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# Design, synthesis and anticancer activities of stilbene-coumarin hybrid compounds: Identification of novel proapoptotic agents

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ABSTRACT

The naturally occurring coumarins and resveratrol, attract great attention due to their wide range of biological properties, including anticancer, antileukemic, antibacterial and anti-inflammatory activities; moreover, their cancer chemopreventive property have been recently emphasized. A novel class of hybrid compounds, obtained by introducing a substituted *trans*-vinylbenzene moiety on a coumarin backbone, was synthesized and evaluated for the antitumor profile. A number of derivatives showed a good antiproliferative activity, in some cases higher to that of the reference compound resveratrol. The most promising compounds in this series were **14** and **17**, endowed with excellent antiproliferative and proapoptotic activities. The present study suggests that the 7-methoxycoumarin nucleus, together with the 3,5-disubstitution pattern of the *trans*-vinylbenzene moiety, are likely promising structural features to obtain excellent antitumor compounds endowed with a apoptosis-inducing capability.



## 1. Introduction

Cancer is at present one of the most leading causes of death in the United States and in the developed countries,[1] and many efforts have been made to discovery agents endowed with cytostatic action.[2] For several chemotherapeutic agents, a direct correlation between the antitumor efficacy and the ability to induce apoptosis was established, and the development of new anticancer approaches, aimed at promoting apoptosis in cancer cells, gained a paramount importance.[3]

Natural products, with their ability to interact with more to one target, represent, in medicinal chemistry, a significant source of inspiration for the design of structural analogues with improved pharmacological profile.

Coumarins are a wide group of naturally occurring compounds that, due to their remarkable array of biological activities, usually associated to low toxicity, form an elite class of compounds which occupy a special role in nature. Indeed, a large number of coumarins have been isolated from a variety of plant sources and underwent extensive investigations aimed to assess their potential therapeutic applications:[4] some relevant actions were reported such as: anticancer,[5,6] anti-HIV,[7,8] anticoagulant,[9] antimicrobial,[10,11] antioxidant and anti-inflammatory.[12] The antitumor effects, which are related to the inhibition of the cellular proliferation, through a variety of mechanisms, were extensively examined.[13–17] In particular, coumarin itself (**1a**) and its metabolite 7-hydroxycoumarin (**1b**, Fig. 1) have been investigated as potential candidates for cancer therapy,[18,17,19] scopoletin (6-methoxy-7-hydroxycoumarin, **1c**) and esculetin (6,7-dihydroxycoumarin, **1d**) have been shown to exhibit an antiproliferative effect in leukaemic cells by inducing apoptosis.[20,21]

This inherent biological relevance has attracted much interest in the drug discovery field, and the benzopyran-2-one nucleus emerged as a valuable molecular template for the design of a variety of analogues. A lot of efforts aimed at the design of properly functionalized synthetic coumarins as effective agents able to potently and selectively affect some cellular functions, have been performed.[22–31]

Recently, considerable attention has been also focused on resveratrol (3,5,4′-*trans*-trihydroxystilbene, **2**, Fig. 1), a well-known natural polyphenol found in large amount in grapes,[32] that has been reported to exert multiple biological activities[33] including anti-inflammatory,[34] anti-oxidant,[35] inhibition of platelet aggregation,[36] antitumor,[37] and induction of apoptosis.[38] Remarkably, the cancer chemopreventive activity[39,40] of **2** represents an important add value and it seems to be strictly connected to the antitumor, and the proapoptotic effects.[41] Structure–activity relationship (SAR) studies, performed on a number of resveratrol analogues, allowed to establish the chemical features responsible for the antitumor and the proapoptotic activities; for example the 3,5-dimethoxyphenyl moiety was identified to play a pivotal role in conferring both the antitumor and the proapoptotic activities.[42–44] Generally, the presence of a number of methoxy groups seems to

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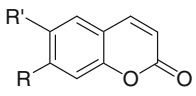
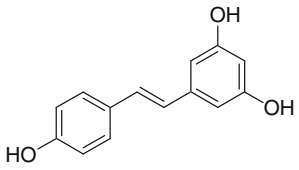
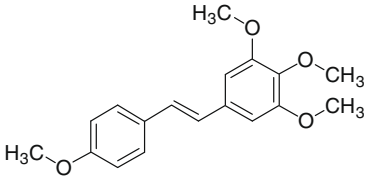


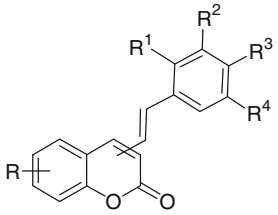
**1a**: R, R' = H
**1b**: R = OH, R' = H
**1c**: R = OH, R' = $OCH_3$
**1d**: R, R' = OH



**2** (resveratrol)



**3** (DMU-212)



**12-21**

| compd | insertion position | R | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|---|
| **12** | 4 | H | H | $OCH_3$ | H | $OCH_3$ |
| **13** | 4 | 6-$OCH_3$ | H | $OCH_3$ | H | $OCH_3$ |
| **14** | 4 | 7-$OCH_3$ | H | $OCH_3$ | H | $OCH_3$ |
| **15** | 4 | 7-$OCH_3$ | H | $OCH_3$ | $OCH_3$ | $OCH_3$ |
| **16** | 4 | 7-$OCH_3$ | $OCH_3$ | $OCH_3$ | $OCH_3$ | H |
| **17** | 4 | 7-$OCH_3$ | H | $CH_3$ | H | $CH_3$ |
| **18** | 4 | 7-$OCH_3$ | H | H | $OC_5H_9$ | H |
| **19** | 4 | 7-$OCH_3$ | H | $OCH_3$ | H | H |
| **20** | 3 | 7-$OCH_3$ | H | $OCH_3$ | H | $OCH_3$ |
| **21** | 4 | 7-OH | H | OH | H | OH |

**Figure 1.** Chemical structures of coumarins and stilbene analogues mentioned in this study and general structure of the synthesized compounds (**12**–**21**).

be a fundamental requirement to obtain potent cytotoxic agents, and the 3,4,5,4′-tetramethoxystilbene (DMU-212, **3**, Fig. 1) proved to be more potent and selective than the parent compound **2** in the inhibition of the cancer cell growth.[45,46]

