ± 5.20	>50
6d		24	41.70 ± 4.66	44.30 ± 0.99	44.15 ± 3.04	>50	44.12 ± 5.30	>50	48.35 ± 5.30
		48	30.80 ± 2.32	30.47 ± 4.40	18.8 ± 3.96	23.57 ± 3.18	13.67 ± 0.88	45.70 ± 4.94	22.68 ± 4.60
6e		24	19.10 ± 0.99	>50	11.25 ± 0.35	>50	4.85 ± 0.78	42.57 ± 4.80	>50
		48	16.85 ± 1.22	20.20 ± 0.60	5.15 ± 0.35	>50	1.52 ± 0.24	17.85 ± 2.35	>50
6f		24	7.90 ± 1.84	15.77 ± 4.06	13.48 ± 5.54	13.75 ± 2.18	4.21.05 ± 0.45	38.42 ± 4.13	24.56 ± 3.75
		48	5.44 ± 0.40	6.30 ± 0.84	4.42 ± 0.76	3.85 ± 0.60	1.78 ± 0.22	12.64 ± 2.17	9.87 ± 1.55
6g		24	26.20 ± 1.30	29.31 ± 21.9	31.45 ± 4.76	25.10 ± 3.19	2.28 ± 0.42	45.68 ± 5.36	>50
		48	17.40 ± 2.81	20.74 ± 6.70	18.56 ± 2.11	8.34 ± 1.26	0.54 ± 0.08	16.45 ± 2.55	26.45 ± 2.93
6h		24	12.40 ± 2.80	>50	>50	>50	7.16 ± 1.04	>50	>50
		48	10.78 ± 1.42	40.57 ± 5.76	>50	43.70 ± 5.16	2.18 ± 0.34	>50	44.70 ± 5.92
6i		24	>50	>50	>50	>50	>50	>50	>50
		48	47.60 ± 6.41	>50	>50	26.70 ± 3.45	>50	>50	>50

[a] IC_{50} values in μM are reported as the mean ± SD from 3 to 4 independent experiments.

38.4 ± 2.8 μM), HeLa and A431 cells (IC_{50} = >50 μM) with respect to analogue **6h**. In contrast the 3-methyl,4-methoxyphenyl derivative **6d** exhibits weak activity against LNCaP, A549 and HepG2 cells and is more potent against MCF7 cells (IC_{50} = 18.8 ± 3.96 μM) and A431 cells (IC_{50} = 13.67 ± 0.88 μM), while the 4-methoxyphenyl derivative **6i** is inactive against A549, MCF7, A431, HepG2 and HT29 cells (IC_{50} = >50 μM), slightly active against LNCaP cells (IC_{50} = 47.60 ± 6.41 μM) and more potent against HeLa cells (IC_{50} = 26.70 ± 3.45 μM). The 2,4,5-trimethylphenyl analogue **6c** exhibits high potency against MCF7 cells (IC_{50} = 7.78 ± 1.72 μM) and A431 cells (IC_{50} = 12.46 ± 1.34 μM), lower activity against HeLa cells (IC_{50} = 32.4 ± 2.80 μM) which is decreased even more against LNCaP cells (IC_{50} = 46.2 ± 4.80 μM) and HepG2 cells (IC_{50} = 48.56 ± 5.20 μM), and the activity is abolished for A549 and HT29 cells. Finally, introduction of a 3-ethynylphenyl substituent, compound **6e**, results in good activity against LNCaP, A549 cells and HepG2 cells (IC_{50} = 16.85 ± 1.22 μM, IC_{50} = 20.20 ± 0.60 μM and IC_{50} = 17.85 ± 2.35 μM, respectively), and excellent against A431 cells (IC_{50} = 1.52 ± 0.24 μM) and MCF7 cells (IC_{50} = 5.15 ± 0.35 μM). In general, electron withdrawing substituents favour activity against LNCaP cells (compounds **6a**, **6e**, **6f**, **6g**, **6h**) while their combination with electron donating groups is detrimental to activity against this cell line (**6b** versus **6e**). Concerning A549 cells no clear conclusion can be drawn, however the trend observed for LNCaP cells is observed also for A549. The majority of the alkynylaryl pregnenolone derivatives of the present study are very potent against MCF7 cells and the activity is very much dependent on the electronegativity and the position of the substituents on the phenyl ring. Compounds **6a**, **6f**, **6g** and **6h** bearing electron withdrawing substituents are very potent against HeLa cells, while, derivatives **6e**, **6f**, **6g** and **6h** exhibit excellent activity against A431 cells. Compounds **6e**, **6f** and **6g** and compound **6f** are the most potent against HepG2 and HT29 cells, respectively.

Even though an incubation period of 48 h is most frequently used for the evaluation of antiproliferative action, we also examined the effects of our compounds at 24 h incubation in order to get an insight on their acute cytotoxic activity. As we observe in Table 1 the potent compounds (i.e., **6a**, **6b**, **6e**, **6f**, **6g**, **6h**) exhibit good activity even after a short incubation period (24 h) which of course improves after 48 h. This is also depicted in Figure 2 which includes the dose response curves of the most potent compound **6f** against LNCaP, A549 and MCF7 cell lines.

The acute cytotoxic activity of the potent derivatives was also corroborated by determination of cell viability using the trypan blue exclusion test. HeLa, MCF7 and LNCaP cells were treated with 10 μM concentration of compounds **6e**, **6f**, **6g** and **6h** whereas A431 cells were treated with 5 μM concentration, in line with the IC_{50} values in Table 1, and the viability was determined after 24 h. (Table 2).

The results obtained by trypan blue were similar to those obtained by the MTT test and reveal a clear cytotoxic effect of these compounds. Morphological alterations, such as rounded cells were also visible as soon as after 8 h of incubation (data not shown). Interestingly, the extent of the cytotoxic effects of the compounds depended on the tumour cell line investigated with A431, the epidermoid carcinoma, being the most sensitive. These cells are highly sensitive to mitogenic stimuli because overexpress the epidermal growth factor receptor. The best-characterized pathway known to transduce growth factor activation and promote cell growth or survival in cancer cells, is the Akt/protein kinase B (Akt), a key effector of phosphatidylinositol 3′-kinase (PI3K).[15] Phosphorylation of Akt at Thr308 and Ser473 is required for activation and—in this form—is found consistently hyperactivated in many tumours. Based on the above, we evaluated the effects of the most active compounds on the levels of phosphorylated Akt (Ser^{473}) (Fig. 3). Cells were treated with compounds **6e**–**6g** at a concentration corresponding to their IC_{50} value. Cell lysates were prepared 24 h later and 50 μg each, were loaded into each well and fractionated by 10% SDS–PAGE. Immunoblotting was performed as described in 'Materials and Methods'. Equal loading was verified by blotting the membranes with an antibody against β-actin. Indeed, western blot analysis demonstrated that cytotoxic compounds **6e**–**6g** interfere with the PI3K-Akt/PKB pathway which is a crucial survival signalling pathway for a variety of human cancers (Fig. 3).