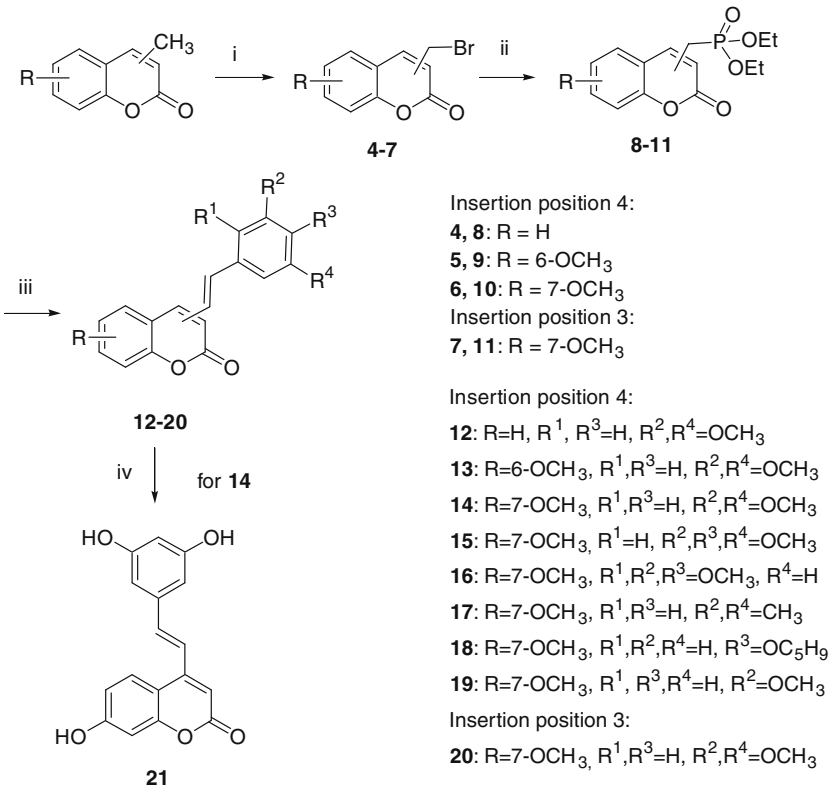
Over the last few years, with the progress of the medicinal chemistry, the hybrid approach has received significant attention since it allowed obtaining molecules with improved biological activity with respect to the corresponding lead compounds. Thus, the design and synthesis of hybrid molecules encompassing two pharmacophores in one molecular scaffold is a well established approach to the synthesis of more potent drugs.[47] Using this approach, several research groups have recently designed and synthesized hybrid molecules by coupling coumarins with a number of bioactive molecules such as: resveratrol, maleimide and alpha-lipoic acid; these efforts resulted in new molecules endowed with vasorelaxant and platelet antiaggregating, imaging, and antioxidant and anti-inflammatory properties, respectively.[48–50] Objective of the present study was to synthesize a novel class of conjugates (compounds **12**–**21**), which general formula was depicted Figure 1, obtained by the insertion of a properly substituted *trans*-vinylbenzene moiety on a coumarin backbone. As first line of search, the *trans*-3,5-dimethoxyphenylvinyl function was introduced on a number of coumarins (compounds **12**–**14** and **20**) and the 7-methoxycoumarin bearing the *trans*-vinylbenzene moiety in the 4 position (**14**) proved to be the derivative endowed with the higher antiproliferative activity. These results prompted to further explore this framework and some structural analogues of **14**, bearing different substitution patterns on the vinylbenzene portion (compounds **15**–**19** and **21**) were prepared. The new coumarin-resveratrol hybrids were tested for their antiproliferative activity against a human lung carcinoma cell line (H460). The two most promising compounds (**14** and **17**) underwent additional biological investigations regarding: the inhibitory effects against proliferation of squamous cell carcinoma (A431) and melanoma (JR8); the ability to induce apoptosis, together with the cellular basis and the molecular events responsible for their anticancer profile.

## 2. Chemistry

Preparation of the derivatives **12**–**21** was accomplished by means of a common synthetic procedure shown in Scheme 1. Bromination of the 3 or 4-methylcoumarin derivatives with *N*-bromosuccinimide (NBS) gave the 3 or 4-bromomethyl intermediates **4**–**7**, that were reacted with triethylphosphate, to obtain the corresponding phosphonic acid diethyl ester derivatives **8**–**11**. Horner–Emmons–Wadsworth reaction of the above obtained compounds with the selected aldehydes in the presence of sodium methoxide as base, afforded the stilbene derivatives **12**–**20** in a 9:1 mixture of *E*/*Z* isomers. The desired E-isomer (*trans*) was achieved by means of the fractionated crystallization from toluene. The formation of the *E* isomers was confirmed by the characteristic $^1$H NMR coupling constants (*J*) of 15–18 Hz observed for the olefinic protons.[51] Cleavage of the methoxy functions of compound **14**, with $BBr_3$ at low temperature, afforded compound **21**.

## 3. Biological results and discussion

The synthesized analogues **12**–**21** were initially tested for their antiproliferative activity in H460 cells and the results were reported in Table 1. The analogues **12**–**14**, obtained by inserting the *trans*-3,5-dimethoxyphenylvinyl moiety in the positions 4 of coumarin, 6-methoxy and 7-methoxycoumarin scaffolds, respectively, showed quite different antiproliferative potencies: compound **12**, with $IC_{50}$ value of 2.6 μM, was about fivefold more active than derivatives **13** and **2**; compound **14**, which showed a $IC_{50}$ value of 0.45 μM, was 6- and 28-fold more active than derivatives **12** and **2**, respectively, and undoubtedly emerged as one of the most active compounds within this subset, thus offering a proof about the importance of the 7-methoxycoumarin nucleus as main backbone. Accordingly, shifting the *trans*-3,5-dimethoxyphenylvinyl moiety of derivative **14** from the C-4 to the C-3 position, to yield compound **20**, a dramatic drop of potency was observed, suggesting that also the C-4 insertion position plays a




**Scheme 1.** Reagents and conditions: (i) NBS, $(PhCOO)_2O$, $CCl_4$, reflux; (ii) $PO(OEt)_3$, 150 °C; (iii) Ar-CHO, $NaOCH_3$, DMF, 0–100 °C; (iv) $BBr_3$, DCM, 0 °C, rt.

**Table 1**
Antiproliferative activity of synthetic coumarins **12**–**21** in human tumour cells from different tumour types (lung carcinoma H460, squamous cell carcinoma A431, melanoma JR8)[a] Figure 4

| Compd | $IC_{50}$ (μM) | | |
|---|---|---|---|
| | **H460** | **A431** | **JR8** |
| Resveratrol | 12.9 ± 3.1 | n.t. | n.t. |
| **12** | 2.6 ± 0.2 | n.t. | n.t. |
| **13** | 11.0 ± 1 | n.t. | n.t. |
| **14** | 0.45 ± 0.09 | 3.44 | 3.2 |
| **15** | >100 | n.t. | n.t. |
| **16** | >50 | n.t. | n.t. |
| **17** | 0.29 ± 0.08 | 3.5 | 3.5 |
| **18** | >50 | n.t. | n.t. |
| **19** | 12.9 ± 1.3 | n.t. | n.t. |
| **20** | >10 | n.t. | n.t. |
| **21** | 27.0 ± 3.0 | n.t. | n.t. |

Chemical structures of coumarins and stilbene mentioned in this study and general structure of the synthesized compounds.
n.t.: not tested.
[a] Drug effects were determined after 72 h exposure.

pivotal role. A detrimental effect was also observed with the replacement of the methoxy groups of **14** with hydroxyls: the derivative **21** was 60-fold less active than **14** and only twofold less active than **2**. Taken these data into account, further structural modifications of **14**, purposely focused on the vinybenzene portion of the molecule, were performed; a noteworthy reduction of the antiproliferative activity was observed for compounds **15** and **16**, bearing a supplementary methoxy group, and for derivative **18**, with a *p*-3,3-dimethylalliloxy side chain. Moreover, the removal of one methoxy group of **14** caused a loss of potency: compound **19** was equipotent to **2**. These findings outlined as the 3,5-dimethoxy groups on the *trans*-vinylbenzene moiety play a pivotal role; their replacement with methyls lead to compound **17**, that proved to be slightly more potent than the corresponding lead molecule **14**.

The two most effective compounds of the series (**14** and **17**) underwent additional biological investigations such as: their potency against A431 and JR8 cells was evaluated; in both these cell systems a comparable activity (at micromolar level) was observed.

Furthermore, in an attempt to better characterize the cellular basis of their antiproliferative effects, the ability of these selected compounds to induce apoptosis in the most sensitive cell line (i.e., H460) was investigated. At concentrations causing comparable antiproliferative effects ($IC_{80}$), **14** and **17** produced a level of apoptosis similar to that of cisplatin and substantially higher to that of **2**, which was a poor inducer of apoptosis in this cell system (Fig. 2A). In particular, the proapoptotic effect of **14** was confirmed by analysis of cleavage of caspase 3 and PARP. As shown in Figure 2B, activation of caspase 3 and cleavage of PARP was already detectable after 24 h exposure.

Besides, to gain insights into the cellular basis of the peculiar behaviour of these selected compounds some aspects of cellular response in H460 cells have been investigated (Fig. 3): when the cells were exposed to antiproliferative concentrations ($IC_{50}$) for 24 h, cell cycle analysis showed a moderate arrest at the G2/M phase, this effect was dose-dependent because the cell accumulation in G2/M phase was increased at more toxic concentrations. This response, in terms of perturbation of cell cycle, was somewhat similar to that produced by resveratrol at substantially higher concentrations (20 μM).

Moreover, the molecular events involved in cellular response to the effective compounds, were investigated and to this end, the levels of regulatory proteins implicated in G2 arrest, including p21, cyclin B1, $p34^{cdc2}$ were examined (Fig. 4A). Protein p21 plays a crucial role in G2 arrest trough accumulation of inactive cyclin $B1/p34^{cdc2}$ complex. In fact, under the same conditions used for analysis of cell cycle perturbation (24 h exposure to 1.5 μM for

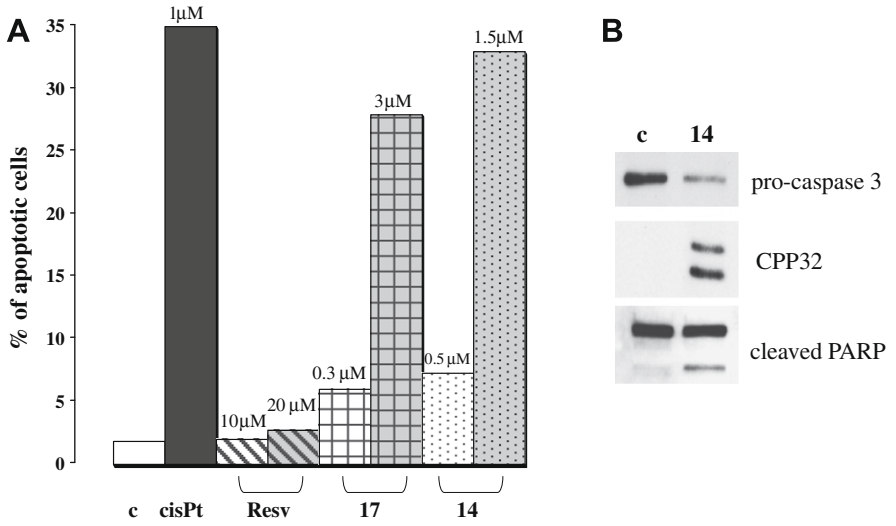



**Figure 2.** Induction of apoptotic cell death in H460 cells by compounds **14** and **17** and effects of **14** on apoptosis-related proteins. (A) Comparison of apoptosis induced by cisplatin (cisPt) resveratrol (Resv), compounds **14** and **17**. After 72 h exposure to equitoxic concentrations of cisPt (1 μM), resveratrol (10 and 20 μM), **17** (0.3 and 3 μM) and **14** (0.5 and 1.5 μM), apoptosis was detected by TUNEL assay. Drug effects are reported as percent of TUNEL-positive cells. (c) control untreated. (B) Biochemical analysis of caspase 3 and PARP cleavage. Total cellular extracts were obtained after 24 h exposure to **14** (1.5 μM). Western blot analyses were then performed to assess protein expression. Actin is shown as a control for protein loading.