Finally, we also examined the effect of dehydroepiandrosterone (DHEA) against the cell lines of the present study in order to get an insight as to whether the biological activity we observe for compounds **6a**–**i** is related to the substitution on position C17. DHEA is inactive against all the cell lines (IC_{50} >50 μM) and marginally active against A549 cells (IC_{50} = 47.8 ± 3.80 μM) and thus, we can

Table 2
Cell viability—expressed as percentage of viable cells over the total number of cells—after 24 h of exposure to compounds **6e**–**6h**

Compound	HeLa (10 μM)	LNCaP (10 μM)	A431 (5 μM)	MCF7 (10 μM)
6e	93.2 ± 4.5	90.3 ± 7.6	0	0
6f	70.8 ± 10.3	0	0	18.9 ± 2.2
6g	92.5 ± 6.2	92.1 ± 6.8	0	94.2 ± 7.3
6h	98.4 ± 1	25.6 ± 4.7	28.6 ± 3.2	90.4 ± 7.7

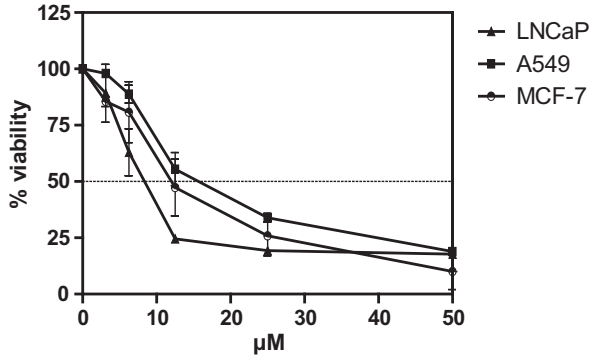


Figure 2. Dose response curves of the cytotoxic activity of compound **6f** on LNCaP, A549 and MCF7 cell lines. Values are the mean ± standard deviation of four independent experiments.

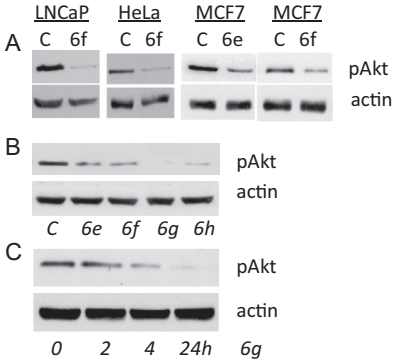


Figure 3. Effect of selected compounds on Akt phosphorylation in cancer cell lines The phosphorylation of Akt (pAkt-Ser^{473}) is shown in the *top panels*, and total levels of actin are shown in the *bottom panels*. (A) effects of **6e**, **6f** on pAkt levels in LNCaP, HeLa and MCF7 cells; (B) effects of **6e**, **6f**, **6g** and **6h** on pAkt in A431 cells; (C) time dependent effect of **6g** on pAkt in A431 cells. *C*: untreated cells.

conclude that the 20-ketoalkynylaryl moiety is responsible for imparting anticancer activity to the corresponding pregnenolone derivatives.

3. Conclusions

A series of novel 21-alkynylaryl derivatives of pregnenolone were synthesized and evaluated for their anticancer activity against seven human cancer cell lines. The majority of the compounds exhibited anticancer activity and the 2-trifluoromethylphenyl derivative **6f** was found to posses promising activity against all seven cell lines. A431 cells were the most sensitive against the compounds of the present study, with derivatives **6e–6h** bearing electron withdrawing substituents exhibiting high potency with IC_{50} values ranging between 2.18 and 0.54 μM. Furthermore, these compounds were found to interfere with the PI3K-Akt/PKB pathway. These results could further support future design of pregnenolone-based anticancer drugs.

4. Experimental section

4.1. Chemistry

^{1}H NMR spectra were recorded at 600 or 300 MHz, ^{13}C NMR spectra at 150.9 or 75.5 MHz and ^{19}F NMR spectra at 564.8 MHz. ^{1}H and ^{13}C NMR spectra are internally referenced to residual solvent signals ($CDCl_3$). ^{19}F NMR spectra are referenced to CCl_3F. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, dd = doublet of doublet, ddd = doublet of doublet of doublet, m = multiplet), coupling constant and integration. Data for ^{13}C NMR are reported in terms of chemical shift (δ ppm). Melting points (°C) are uncorrected. IR spectra were obtained on an ATR-IR spectrometer. HR-mass spectra were recorded on UHPLC LC-MSn Orbitrap Velos-Thermo instrument. Elemental analyses were obtained using a Perkin Elmer Series II CHNS/O 2400 instrument. THF was distilled over sodium in the presence of benzophenone, DCM was distilled over calcium hydride and DMF was dried over 4 Å molecular sieves. Thin-layer chromatography (TLC) was performed on glass plates coated with silica gel (0.2 mm, 60 F254) and flash chromatography using silica gel (200–400 mesh).

4.1.1. 3β-(*t*-Butyldiphenylsilyloxy)-5-pregnen-20-one (2)[16]

To a solution of pregnenolone (**1**) (2.0 g, 6.32 mmol) in DMF (40 mL) imidazole (1.1 g, 15.8 mmol) was added at 0 °C and the reaction was stirred at that temperature for 30 min, and TBDPSCl (4.7 mL, 15.8 mmol) was added. The reaction was warmed 50 °C and was stirred overnight. Subsequently, the reaction was cooled to room temperature, was quenched with saturated NH_4Cl, and the solvent was evaporated under reduced pressure. The residue was diluted with H_2O and was extracted with ethyl acetate. The organic phase was washed with brine, was dried over anhydrous Na_2SO_4 and was evaporated in vacuo. The residue was recrystallized from ethanol to afford **2** in quantitative yield (3.5 g, 100%). mp 125–129 °C; $[\alpha]_D^{20}$ 6.04° (ca. 0.00149 g/mL, $CHCl_3$); R_f: 0.68 (cyclohexane/ethyl acetate 8:2); ^{1}H NMR ($CDCl_3$): δ 0.60 (s, 3H), 0.81–0.91 (m, 3H), 0.98 (s, 3H), 1.06 (s, 9H), 1.15–2.03 (m, 14H), 2.10 (s, 3H), 2.14–2.38 (m, 2H), 2.49 (t, 1H, J = 9.0 Hz), 3.48–3.58 (m, 1H), 5.12 (d, 1H, J = 4.9 Hz), 7.33–7.44 (m, 6H), 7.66–7.72 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.4, 19.3, 19.6, 21.2, 23.0, 24.6, 27.2, 31.7, 31.9, 32.0, 32.1, 36.7, 37.4, 39.1, 42.6, 44.2, 50.1, 57.1, 63.9, 73.3, 121.0, 127.63, 127.65, 129.61, 129.64, 134.94, 134.98, 135.95, 135.96, 141.4, 209.8; Anal. Calcd for $C_{37}H_{50}O_2Si$: C, 80.09; H, 9.08. Found C, 79.68; H, 8.79.