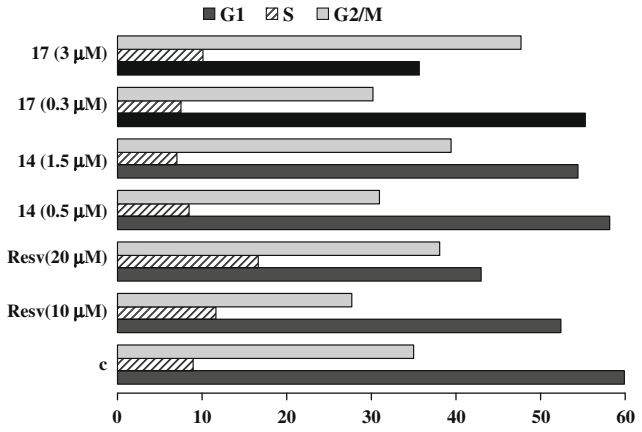



**Figure 3.** Cell cycle analysis. H460 cells were treated for 24 h with equitoxic concentrations of resveratrol (Resv) (10 and 20 μM) or **14** (0.5 and 1.5 μM) or **17** (0.3 and 3 μM).

**14** and 3 μM for **17**, respectively), there was a marked increase of p21 expression concomitant with upregulation of cyclin B1 and $p34^{cdc2}$ (Fig. 4A). All together these events are consistent with a critical role of p21 in G2 arrest as a consequence of its association with $p34^{cdc2}$ and inactivation of cyclin B1–$p34^{cdc2}$ complex. Indeed, Western blot analysis of immunoprecipitates with $p34^{cdc2}$ antibody revealed an accumulation of p21–$p34^{cdc2}$ complexes (Fig. 4B).

Thus, a tentative explanation of the proapoptotic activity of **14** and **17** could be related to activation of apoptotic signals, as a consequence of arrest in G2 phase, and inhibition of G2→M transition, which favours apoptosis activation.

On the basis of the promising features at cellular level, compound **14** was also tested in the human lung carcinoma H460 xenograft model. In this preliminary experiment, when administered orally at 10–20 mg/kg with a daily treatment schedule, the derivative produced an appreciable tumour growth inhibition (by ~50%) without evidence of toxic effects. The optimal treatment conditions remain to be defined.

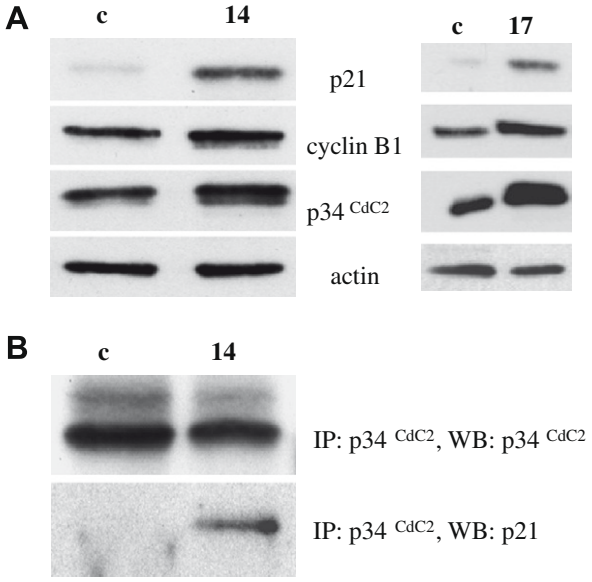



**Figure 4.** Modulation of cell cycle regulatory proteins in H460 cells treated with **14** or **17**. (A) Cyclin B1, $p34^{cdc-2}$ and p21 protein expression. Total cellular extracts were obtained after 24 h exposure to **14** (1.5 μM) or **17** (3 μM) and analyzed by Western blotting. Actin is shown as a control for protein loading. (B) Co-immunoprecipitation analysis of $p34^{cdc-2}$/p21 in H460 cells after 24 h exposure to **14** (1.5 μM). After treatment cell lysates were harvested and immunoprecipitated with anti-$p34^{cdc-2}$ rabbit polyclonal antibody. Immunoprecipitates were immunoblotted for p21. Blots were stripped and probed for $p34^{cdc-2}$.

## 4. Conclusions

A series of hybrid molecules (derivatives **12**–**21**), composed by a coumarin scaffold and a properly substituted *trans*-vinylbenzene moiety, were synthesized and tested to assess their antiproliferative activity on H460 lung carcinoma cells. For the most potent compounds the proapoptotic effects were also evaluated. The derivatives **15**, **16** and **18**, in which the *para* position of the vinylbenzene moiety was occupied by a substituent, proved to be the less active of the series; in particular, **18**, with an hindering substi-

tuent in this position was designed as the negative control compound. An increment of potency was observed for compound **19**, bearing a *meta* methoxyl, that showed the same potency of the reference compound **2**. The presence of the methoxyls at specific positions, in both fragments of the hydrid molecules, demonstrated to be critical in conferring the antiproliferative activity: the subset of compounds **12**–**14** and **20**, characterized by the presence of a 3,5-dimethoxyphenylvinyl moiety, demonstrated that the substitution pattern on the coumarin scaffold significantly affected potency. Specifically, **12**, without substituent on the coumarin nucleus, was fivefold more active than **2**, **13**, bearing a methoxy group in the 6 position of the coumarin scaffold, demonstrated the same potency of **2**, and **14**, the C-7 methoxylated analogue, showed a substantial enhancement of activity, proving to be a good antiproliferative agent with $IC_{50}$ values of 0.45 μM. A decrease of activity was observed by shifting the 3,5-dimethoxy *trans*-vinylbenzene function of **14** from C-4 to the C-3 position (**20**). Replacement of both the methoxyls of **14** with the hydroxyls, to obtain **21**, resulted in about a twofold reduction in activity with respect to the strictly related molecule **2**; on the contrary, promising results were obtained by replacing the methoxyls at the 3 and 5 positions of the vinylbenzene moiety with methyls, as documented by the effects of **17**, characterized by submicromolar $IC_{50}$ value. Derivatives **14** and **17** exhibited pharmacologically relevant antiproliferative activity and underwent deep biological investigations, thus revealing their ability to induce an appreciable level of apoptosis (~30% of TUNEL-positive cells), comparable to that of cisplatin, and to a great extent superior to that of **2**. This effect was associated with a partial cell accumulation in G2/M phase of cell cycle. Indeed, the presence of particular substituents in specific positions of both fragments of the hydrid molecule, demonstrated to be critical in conferring the antiproliferative activity; the 7, 3′ and 5′ substitution pattern demonstrated to be a favourable feature for both the antitumor and the proapoptotic activities.

In conclusion, in this study, among the newly synthesized hybrid molecules, compounds **14** and **17** were identified to be very promising candidates; their excellent antiproliferative potency, together with their remarkable apoptosis-inducing activity, make them leads of great interest for further studies.

## 5. Experimental section

### 5.1. Chemistry

#### 5.1.1. Materials and methods

Chemicals and reagents were obtained from commercial sources and used without further purification. All melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. Product mixtures were monitored by thin-layer chromatography (TLC) on Merk precoated Silica Gel $F_{254}$ plates. Nuclear magnetic resonance ($^1$H NMR) spectra were recorded with a Varian VXR 200 and 300 MHz spectrometers, peaks positions are given in parts per million downfield from tetramethylsilane (TMS) as the internal standard and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet) or br (broad). Coupling constants (*J*) are reported in hertz (Hz). OH protons were detected upon proton exchange with $D_2O$. Mass spectra were obtained with Waters Micromass ZQ 4000 (ES-MS spectra) or V. G. 7070 E (EI-MS spectra) apparatuses. The purity of the tested compounds was determined by HPLC analysis, performed on a Jasco LC 1500 PU-1587; the column used was a Phenomenex Luna C18(2) 5 μm 4.60 × 150 mm; elution conditions: mobile phase 70:30 $CH_3CN/H_2O$; the flow-rate was 0.8 ml/min and the injection volume was 5 μl; peaks were detected at 250 nm and results were >95% purity.Wherever analyses are only indicated with elements symbols, analytical results obtained for those elements are within 0.4% of the theoretical values. Column chromatography was carried out with silica gel (Kiesel gel 40, 0.040–0.063 mm; Merck) using the flash technique. Yields were reported after crystallization or chromatographic purification. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry.

### 5.2. General procedure for the preparation of bromomethyl-2*H*-chromen-2-one derivatives (4–7)

To a suspension of a suitable methylcoumarin derivative (10 mmol) and NBS (11 mmol) in $CCl_4$ (100 mL), a catalytic amount of benzoyl peroxide was added. The reaction mixture was refluxed for 8 h, the succinimide produced during the reaction was filtered off, and the solvent was washed with $H_2O$, dried and removed under reduced pressure. The crude was crystallized from ligroin to afford the desired bromomethyl derivative as a pure crystalline compound.

#### 5.2.1. 4-Bromomethyl-2*H*-chromen-2-one (4)

Starting from 4-methyl-2*H*-chromen-2-one[52] (1.6 g, 10 mmol), the desired derivative **4** was obtained (2.0 g, 85% yield); mp 175–177 °C. $^1$H NMR ($CDCl_3$): $\delta$ 4.58 (s, 2H), 6.35 (s, 1H), 7.25–7.42 (m, 2H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.62 (d, *J* = 8.8 Hz, 1H).

#### 5.2.2. 4-Bromomethyl-6-methoxy-2*H*-chromen-2-one (5)

Starting from 6-methoxy-4-methyl-2*H*-chromen-2-one[52] (1.9 g, 10 mmol), **5** was obtained (2.1 g, 75% yield); mp 198–200 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.84 (s, 3H), 4.5 (s, 2H), 6.33 (s, 1H), 6.95 (s, *J* = 2.0 Hz, 1H), 7.05 (dd, *J* = 2.0 and 8.4 Hz, 1H), 7.42 (d, *J* = 8.4 Hz, 1H).

#### 5.2.3. 4-Bromomethyl-7-methoxy-2*H*-chromen-2-one (6)

Staring from 7-methoxy-4-methyl-2*H*-chromen-2-one[53] (1.9 g, 10 mmol), **6** was obtained (1.95 g, 73% yield); mp 214–215 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.90 (s, 3H), 4.48 (s, 2H), 6.39 (s, 1H), 6.88 (d, *J* = 2.2 Hz, 1H), 6.92 (dd, *J* = 2 and 8.8 Hz, 1H, 7.64 (d, *J* = 8.5 Hz, 1H).

#### 5.2.4. 3-Bromomethyl-7-methoxy-2*H*-chromen-2-one (7)

Starting from 7-methoxy-3-methylchromen-2-one[28] (1.9 g, 10 mmol), **7** was obtained (1.9 g, 71% yield); mp 131–132 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.89 (s, 3H), 4.43 (s, 2H), 6.80–6.95 (m, 2H), 7.40 (d, *J* = 8.8 Hz, 1H), 7.80 (s, 1H).

### 5.3. General procedure for the preparation of the phosphonic acid diethyl ester derivatives (8–11)

A mixture of bromomethyl derivative 4–7 (10 mmol) and triethylphosphate (10 mL) was heated at 130 °C for 2 h, then cooled at room temperature and left to stand overnight. The desired compound precipitates as a white solid that was collected by filtration, washed with diethyl ether and dried.

#### 5.3.1. (2-Oxo-2*H*-chromen-4-ylmethyl)phosphonic acid diethyl ester (8)

Starting from **4** (2.39 g, 10 mmol) **8** was obtained (1.4 g, 60% yield), mp 195–197 °C. $^1$H NMR ($CDCl_3$): $\delta$ 1.27 (t, *J* = 6.9 Hz, 6H), 3.36 (d, *J* = 22.8 Hz, 2H), 4.16 (q, *J* = 7.2 Hz, 4H), 6.30 (s, 1H), 7.01–7.35 (m, 2H), 7.49–7.58 (m, 1H), 7.76 (d, *J* = 8.2 Hz, 1H). ES-MS *m*/*z*: 319 (M+23). Anal. Calcd for $C_{14}H_{17}O_5P$: C, 56.76; H, 5.78. Found: C, 56.81; H, 5.72.