4.1.2. 3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-carboxylic acid (3)

To a solution of compound **2** (3.2 g, 5.83 mmol) in dioxane (77 mL) and water (23 mL) was added at 0 °C a solution of NaOBr (158 mL) (prepared by mixing a solution of 15.3 g NaOH in 131 mL water with 84.6 mL dioxane and 5.08 mL Br_2 at 0 °C). The resulting mixture was stirred at ambient temperature overnight, and the mixture was cooled to 0 °C and was quenched with saturated Na_2SO_3, followed by an additional 10 min stirring at the same temperature. The mixture was extracted with ethyl acetate and the organic phase was washed with water and brine, was dried over anhydrous Na_2SO_4 and was evaporated in vacuo. Compound **3** was obtained as white crystals (3.22 g, 99%) and was pure enough to be employed in the next step without any further purification. mp 195–198 °C; $[\alpha]_D^{20}$ −19.33° (ca. 0.00119 g/mL, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 3237, 2935, 1720; R_f: 0.46, cyclohexane/ethyl acetate 9:1; ^{1}H NMR ($CDCl_3$): δ 0.76 (s, 3H), 0.85–0.96 (m, 3H), 1.02 (s, 3H), 1.10 (m, 9H), 1.18–2.21 (m, 16H), 2.38 (t, 1H, J = 9.3 Hz), 3.52–3.63 (m, 1H), 5.15 (d, 1H, J = 4.9 Hz), 7.36–7.47 (m, 6H), 7.70–7.74 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.1, 19.1, 19.4, 20.6, 23.3, 24.5, 26.9, 27.0, 30.2, 31.7, 31.8, 31.9, 36.5, 37.2, 37.9, 42.4, 44.1, 49.9, 55.1, 56.3, 73.2, 120.8, 127.42, 127.44, 129.41, 129.43, 134.74, 134.75, 135.73, 135.74, 141.3, 180.5; Anal. Calcd for $C_{36}H_{48}O_3Si$: C, 77.65; H, 8.69. Found C, 77.34; H, 9.07.

4.1.3. *N*-Methoxy,*N*-methyl-3β-(*t*-butyldiphenylsilyloxy)-androst-5-en-17β-carboxamide (4)[17]

To a stirred solution of **3** (1.2 g, 2.16 mmol) in dry CH_2Cl_2 (12 mL) were sequentially added *N*,*O*-dimethylhydroxylamine hydrochloride (316 mg, 3.23 mmol), 4-dimethylaminopyridine (395 mg, 3.23 mmol) and EDCI (620 mg, 3.23 mmol) at room temperature and the reaction mixture was stirred overnight. When the reaction was completed, brine was added and the mixture was diluted with ethyl acetate. The organic phase was washed with 5% HCl solution and brine, it was dried over anhydrous Na_2SO_4 and was evaporated in vacuo. The residue was purified by flash column chromatography (petroleum ether–acetone, 95:5) to afford compound **4** (1.21 g, 94%) as a white crystalline solid. mp 143–46 °C; $[\alpha]_D^{20}$ −45.73° (ca. 0.00164 g/mL, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 2932, 1652; R_f: 0.26, petroleum ether/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.73 (s, 3H), 0.77–0.88 (m, 3H), 0.98 (s, 3H), 1.05 (s, 9H), 1.14–2.38 (m, 16H), 2.72–2.75 (m, 1H), 3.18 (s, 3H), 3.48–3.58 (m, 1H), 3.63 (s, 3H), 5.13 (d, 1H, J = 5.0 Hz), 7.32–7.44 (m, 6H), 7.66–7.69 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.8, 19.1, 19.4, 20.9, 24.8, 24.9, 27.0, 31.8, 31.9, 32.0, 36.5, 37.2, 38.9, 42.3, 42.4, 45.1, 50.1, 50.7, 56.7, 60.9, 73.2, 120.9, 127.4, 127.5, 129.3 129.4, 134.7, 134.8, 135.5, 135.6, 141.3, 174.7; MS (ESI^+), m/z 600.39 $[M+H]^+$; Anal. Calcd for $C_{38}H_{53}NO_3Si$: C, 76.08; H, 8.90; N, 2.33. Found C, 76.24; H, 9.07; N, 2.63.

4.1.4. General procedures for the synthesis of the alkynylaryl pregnenolone derivatives 5a–i

Method A. To a solution of *n*-BuLi (1.6 M solution in hexanes) in anhydrous THF (0.33 M, 2 equiv) at −40 °C was added TMEDA (2 equiv) and the resulting solution was stirred at that temperature for 15 min. Subsequently, the appropriate alkyne (3 equiv) was added at −40 °C and the reaction was stirred at that temperature for 1 h after which a solution of the Weinreb amide **4** (1 equiv) in THF (0.055 M) was added and the temperature was allowed to rise to −10 °C over 3 h and the reaction was stirred at −10 °C until completion. The reaction was quenched with saturated NH_4Cl solution (3 mL) and was diluted with diethyl ether. The organic layer was washed with brine, was dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. The residue was purified by flash column chromatography to afford the desired compounds **5a–e**.

Method B. To a solution of *n*-BuLi (1.6 M solution in hexanes) in anhydrous THF (0.33 M, 2 equiv) was added at −40 °C the appropriate alkyne (3 equiv) and the reaction was stirred at that temperature for 1 h. Subsequently a solution of the Weinreb amide **4** (1 equiv) in THF (0.055 M) was added and the temperature was allowed to rise to −10 °C over 3 h and the reaction was stirred at −10 °C until completion. The reaction was quenched with saturated NH_4Cl solution (3 mL) and was diluted with diethyl ether. The organic phase was washed with brine, was dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. The residue was purified by flash column chromatography to afford the desired compounds **5f**–**i**.

4.1.4.1. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(3-fluorophenyl)-2-propyn-1-one (5a). Compound **5a** was prepared according to *Method A* from Weinreb amide **4** (0.4 g, 0.66 mmol) and 1-ethynyl-3-fluorobenzene (0.24 mL, 2.00 mmol) as an oil after purification by flash column chromatography (petroleum ether–acetone, 95:5) (300 mg, 68%). IR: 2932, 2198, 1663 cm^{-1}; R_f: 0.47, petroleum ether/acetone 95:5; ^1H NMR ($CDCl_3$): δ 0.71 (s, 3H), 0.83–0.92 (m, 3H), 0.98 (s, 3H), 1.06 (s, 9H), 1.12–2.38 (m, 16H), 2.69 (t, J = 8.6 Hz, 1H), 3.50–3.58 (m, 1H), 5.13 (d, J = 4.4 Hz, 1H), 7.07–7.23 (m, 2H), 7.28–7.42 (m, 8H), 7.66–7.69 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.2, 19.1, 19.3, 20.9, 22.2, 24.3, 26.9, 31.6, 31.7, 36.4, 37.2, 38.4, 42.4, 45.1, 49.9, 56.9, 64.8, 73.1, 88.9 (d, J_{C-F} = 3.5 Hz), 89.4, 117.9 (d, J_{C-F} = 21.1 Hz), 119.3 (d, J_{C-F} = 23.1 Hz), 120.7, 122.0 (d, J_{C-F} = 9.2 Hz), 127.3, 127.4, 128.6 (d, J_{C-F} = 3.2 Hz), 129.37, 129.39, 130.2 (d, J_{C-F} = 8.5 Hz), 134.6, 134.7, 135.6, 135.7, 141.1, 162.1 (d, J_{C-F} = 248.2 Hz), 188.5; ^{19}F NMR ($CDCl_3$): δ (−111.94)–(−111.86) (m); HR-MS (ESI^+): $[M+H]^+$, found 659.3709. $C_{44}H_{52}FO_2Si$ requires 659.3715; $[M+Na]^+$, found 681.3523. $C_{44}H_{51}FO_2SiNa$ requires 681.3535.

4.1.4.2. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(3-methyl-4-fluorophenyl)-2-propyn-1-one (5b). Compound **5b** was prepared according to *Method A* from Weinreb amide **4** (0.1 g, 0.16 mmol) and 4-ethynyl-1-fluoro-2-methylbenzene (0.06 mL, 0.50 mmol) as yellow crystals after purification by flash column chromatography (petroleum ether 40–60 °C/acetone, 95:5) (80 mg, 71%). mp 143–145 °C; IR: 2932, 2190, 1651 cm^{-1}; R_f: 0.26, petroleum ether 40–60 °C/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.72 (s, 3H), 0.84–0.95 (m, 3H), 1.00 (s, 3H), 1.07 (s, 9H), 1.14–2.19 (m, 16H), 2.28 (d, J = 1.9 Hz, 3H), 2.69 (t, J = 8.8 Hz, 1H), 3.50–3.60 (m, 1H), 5.14 (d, J = 5.1 Hz, 1H), 7.01 (dd, J = 8.5, 9.1 Hz, 1H), 7.34–7.46 (m, 8H), 7.68–7.71 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.2, 14.3 (d, J_{C-F} = 3.7 Hz), 19.1, 19.4, 20.9, 22.3, 24.3, 26.9, 31.7, 31.82, 31.85, 36.5, 37.2, 38.4, 42.4, 45.1, 49.9, 56.9, 64.9, 73.1, 88.9 (d, J_{C-F} = 1.4 Hz), 90.4 (d, J_{C-F} = 0.9 Hz), 115.7 (d, J_{C-F} = 23.4 Hz), 115.9 (d, J_{C-F} = 3.9 Hz), 120.7, 125.8 (d, J_{C-F} = 18.3 Hz), 127.4, 127.5, 129.4, 129.5, 132.5 (d, J_{C-F} = 8.8 Hz), 134.7, 134.8, 135.7, 135.8, 136.2 (d, J_{C-F} = 6.0 Hz), 141.2, 162.5 (d, J_{C-F} = 252.1 Hz), 188.9; ^{19}F NMR ($CDCl_3$): δ (−111.43)–(−111.34) (m).; HR-MS (ESI^+): $[M+H]^+$, found 673.3878. $C_{45}H_{54}FO_2Si$ requires 673.3972; $[M+Na]^+$, found 695.3696. $C_{45}H_{53}FO_2SiNa$ requires 695.3691.

4.1.4.3. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(2,4,5-trimethylphenyl)-2-propyn-1-one (5c). Compound **5c** was prepared according to *Method A* from Weinreb amide **4** (0.2 g, 0.33 mmol) and 1-ethynyl-2,4,5-trimethylbenzene (0.148 g, 1.0 mmol) as a crystalline solid after purification by flash column chromatography (petroleum ether 40–60 °C/acetone, 95:5) (103 mg, 45% yield). mp 74–76 °C; IR: 2932, 2184, 1656 cm^{-1}; R_f: 0.60, petroleum ether 40–60 °C/acetone 95:5; ^1H NMR ($CDCl_3$): δ 0.75 (s, 3H), 0.85–0.93 (m, 3H), 1.01 (s, 3H), 1.09 (s, 9H), 1.13–2.16 (m, 14H), 2.22 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 2.30–2.36 (m, 2H), 2.42 (s, 3H, CH_3), 2.71 (t, J = 8.7 Hz, 1H), 3.51–3.61 (m, 1H), 5.15 (d, J = 4.3 Hz, 1H), 7.02 (s, 1H), 7.31 (s, 1H), 7.36–7.45 (m, 6H), 7.69–7.71 (m, 4H); ^3C NMR ($CDCl_3$): δ 13.3, 19.0, 19.1, 19.4, 19.8, 20.1, 20.9, 22.6, 24.5, 26.9, 31.7, 31.8, 31.9, 36.5, 37.2, 38.7, 42.4, 45.1, 50.0, 56.9, 64.8, 73.2, 91.0, 92.6, 117.1, 120.7, 127.41, 127.43, 129.40, 129.42, 131.1, 134.2, 134.4, 134.7, 134.8, 135.72, 135.73, 139.4, 139.9, 141.3, 189.0; HR-MS (ESI^+): $[M+H]^+$ found 683.4289. $C_{47}H_{59}O_2Si$ requires 683.4279; $[M+Na]^+$ found 705.4108. $C_{47}H_{58}O_2SiNa$ requires 705.4098.

4.1.4.4. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(2-methyl-4-methoxyphenyl)-2-propyn-1-one (5d). Compound **5d** was prepared according to *Method A* from Weinreb amide **4** (0.2 g, 0.33 mmol) and 1-ethynyl-2-methyl-4-methoxybenzene (146 mg, 1.0 mmol) as a crystalline solid after purification by flash column chromatography (petroleum ether–acetone, 98:2) (207 mg, 91% yield). mp 66–69 °C; IR: 2932, 2183, 1654 cm^{-1}; R_f: 0.43, petroleum ether 40–60 °C/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.76 (s, 3H), 0.88–0.90 (m, 3H), 1.02 (s, 3H), 1.09 (s, 9H), 1.16–2.38 (m, 16H), 2.49 (s, 3H), 2.70–2.72 (m, 1H), 3.58 (br s, 1H), 3.81 (s, 3H), 5.17 (br s, 1H), 6.72–6.78 (m, 2H), 7.41–7.51 (m, 7H), 7.71 (br s, 4H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.0, 19.3, 20.9, 21.0, 22.5, 24.4, 26.9, 31.7, 31.8, 36.5, 37.2, 38.6, 42.4, 45.0, 49.9, 55.2, 56.8, 64.8, 73.1, 91.2, 92.9, 111.6, 112.0, 115.3, 120.7, 127.3, 127.4, 129.3, 129.4, 134.6, 134.62, 134.7, 135.4, 135.7, 141.2, 144.3, 161.4, 188.9; HR-MS (ESI^+): $[M+H]^+$ found 685.4096. $C_{46}H_{57}O_3Si$ requires 685.4071; $[M+Na]^+$ found 707.3916. $C_{46}H_{56}O_3SiNa$ requires 707.3891.

4.1.4.5. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(3-ethynylphenyl)-2-propyn-1-one (5e). Compound **5e** was prepared according to *Method A* from Weinreb amide **4** (0.2 g, 0.33 mmol) and 1,3-diethynylbenzene (0.14 mL, 1.0 mmol) as an oil after purification by flash column chromatography (petroleum ether 40–60 °C/ethyl acetate, 95:5) (104 mg, 47% yield). IR: 2932, 2194, 1661 cm^{-1}; R_f: 0.58, cyclohexane/ethyl acetate 9:1; ^1H NMR ($CDCl_3$): δ 0.73 (s, 3H), 0.85–0.92 (m, 3H), 1.00 (s, 3H), 1.08 (s, 9H), 1.14–2.40 (m, 16H), 2.71 (t, 1H, J = 8.6 Hz), 3.13 (s, 1H), 3.51–3.61 (m, 1H), 5.15 (d, 1H, J = 4.7 Hz), 7.32–7.72 (m, 14H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.1, 19.3, 20.9, 22.3, 24.3, 27.0, 31.7, 31.80, 31.82, 36.4, 37.1, 38.5, 42.4, 45.1, 49.9, 56.9, 64.9, 73.1, 78.6, 82.1, 89.4, 89.5, 120.7, 120.8, 122.9, 127.4, 127.5, 128.7, 129.4, 129.41, 132.9, 133.9, 134.7, 134.8, 135.7, 135.8, 136.0, 141.2, 188.8; HR-MS (ESI^+): $[M+H]^+$ found 665.3824. $C_{46}H_{53}O_2Si$ requires 665.3809; $[M+Na]^+$ found 687.3641. $C_{46}H_{52}O_2SiNa$ requires 687.3629.