#### 5.3.2. (6-Methoxy-2-oxo-2*H*-chromen-4-ylmethyl)phosphonic acid diethyl ester (9)

Starting from **5** (2.7 g, 10 mmol), **9** was obtained (2.6 g, 81% yield); mp 265–268 °C. $^1$H NMR ($CDCl_3$): $\delta$ 1.27 (t, *J* = 6.9 Hz, 6H),

3.28 (d, $J$ = 22.8 Hz, 2H), 3.88 (s, 3H), 4.11 (q, $J$ = 7.2 Hz, 4H), 6.30 (s, 1H), 7.02 (d, $J$ = 2.0 Hz, 1H), 7.08 (dd, $J$ = 2.0 and 8.4 Hz, 1H), 7.25 (d, $J$ = 8.4 Hz, 1H). ES-MS $m/z$: 327 (M+1). Anal. Calcd for $C_{15}H_{19}O_6P$: C, 55.22; H, 5.87. Found: C, 55.29; H, 5.82.

#### 5.3.3. (7-Methoxy-2-oxo-2*H*-chromen-4-ylmethyl)phosphonic acid diethyl ester (10)

Staring from **6** (2.7 g, 10 mmol), **10** was prepared (2.3 g, 71% yield); mp 238–241 °C. $^1$H NMR ($CDCl_3$): $\delta$ 1.31 (t, $J$ = 6.9 Hz, 6H), 3.90 (s, 3H), 3.25 (d, $J$ = 22.7 Hz, 2H), 4.07 (q, $J$ = 7.2 Hz, 4H), 6.39 (s, 1H), 6.88 (d, $J$ = 2.2 Hz, 1H), 6.92 (dd, $J$ = 2 and 8.8 Hz, 1H, 7.64 (d, $J$ = 8.5 Hz, 1H). ES-MS $m/z$: 349 (M+23). Anal. Calcd for $C_{15}H_{19}O_6P$: C, 55.22; H, 5.87. Found: C, 55.25; H, 5.83.

#### 5.3.4. (7-Methoxy-2-oxo-2H-chromen-3-ylmethyl) phosphonic acid diethyl ester (11)

Starting from **7** (2.7 g, 10 mmol), **11** was prepared (2.1 g, 65% yield); mp 225–228 °C. $^1$H NMR ($CDCl_3$): $\delta$ 1.31 (t, $J$ = 7.4 Hz, 6H), 3.15 (d, $J$ = 21.6 Hz, 2H), 3.87 (s, 3H), 4.13 (q, $J$ = 7.4 Hz, 4H), 6.80–6.87 (m, 2H), 7.37 (d, $J$ = 8.4 Hz, 1H), 7.77 (d, $J$ = 4.0 Hz, 1H). ES-MS $m/z$: 327 (M+1). Anal. Calcd for $C_{15}H_{19}O_6P$: C, 55.22; H, 5.87. Found: C, 55.17; H, 5.88.

### 5.4. General procedure for the synthesis of stilbene derivatives (12–20)

To a solution of a suitable phosphonic acid diethyl ester (**8**–**11**) (3.0 mmol) in DMF (1.5 mL) at 0 °C, $NaOCH_3$ (4.5 mmol) was carefully added. The mixture was stirred at the same temperature for 1 h, then the selected aldehyde (3.0 mmol) in DMF (2.5 mL) was added dropwise and the reaction mixture was stirred at room temperature for 1.5 h and heated at 95 °C for 1 h. After cooling, the mixture was poured in ice water and left to stand overnight. The solid formed was collected by filtration, washed with water and dried. Crystallization from toluene gave the desired stilbene derivative as a pure E-isomer.

#### 5.4.1. 4-(*E*)-[2-(3,5-Dimethoxyphenyl)vinyl]-2*H*-chromen-2-one (12)

Starting from **8** (0.9 g, 3.0 mmol) and 3,5-dimethoxybenzaldehyde (0.49 g, 3.0 mmol), **12** was obtained (0.6 g, 65% yield); mp 143–145 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.85 (s, 6H), 6.53 (t, $J$ = 2.2 Hz, 1H), 6.62 (s, 1H), 6.75 (d, $J$ = 2.2 Hz, 2H), 7.25 (d, $J$ = 16.0 Hz, 1H), 7.30 (d, $J$ = 8.6 Hz, 1H), 7.37 (d, $J$ = 16.0 Hz, 1H), 7.35–7.45 (m, 1H), 7.55–7.65 (m, 1H), 7.82 d, $J$ = 8.2 Hz, 1H. ES-MS $m/z$: 331 (M+23). Anal. Calcd for $C_{19}H_{16}O_4$: C, 74.01; H, 5.23. Found: C, 73.95; H, 5.16.

#### 5.4.2. 4-[(*E*)-2-(3,5-Dimethoxyphenyl)vinyl]-6-methoxy-2*H*-chromen-2-one (13)

Starting from **9** (0.9 g, 3.0 mmol) and 3,5-dimethoxybenzaldehyde (0.49 g, 3.0 mmol), **13** was obtained (0.51 g. 51% yield); mp 191–193 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.85 (s, 6H), 3.88 (s, 3H), 6.51 (t, $J$ = 2.2 Hz, 1H), 6.59 (s, 1H), 6.71 (d, $J$ = 2.2 Hz, 2H), 6.15–6.25 m, 3H), 7.27 (d, $J$ = 8.8 Hz, 1H), 7.29 (d, $J$ = 17.6 Hz, 1H). ES-MS $m/z$: 338 (M-1) and 361 (M+23). Anal. Calcd for $C_{20}H_{18}O_5$: C, 70.99; H, 5.36. Found: C, 70.90; H, 5.42.