4.1.4.6. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(2-trifluoromethylphenyl)-2-propyn-1-one (5f). Compound **5f** was prepared according to *Method B* from Weinreb amide **4** (0.1 g, 0.16 mmol) and 1-ethynyl-2-trifluoromethylbenzene (0.07 mL, 0.50 mmol) as an oil after purification by flash column chromatography (cyclohexane–ethyl acetate, 98:2) (112 mg, 95% yield). IR: 2931, 2206, 1664 cm^{-1}; R_f: 0.44, petroleum ether 40–60 °C/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.75 (s, 3H), 0.86–0.92 (m, 3H), 1.00 (s, 3H), 1.09 (s, 9H), 1.15–2.72 (m, 16H), 2.73 (t, J = 8.8 Hz, 1H), 3.52–3.62 (m, 1H), 5.15 (d, J = 4.4 Hz, 1H), 7.35–7.45 (m, 6H), 7.51–7.59 (m, 2H), 7.69–7.73 (m, 6H); ^{13}C NMR ($CDCl_3$): δ 13.6, 19.3, 19.7, 21.1, 22.9, 24.8, 27.2, 32.0, 32.11, 32.15, 36.8, 37.4, 38.6, 42.7, 45.7, 50.2, 57.1, 65.1, 73.4, 85.9, 93.1 (q, J_{C-F} = 0.9 Hz), 118.4 (q, J_{C-F} = 2.1 Hz), 120.7, 123.1 (q, J_{C-F} = 272.5 Hz), 126.1 (q, J_{C-F} = 5.0 Hz), 127.4, 127.5, 129.4, 129.5, 130.1, 131.6 (q, J_{C-F} = 0.9 Hz), 132.5 (q, J_{C-F} = 31.1 Hz), 134.7, 134.8, 135.4, 135.7, 135.8, 141.3, 188.9; ^{19}F NMR ($CDCl_3$): δ −62.1 (s); HR-MS (ESI^+): $[M+H]^+$ found 709.3689. $C_{45}H_{52}F_3O_2Si$

requires 709.3683; $[M+Na]^+$ found 731.3510. $C_{45}H_{51}F_3O_2SiNa$ requires 731.3503.

4.1.4.7. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(3-trifluoromethylphenyl)-2-propyn-1-one (5g). Compound **5g** was prepared according to *Method B* from Weinreb amide **4** (0.1 g, 0.16 mmol) and 1-ethynyl-3-trifluoromethylbenzene (0.07 mL, 0.50 mmol) as an oil after purification by flash column chromatography (cyclohexane/ethyl acetate, 98:2) (70 mg, 59% yield). IR 2931, 2206, 1663 cm^{-1}; R_f: 0.61, petroleum ether/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.74 (s, 3H), 0.84–0.94 (m, 3H), 1.00 (s, 3H), 1.07 (m, 9H), 1.15–2.40 (m, 16H), 2.72 (t, J = 8.7 Hz, 1H), 3.50–3.60 (m, 1H), 5.14 (d, J = 4.8 Hz, 1H), 7.34–7.45 (m, 6H), 7.52 (t, J = 7.8 Hz, 1H), 7.68–7.74 (m, 6H), 7.80 (br s, 1H); ^{13}C NMR ($CDCl_3$): δ 13.6, 19.4, 19.6, 21.2, 22.6, 24.6, 27.2, 31.99, 32.08, 32.1, 36.8, 37.4, 38.8, 42.7, 45.5, 50.2, 57.2, 65.2, 73.4, 88.6, 90.1, 120.9, 121.5, 123.6 (q, J_{C-F} = 272.6 Hz), 127.3 (q, J_{C-F} = 3.7 Hz), 127.4, 127.5, 129.2, 129.4 (q, J_{C-F} = 3.9 Hz), 129.42, 129.5, 131.6 (q, J_{C-F} = 33.0 Hz), 134.7, 134.8, 135.74, 135.75, 135.8 (q, J_{C-F} = 1.2 Hz), 141.6, 188.9; ^{19}F NMR ($CDCl_3$): δ −63.4 (br s); HR-MS (ESI^+): $[M+H]^+$ found 709.3702. $C_{45}H_{52}F_3O_2Si$ 709.3683; $[M+Na]^+$ found 731.3525. $C_{45}H_{51}F_3O_2SiNa$ 731.3503.

4.1.4.8. 1-[3β-(*t*-Butyldiphenylsilyloxy)-androst-5-en-17β-yl]-3-(4-fluorophenyl)-2-propyn-1-one (5h). Compound **5h** was prepared according to *Method B* from Weinreb amide **4** (0.1 g, 0.16 mmol) and 1-ethynyl-4-trifluoromethylbenzene (60 mg, 0.50 mmol) as an oil after purification by flash column chromatography (cyclohexane–ethyl acetate, 98:2) (72 mg, 66% yield). IR: 2933, 2203, 1658 cm^{-1}; R_f: 0.68, cyclohexane/ethyl acetate 9:1; ^1H NMR ($CDCl_3$): δ 0.73 (s, 3H), 0.85–0.94 (m, 3H), 1.00 (s, 3H), 1.06 (m, 9H), 1.14–2.39 (m, 16H), 2.70 (t, J = 8.7 Hz, 1H), 3.50–3.60 (m, 1H), 5.14 (d, J = 4.8 Hz, 1H), 7.07 (t, J = 8.6 Hz, 2H), 7.35–7.45 (m, 6H), 7.53–7.58 (m, 2H), 7.68–7.71 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.2, 19.1, 19.4, 21.0, 22.3, 24.3, 26.9, 27.0, 31.7, 31.8, 36.5, 37.2, 38.5, 42.4, 45.2, 49.9, 57.0, 64.8, 73.1, 89.1 (d, J_{C-F} = 1.4 Hz), 89.8, 116.1 (d, J_{C-F} = 22.3 Hz), 116.3 (d, J_{C-F} = 3.5 Hz), 120.8, 127.4, 127.5, 129.4, 129.5, 134.7, 134.8, 135.1 (d, J_{C-F} = 8.8 Hz), 135.7, 135.8, 141.2, 163.8 (d, J_{C-F} = 253.6 Hz), 188.8; ^{19}F NMR ($CDCl_3$): δ (−106.92)–(−106.83) (m).

4.1.4.9. 1-[3β-(*t*-Butyldiphenylsilyoxy)-androst-5-en-17β-yl]-3-(4-methoxyphenyl)-2-propyn-1-one (5i). Compound **5i** was prepared according to *Method B* from Weinreb amide **4** (0.1 g, 0.16 mmol) and 1-ethynyl-4-methoxybenzene (0.12 mL, 0.83 mmol) as a crystalline solid after purification by flash column chromatography (cyclohexane–ethyl acetate, 95:5) (99 mg, 89% yield). mp 159–160 °C; IR 2932, 2180, 1636 cm^{-1}; R_f: 0.29, cyclohexane/ethyl acetate 95:5; ^1H NMR ($CDCl_3$): δ 0.71 (s, 3H), 0.82–0.92 (m, 3H), 0.98 (s, 3H), 1.06 (s, 9H), 1.12–2.38 (m, 16H), 2.68 (t, J = 8.7 Hz, 1H), 3.48–3.59 (m, 1H), 3.84 (s, 3H), 5.13 (d, J = 4.7 Hz, 1H,), 6.88 (d, J = 8.8 Hz, 2H), 7.34–7.44 (m, 6H), 7.51 (d, J = 8.8 Hz, 2H), 7.67–7.69 (m, 4H).; ^{13}C NMR ($CDCl_3$): δ 13.2, 19.1, 19.4, 20.9, 22.4, 24.4, 27.0, 31.78, 31.85, 31.88, 36.5, 37.2, 38.5, 42.4, 45.1, 50.0, 55.4, 56.9, 64.8, 73.1, 89.2, 92.1, 112.1, 114.3, 120.8, 127.4, 127.5, 129.4, 129.5, 134.7, 134.8, 134.9, 135.7, 135.8, 141.3, 161.5, 189.0; HR-MS (ESI^+): $[M+H]^+$, found 671.3913. $C_{45}H_{55}O_3Si$ requires 670.3915.; $[M+Na]^+$, found 693.3731 $C_{45}H_{54}O_3SiNa$ requires 693.3734.

4.1.5. General procedure for the synthesis of compounds 6a–i

To a solution of the appropriate steroid **5a–i** (1 mmol) in dry CH_2Cl_2 (0.06 M) was added dropwise at 0 °C HF-pyridine (10 equiv) and the mixture was stirred at ambient temperature for 12 h. After completion the reaction was cooled to 0 °C, was quenched by addition of water (3 mL), was stirred for 5 min and was extracted with CH_2Cl_2. The organic layer was washed with brine, was dried over anhydrous Na_2SO_4 and was evaporated in vacuo. The residue was purified by flash column chromatography to obtain the desired products **6a–i**.

4.1.5.1. 17β-[1-Oxo-3-(3-fluorophenyl)-2-propynyl]-androst-5-en-3β-ol (6a). Compound **6a** was prepared according to the general method described above from compound **5a** (293 mg, 0.45 mmol) as a crystalline solid after purification by flash column chromatography (petroleum ether 40–60 °C/acetone, 9:1) as crystalline solid (75 mg, 40% yield). mp 131–132 °C; $[\alpha]_D^{20}$ −29.21° (c = 0.09583, $CHCl_3$); IR: 3460, 2933, 2202, 1650 cm^{-1}; R_f: 0.33, petroleum ether 40–60 °C/acetone 8:2; ^1H NMR ($CDCl_3$): δ 0.73 (s, 3H), 0.99 (s, 3H), 1.05–2.38 (m, 19H), 2.71 (t, J = 8.6 Hz, 1H), 3.46–3.53 (m, 1H), 5.33 (d, J = 4.8 Hz, 1H), 7.10–7.38 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.3, 20.9, 22.3, 24.3, 31.5, 31.7, 31.8, 36.5, 37.2, 38.4, 42.1, 45.1, 49.9, 56.9, 64.9, 71.5, 89.0 (d, J_{C-F} = 3.4 Hz), 89.3, 118.0 (d, J_{C-F} = 21.2 Hz), 119.4 (d, J_{C-F} = 23.0 Hz), 121.2, 121.9 (d, J_{C-F} = 9.3 Hz), 128.6 (d, J_{C-F} = 3.3 Hz), 130.3 (d, J_{C-F} = 8.4 Hz), 140.7, 162.2 (d, J_{C-F} = 248.1 Hz), 188.8; ^{19}F NMR ($CDCl_3$): δ (−112.15)–(−112.09) (m); HR-MS (ESI^+): $[M+H]^+$ found 421.2525. $C_{28}H_{34}FO_2$ requires 421.2537.

4.1.5.2. 17β-[1-Oxo-3-(3-methyl-4-fluorophenyl)-2-propynyl]-androst-5-en-3β-ol (6b). Compound **6b** was prepared according to the general method described above from compound **5b** (36 mg, 0.05 mmol) as a crystalline solid after purification by flash column chromatography (cyclohexane-ethyl acetate, 95:5) (11 mg, 47% yield). mp 138–140 °C; $[\alpha]_D^{20}$ −57.61° (c = 0.04166, $CHCl_3$); IR: 3307, 2928, 2193, 1643 cm^{-1}; R_f = 0.57, cyclohexane/ethyl acetate 95:5; ^1H NMR ($CDCl_3$): δ 0.74 (s, 3H), 1.01 (s, 3H), 1.05–2.04 (m, 16H), 2.18–2.23 (m, 1H), 2.28 (s, 3H), 2.32–2.40 (m, 2H), 2.71 (t, J = 8.6 Hz, 1H), 3.48–3.58 (m, 1H), 5.36 (d, J = 4.2 Hz, 1H), 7.00 (t, J = 8.8 Hz, 1H,), 7.36–7.41 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 13.3, 14.3 (d, J_{C-F} = 3.4 Hz), 19.4, 21.0, 22.4, 24.4, 31.6, 31.8, 31.9, 36.5, 37.3, 38.5, 42.2, 45.1, 50.1, 57.0, 64.8, 71.7, 88.8 (d, J_{C-F} = 1.4 Hz), 90.5, 115.7 (d, J_{C-F} = 23.4 Hz), 115.9 (d, J_{C-F} = 3.9 Hz), 121.3, 125.8 (d, J_{C-F} = 18.3 Hz), 132.5 (d, J_{C-F} = 8.8 Hz), 136.2 (d, J_{C-F} = 6.0 Hz), 140.7, 162.6 (d, J_{C-F} = 252.1 Hz), 189.0; ^{19}F NMR ($CDCl_3$): δ (−111.34)–(−111.30) (m); HR-MS ($APCI^+$): $[M+H]^+$ found 435.2683. $C_{29}H_{36}FO_2$ requires 435.2694.

4.1.5.3. 17β-[1-Oxo-3-(2,4,5-trimethylphenyl)-2-propynyl]- androst-5-en-3β-ol (6c). Compound **6c** was prepared according to the general method described above from compound **5c** (99 mg, 0.15 mmol) as a crystalline solid after purification by flash column chromatography (petroleum ether 40–60 °C/acetone, 9:1) (41 mg, 64% yield). mp 101–102 °C; $[\alpha]_D^{20}$ −79.59° (c = 0.07916, $CHCl_3$); IR: 3506, 2931, 2184, 1644 cm^{-1}; R_f: 0.13, petroleum ether 40–60 °C/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.75 (s, 3H), 1.00 (s, 3H), 1.05–2.04 (m, 16H), 2.20 (s, 3H), 2.25 (s, 3H), 2.28–2.36 (m, 3H), 2.41 (s, 3H), 2.72 (t, J = 8.6 Hz, 1H), 3.48–3.56 (m, 1H), 5.35 (d, J = 3.9 Hz, 1H), 7.01 (br s, 1H), 7.29 (br s, 1H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.0, 19.4, 19.8, 20.1, 21.0, 22.6, 24.4, 31.5, 31.7, 31.9, 36.5, 37.2, 38.6, 42.2, 45.1, 50.0, 56.8, 64.9, 71.7, 91.1, 92.7, 117.0, 121.3, 131.1, 134.2, 134.4, 139.5, 140.0, 140.7, 189.1; HR-MS (ESI^+): $[M+Na]^+$ found 467.2903. $C_{31}H_{40}O_2Na$ requires 467.2921.