#### 5.4.3. 7-Methoxy-4-[(*E*)-2-(3,5-dimethoxyphenyl)vinyl]-2*H*-chromen-2-one (14)

Starting from **10** (0.9 g, 3.0 mmol) and 3,5-dimethoxybenzaldehyde (0.49 g, 3 mmol), **14** was obtained (0.69 g, 68% yield); mp 176–178 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.85 (s, 6H), 3.89 (s, 3H), 6.43 (s, 1H), 6.49–6.51 (m, 1H), 6.71 (d, $J$ = 2 Hz, 2H), 6.86 (d, $J$ = 2 Hz, 1H), 6.91 (dd, $J$ = 2.2 and 8.8 Hz, 1H), 7.21 (d, $J$ = 15.6 Hz, 1H), 7.27 (d, $J$ = 15.6 Hz, 1H), 7.68 (dd, $J$ = 2.4 and 8.8 Hz, 1H). ES-MS $m/z$: 361 (M+23). EI-MS $m/z$: 338.12 (100%), 339.12 (23%). Anal. Calcd for $C_{20}H_{18}O_5$: C, 70.99; H, 5.36. Found: C, 70.95; H, 5.40.

#### 5.4.4. 7-Methoxy-4-[(*E*)-2-(3,4,5-trimethoxyphenyl)vinyl]-2*H*-chromen-2-one (15)

Starting from **10** (0.9 g, 3.0 mmol) and 3,4,5-trimethoxybenzaldehyde (0.57 g, 3.0 mmol), **15** was obtained (0.80 g, 72% yield); mp 181–182 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.90 (s, 3H), 3.91 (s, 3H), 3.94 (s, 3H), 3.95 (s, 3H), 6.44 (s, 1H), 6.77 (d, $J$ = 8.6 Hz, 1H), 6.88 (d, $J$ = 2.2 Hz, 1H), 6.91 (dd, $J$ = 2.2 and 8.8 Hz, 1H), 7.29 (d, $J$ = 15.9 Hz, 1H), 7.33 (d, $J$ = 8.6 Hz, 1H), 7.50 (d, $J$ = 15.9 Hz, 1H), 7.71 (d, $J$ = 8.8 Hz, 1H). EI-MS $m/z$: 368 (100%), 369 (24%). Anal. Calcd for $C_{21}H_{20}O_6$: C, 68.47; H, 5.47. Found: C, 68.44; H, 5.38.

#### 5.4.5. 7-Methoxy-4-[(*E*)-2-(2,3,4-trimethoxyphenyl)vinyl]-2*H*-chromen-2-one (16)

Starting from **10** (0.9 g, 3.0 mmol) and 2,3,4-trimethoxybenzaldehyde (0.57 g, 3.0 mmol), **16** was obtained (0.73 g, 66% yield); mp 137–139 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.89 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 3.95 (s, 3H), 6.41 (s, 1H), 6.71 (d, $J$ = 8.8 Hz, 1H), 6.77 (d, $J$ = 2.2 Hz, 1H), 6.83 (dd, $J$ = 2.2 and 8.8 Hz, 1H), 7.25 (d, $J$ = 15.9 Hz, 1H), 7.32 (d, $J$ = 8.6 Hz, 1H), 7.50 (d, $J$ = 15.9 Hz, 1H), 7.65 (d, $J$ = 8.8 Hz, 1H). EI-MS $m/z$: 368 (100%), 369 (24%). Anal. Calcd for $C_{21}H_{20}O_6$: C, 68.47; H, 5.47. Found: C, 68.49; H, 5.55.

#### 5.4.6. 4-[(*E*)-2-(3,5-Dimethylphenyl)ethyl]-7-methoxy-2H-chromen-2-one (17)

Starting from **10** (0.9 g, 3.0 mmol) and 3,5-dimethylbenzaldehyde (0.40 g, 3.0 mmol), **17** was obtained (0.5 g, 66% yield); mp 146–148 °C. $^1$H NMR ($CDCl_3$):$\delta$ 2.37 (s, 6H), 3.89 (s, 3H), 6.43 (s, 1H), 6.86 (d, $J$ = 1.8 Hz, 1H), 6.88 (dd, $J$ = 1.8 and 8.7 Hz, 1H), 7.02 (s, 1H), 7.20 (s, 2H), 7.24 (d, $J$ = 16.2 Hz, 1H), 7.30 (d, $J$ = 16.2 Hz, 1H), 7.70 (d, $J$ = 8.4 Hz, 1H). ES-MS $m/z$: 329 (M+23). Anal. Calcd for $C_{20}H_{18}O_3$: C, 78.41; H, 5.92. Found: C, 78.31; H, 6.00.

#### 5.4.7. 7-Methoxy-4-[(*E*)-{2-[4-(3-methylbut-2-enyloxy)phenyl]-vinyl}-2*H*-chromen-2-one (18)

Starting from **10** (0.9 g, 3.0 mmol) and 4-(3-methylbut-2-enyloxy)benzaldehyde[36] (0.57 g, 3.0 mmol), **18** was obtained (0.77 g, 71% yield); mp 134–136 °C. $^1$H NMR ($CDCl_3$): $\delta$ 1.75 (s, 3H), 1.85 (s, 3H), 3.95 (s, 3H), 4.60 (d, $J$ = 6.9 Hz, 2H), 5.48–5.58 (m, 1H), 6.44 (s, 1H), 6.89 (d, $J$ = 8.4 Hz, 1H), 6.91 (d, $J$ = 2.5 Hz, 1H), 6.97 (d, $J$ = 8.8 Hz, 2H, 7.18 (d, $J$ = 16.0 Hz, 1H), 7.28 (d, $J$ = 16.0 Hz, 1H), 7.53 (d, $J$ = 8.6 Hz, 2H), 7.72 (d, $J$ = 8.5 Hz, 1H). ES-MS $m/z$: 385 (M+23). Anal. Calcd for $C_{23}H_{22}O_4$: C, 76.22; H, 6.12. Found: C, 76.21; H, 6.05.