4.1.5.4. 17β-[1-Oxo-3-(2-methyl-4-methoxyphenyl)-2-propynyl]-androst-5-en-3β-ol (6d). Compound **6d** was prepared according to the general method above from **5d** (200 mg, 0.29 mmol) as a crystalline solid after purification by flash column chromatography (petroleum ether 40–60 °C/acetone, 9:1) (48 mg, 37%). mp 63–64 °C; $[\alpha]_D^{20}$ −25.07° (c = 0.05583, $CHCl_3$); IR: 3374, 2932, 2179, 1650 cm^{-1}; R_f: 0.11, petroleum ether 40–60 °C/acetone 9:1; ^1H NMR ($CDCl_3$): δ 0.73 (s, 3H), 0.84–0.90 (m, 1H), 0.99

(s, 3H), 1.03–2.41 (m, 18H), 2.46 (s, 3H), 2.72 (t, J = 8.6 Hz, 1H), 3.49–3.55 (m, 1H), 3.81 (s, 3H), 5.35 (d, J = 3.9 Hz, 1H), 6.70–6.76 (m, 2H), 7.47 (d, J = 8.4 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.3, 20.9, 21.0, 22.6, 24.5, 31.5, 31.7, 31.8, 36.5, 37.2, 38.6, 42.1, 45.1, 50.0, 55.3, 56.8, 64.7, 71.6, 91.3, 92.8, 111.7, 112.0, 115.3, 121.3, 135.5, 140.7, 144.4, 161.4, 189.0; HR-MS (ESI^+): $[M+H]^+$ found 447.2878. $C_{30}H_{39}O_3$ requires 447.2894; $[M+Na]^+$ found 469.2694. $C_{30}H_{38}O_3Na$ requires 469.2713.

4.1.5.5. 17β-[1-Oxo-3-(3-ethynylphenyl)-2-propynyl]-androst-5-en-3β-ol (6e). Compound **6i** was prepared according to the general method above from **5i** (99 mg, 0.14 mmol) as an oil after purification by flash column chromatography (petroleum ether–acetone, 9:1) (33 mg, 52%). $[\alpha]_D^{20}$ −41.67° (c = 0.13916, $CHCl_3$); IR: 3299, 2933, 2194, 1658 cm^{-1}; R_f: 0.10, petroleum ether/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.74 (s, 3H), 0.84–0.91 (m, 1H), 1.01 (s, 3H), 1.04–2.40 (m, 18H), 2.72 (t, J = 8.7 Hz, 1H), 3.13 (s, 1H), 3.48–3.58 (m, 1H), 5.35 (d, J = 5.0 Hz, 1H), 7.34 (t, J = 7.8 Hz, 1H), 7.51–7.56 (m, 2H), 7.66 (br s, 1H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.3, 21.0, 22.3, 24.4, 31.5, 31.8, 31.9, 36.5, 37.2, 38.4, 42.2, 45.2, 50.0, 56.9, 64.9, 71.7, 78.5, 82.1, 89.3, 89.5, 120.7, 121.3, 122.9, 128.7, 132.9, 134.0, 136.0, 140.8, 188.8; HR-MS ($APCI^+$): $[M+H]^+$ found 427.2622. $C_{30}H_{35}O_2$ requires 427.2632.

4.1.5.6. 17β-[1-Oxo-3-(2-trifluoromethylphenyl)-2-propynyl]-androst-5-en-3β-ol (6f). Compound **6f** was prepared according to the general method described above from 0.14 compound **5f** (100 mg, 014 mmol) as a crystalline solid after purification by flash column chromatography (cyclohexane–ethyl acetate, 75:25) (35 mg, 53%). mp 61–62 °C; $[\alpha]_D^{20}$ −38.18° (c = 0.01833, $CHCl_3$); IR: 3364, 2932, 2205, 1661 cm^{-1}; R_f: 0.13, petroleum ether 40–60 °C/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.75 (s, 3H), 1.00 (s, 3H), 1.04–2.41 (m, 19H), 2.74 (t, J = 8.8 Hz, 1H), 3.47–3.57 (m, 1H), 5.35 (d, J = 5.1 Hz, 1H), 7.51–7.59 (m, 2H), 7.70–7.73 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 13.5, 19.6, 21.2, 22.8, 24.8, 31.8, 32.0, 32.1, 36.8, 37.4, 38.5, 42.4, 45.8, 50.2, 57.1, 65.0, 71.9, 85.9, 93.3 (q, J_{C-F} = 0.9 Hz), 118.8 (q, J_{C-F} = 2.1 Hz), 121.5, 123.4 (q, J_{C-F} = 273.6 Hz), 126.4 (q, J_{C-F} = 5.1 Hz), 130.4, 131.9 (q, J_{C-F} = 0.9 Hz), 132.5 (q, J_{C-F} = 31.2 Hz), 135.6, 141.0, 189.3; ^{19}F NMR ($CDCl_3$): δ −62.2 (m); HR-MS ($APCI^+$): $[M+H]^+$ found 471.2497. $C_{29}H_{34}F_3O_2$ requires 471.2505.

4.1.5.7. 17β-[1-Oxo-3-(3-trifluoromethylphenyl)-2-propynyl]-androst-5-en-3β-ol (6g). Compound **6g** was prepared according to the general method above from **5g** (60 mg, 0.08 mmol) as a crystalline solid after purification by flash column chromatography (cyclohexane–ethyl acetate, 75:25) (18 mg, 47%). mp 175–178 °C; $[\alpha]_D^{20}$ −29.19° (c = 0.03083, $CHCl_3$); IR: 3489, 2922, 2206, 1648 cm^{-1}; R_f = 0.13, petroleum ether/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.75 (s, 3H), 1.01 (s, 3H), 1.07–2.41 (m, 19H), 2.74 (t, J = 8.7 Hz, 1H), 3.48–3.58 (m, 1H), 5.35 (d, J = 4.9 Hz, 1H), 7.53 (t, J = 7.8 Hz, 1H), 7.68–7.75 (m, 2H), 7.80 (br s, 1H); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.4, 21.0, 22.4, 24.4, 31.6, 31.7, 31.9, 36.5, 37.2, 38.5, 42.2, 45.3, 50.0, 56.9, 64.9, 71.7, 88.4, 89.7, 121.3, 123.4 (q, J_{C-F} = 272.7 Hz), 127.0 (q, J_{C-F} = 3.7 Hz), 129.3, 129.4 (q, J_{C-F} = 3.9 Hz), 131.3 (q, J_{C-F} = 33.1 Hz), 135.8 (q, J_{C-F} = 1.1 Hz), 140.8, 188.7; ^{19}F NMR ($CDCl_3$): δ −63.5 (m); HR-MS ($APCI^+$): $[M+H]^+$ found 471.2496. $C_{29}H_{34}F_3O_2$ requires 471.2505.

4.1.5.8. 17β-[1-Oxo-3-(4-fluorophenyl)-2-propynyl]-androst-5-en-3β-ol (6h). Compound **6h** was prepared according to the general method above from **5h** (65 mg, 0.09 mmol) as a viscous oil after purification by column chromatography (cyclohexane–ethyl acetate, 75:25) (24 mg, 58%). $[\alpha]_D^{20}$ −48.57° (c = 0.03500, $CHCl_3$); IR ($CHCl_3$,): 3256, 2935, 2213, 1638 cm^{-1}; R_f: 0.16, petroleum ether 40–60 °C/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.75 (s, 3H), 1.01 (s, 3H), 1.05–2.33 (m, 19H), 2.72 (t, J = 8.6 Hz, 1H), 3.49–3.55 (m, 1H), 5.36 (d, J = 5.1 Hz, 1H), 7.05–7.11 (m, 2H), 7.54–7.59 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 13.6, 19.6, 21.3, 22.6, 24.7, 31.9, 32.0, 32.1, 36.7, 37.5, 38.8, 42.4, 45.4, 50.3, 57.2, 65.1, 71.9, 89.3 (d, J_{C-F} = 1.42 Hz), 90.2, 116.4 (d, J_{C-F} = 22.3 Hz), 116.6 (d, J_{C-F} = 3.51 Hz), 121.5, 135.3 (d, J_{C-F} = 8.85 Hz), 141.1, 164.1 (d, J_{C-F} = 253.6 Hz), 189.1; ^{19}F NMR ($CDCl_3$): δ −106.9 (m); HR-MS (ESI^+): $[M+Na]^+$ found 443.2339. $C_{28}H_{33}FNaO_2$ requires 443.2357.

4.1.5.9. 17β-[1-Oxo-3-(4-methoxyphenyl)-2-propynyl]-androst-5-en-3β-ol (6i). Compound **6i** was prepared according to the general method described above from compound **5i** (76 mg, 0.11 mmol) as an oil after purification by flash column chromatography (cyclohexane–ethyl acetate, 95:5) as oil (11 mg, 23% yield). IR: 3395, 2928, 2180, 1654 cm^{-1}: R_f: 0.40, petroleum ether 40–60 °C/acetone 9:1; ^{1}H NMR ($CDCl_3$): δ 0.75 (s, 3H), 1.01 (s, 3H), 1.05–2.41 (m, 19H), 2.71 (t, J = 8.8 Hz, 1H,), 3.48–3.59 (m, 1H), 3.84 (s, 3H), 5.36 (d, J = 5.0 Hz, 1H,), 6.90 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H,); ^{13}C NMR ($CDCl_3$): δ 13.3, 19.4, 21.0, 22.4, 24.4, 31.6, 31.8, 31.9, 36.6, 37.2, 38.5, 42.2, 45.1, 50.1, 55.4, 57.0, 64.8, 71.7, 89.2, 92.2, 112.0, 114.3, 121.4, 134.9, 140.8, 161.6, 188.9; HR-MS (ESI^+): $[M+H]^+$ found 433.2734. $C_{29}H_{37}O_3$ requires 433.2664.

4.2. Pharmacology

4.2.1. Cells and culture conditions

Seven human cancer cell lines were used for the evaluation of the anticancer activity of the new analogues: A549 (alveolar adenocarcinoma), A431 (epidermoid carcinoma), HeLa (cervical adenocarcinoma), HepG2 (hepatocellular carcinoma), HT29 (colorectal adenocarcinoma), LNCaP (prostatic carcinoma), and MCF7 (breast adenocarcinoma). Cells were cultured as monolayers in DMEM medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and were grown in 100 mm or 60 mm tissue culture dishes in a humidified 5% CO_2 atmosphere at 37 °C. When 80–90% confluence was attained, the monolayers were subcultured by means of 0.25% trypsin-1 mM EDTA or used for experiments.

4.2.2. Cell viability assay

The cytotoxic potential of the C21-modified steroidal analogues was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells in their log phase of growth were harvested, counted and seeded (10^4 cells/well in 100 μL DMEM) in 96-well tissue culture dishes. After 24 h of incubation at 37 °C and 5% CO_2 to allow cell attachment, cultures were treated with varying concentrations (0.1–100 μM) of test samples (steroidal derivatives) made with 1:2 serial dilutions. The different steroidal derivatives were dissolved in a mixture of DMSO/water (1:1) and then introduced into the medium containing the cancer cell lines. Four replicates were set up for each experimental condition. Test samples were left in contact with the cells for 24 or 48 h. Untreated cells served as a control group. MTT solution (at a final concentration of 0.5 mg/ml) was added in each well 4 h before the end of incubation period and the assay was stopped by replacement of the MTT-containing culture medium with 100 μl of 0.1 N HCl in anhydrous isopropanol. Cell viability was determined by measuring absorbance at 545 nm using a microplate reader. The mean OD value of respective blank was subtracted from the mean OD value of each experimental set. Percent growth in the presence of test material was calculated considering the growth in the absence of any test material as 100% and, in turn, percent growth inhibition in the presence of test material was calculated. Finally the IC_{50} values (Table 1) were

calculated from three to four independent experiments, using Microsoft Office Excel software.

4.2.3. Trypan blue exclusion test

Cells were incubated for 24 h with the most active compounds (**6e**, **6f**, **6g**, **6h**) at a concentration of 10 μM or 5 μM. Following treatments, the medium in each plate was collected. Cells attached to wells were removed by trypsinization and pooled with their corresponding medium. Cells were centrifuged at 1200 rpm for 5 min and the pellet was resuspended in PBS containing 0.2% trypan blue. A total of 200–300 cells were counted in each out of three separate experiments and the mean was used for calculations. Viability was determined as the percentage of viable cells over the total number of cells.

4.2.4. Preparation of total protein extracts and Western blotting

Cells were washed twice with ice-cold PBS, harvested by scraping from dishes, lysed in ice-cold Buffer G [20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM NA_3VO_4, 10 mM benzamidine, 20 mM HEPES, pH 7.5] supplemented with 0.5% (v/v) Triton X-100 and a mixture of protease inhibitors [200 μM leupeptin, 5 mM DTT, 300 μM PMSF and 10 μM E64]; and incubated for 30 min at −80 °C. Lysates were then centrifuged (10,000 rpm, 10 min, 4 °C) and the supernatant (total protein extract) was collected. Protein concentration was determined by the Bradford assay (BioRad Laboratories). After quantification the samples were supplemented with 0.33 volumes of SDS–PAGE sample buffer [330 mM Tris–HCl, pH6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue] and boiled for 3 min before electrophoretic separation.

Equal amounts of protein (50 μg per lane) were separated by SDS–PAGE on 10% (w/v) polyacrylamide gels and electrotransfered onto nitrocellulose membranes. The membranes were then blocked in a solution of 5% (w/v) nonfat dry milk in TBS-T [20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween-20] for 1 h at room temperature and probed overnight at 4 °C with primary antibodies against pAkt (1:1000, cell signalling) or β-actin (1:2500, Sigma-Aldrich). After washing with TBS-T (4 × 5 min), blots were incubated with the appropriate HRP-conjugated secondary antibody [1:5000 in TBS-T containing 1% (w/v) nonfat dry milk] for 1 h at room temperature. After washing the blots in TBS-T (4 × 5 min), bands were detected using enhanced chemiluminescence (ECL; Amersham Biosciences) reagent, exposed to super RX film.

Acknowledgement

This work was supported in part by the European Union under grant agreement MEST-CT-2005-0020575 (2006–2010).

Supplementary data

Supplementary data (Copies of ^{1}H and ^{13}C NMR spectra of compounds **6a–6i** and **5a–5i**. Copies of HR-MS spectra of compounds **6a–6i**.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.10.012.

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