#### 5.4.8. 7-Methoxy-4-[(*E*)-2-(3-methoxyphenyl)vinyl]-2*H*-chromen-2-one (19)

Starting from **10** (0.9 g, 3.0 mmol) and 3-methoxylbenzaldehyde (0.41 g, 3.0 mmol), **19** was obtained (0.49 g, 54% yield), mp 137–139 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.78 (s, 3H), 3.81 (s, 3H), 6.46 (s, 1H), 6.85–6.97 (m, 3H), 7.08 (s, 1H), 7.18 (d, $J$ = 7.6 Hz, 1H); 7.22–7.38 (m, 3H), 7.69 (d, $J$ = 8.1 Hz, 1H). ES-MS $m/z$: 331 (M+23). Anal. Calcd for $C_{19}H_{16}O_4$: C, 74.01; H, 5.23. Found: C, 73.95; H, 5.19.

#### 5.4.9. 3-[(*E*)-2-(3,5-Dimethoxyphenyl)vinyl]-7-methoxy-2*H*-chromen-2-one (20)

Starting from **11** (0.9 g, 3.0 mmol) and 3,5-dimethoxybenzaldehyde (0.49 g, 3 mmol), **20** was obtained (0.56 g, 55% yield); mp 198–200 °C. $^1$H NMR ($CDCl_3$) : $\delta$ 3.82 (s, 6H), 3.88 (s, 3H), 6.41 (t, $J$ = 2.2 Hz, 1H), 6.69 (d, $J$ = 2.2 Hz, 2H), 6.82 (d, $J$ = 2.2 Hz, 1H), 6.86 (dd, $J$ = 2.2 and 8.8 Hz, 1H), 7.07 (d, $J$ = 16.2 Hz, 1H), 7.42 (d, $J$ = 8.8 Hz, 1H), 7.48 (d, $J$ = 16.4 Hz, 1H), 7.75 (s, 1H). ES-MS $m/z$:

361 (M+23). Anal. Calcd for $C_{20}H_{18}O_5$: C, 70.99; H, 5.36. Found: C, 71.05; H, 5.29.

#### 5.4.10. 4-[(*E*)-2-(3,5-Dihydroxyphenyl)vinyl]-7-hydroxychromen-2*H*-2-one (21)

$BBr_3$ (1 M solution in $CH_2Cl_2$, 1.35 mL, 1.35 mmol) was added dropwise to a solution of **14** (0.1 g, 0.3 mmol) in $CH_2Cl_2$ (5 mL) at 0 °C. The solution was warmed to room temperature and stirred at the same temperature for 4 h. The solid formed was collected by filtration and purified by flash chromatography on silica gel ($CH_2Cl_2$/methyl alcohol: 9.5/0.5) to obtain **21** (0.028 g, 31% yield); mp 280–282 °C (dec). $^1$H NMR (DMSO-$d_6$): $\delta$ 2.86 (br, 3H), 6.41–6.43 (m, 2H), 6.74 (d, $J$ = 2.2 Hz, 1H), 6.78 (d, $J$ = 2.4 Hz, 1H), 6.86 (d, $J$ = 2 Hz, 1H, 6.88 dd, $J$ = 2.4 and 8.8 Hz, 1H), 7.36 (d, $J$ = 16.0 Hz, 1H), 7.49 (d, $J$ = 15.6 Hz, 1H), 7.92 (d, $J$ = 8.8 Hz, 1H). ES-MS $m/z$: 297 (M+1). Anal. Calcd for $C_{17}H_{12}O_5$: C, 68.92; H, 4.08. Found: C, 69.05; H, 4.02.

### 5.5. Cellular sensitivity to drugs

Cellular sensitivity to drugs was evaluated by growth-inhibition assay 72 h after drug exposure. Tumour cells, in the logarithmic phase of growth, were seeded in duplicate into 6-well plates. Twenty-four hours after seeding, the drug was added to the medium and 72 h after drug exposure cells were harvested and counted with a cell counter. $IC_{50}$ is defined as the drug concentration causing a 50% reduction of cell number compared with that of untreated control.

### 5.6. Determination of apoptosis

Apoptosis was determined in H460 lung carcinoma cells by TUNEL assay following a 72 h exposure to the drug. Floating and adherent cells were fixed in 4% paraformaldehyde, for 60 min, at room temperature, washed and resuspended in ice-cold PBS. The in situ cell death detection kit fluorescein (Roche, Mannheim, Germany) was used according to the manufacturers' instructions and the samples were analyzed by flow cytometry (Becton Dickinson).

### 5.7. Cell cycle analysis

Early events associated with drug effects (cell cycle perturbation and biochemical modulation) were examined within 24 h of drug exposure to avoid any influence of indirect effects of cellular response. Cell cycle analyses were performed in H460 cells 24 h after drug treatments. Briefly, cells were washed, fixed in ice-cold 70% ethanol, and stored at −20 °C. Subsequently, samples were rehydratated with PBS and cellular DNA was stained with 10 μg/ml propidium iodide in PBS, containing RNase A (66 U/ml). Cell cycle distribution was determined by flow cytometry, and data were analyzed by Cell Quest® software; for each sample 40,000 events were collected.

### 5.8. Protein expression analysis

Total cell lysates were prepared rinsing cells twice with ice-cold PBS supplemented with 0.1 mM sodium orthovanadate, and then lysing them in hot sample buffer.[54] After determination of protein concentration, whole-cell extracts were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Detection of proteins was accomplished using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence purchased from Amersham Biosciences, Rockford, IL. Primary antibodies used in this study are: anti-p21 (NeoMarker, USA), anti-p34$^{cdc2}$ (GIBCO, Invitrogen, NY, USA), anti-cyclin B1 (Santa Cruz Biotechnology, USA), anti-pro-caspase 3 (BD Transduction Laboratories, Lexington, USA), anti-cleaved CCP32 (Asp175) (Cell Signalling Technology, USA), anti-PARP-1 (Calbiochem, USA), anti-actin (Sigma).

### 5.9. Co-immunoprecipitation and immunoblot analysis

Following the designated treatments, cells were lysed in NET buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L sodium chloride, 0.1% NP40,1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 2.5 μg/mL leupeptin, 5 μg/mL aprotinin] first for 30 min on ice and then for 30 min at 4 °C in rotation. The nuclear and cellular debris were cleared by centrifugation (10,000*g*, 10 min, 4 °C). Total cellular proteins were then quantified using the BCA protein assay. Cell lysates (500 μg) were incubated with the cdc-2-specific monoclonal antibody for 2 h at 4 °C. Protein A-sepharose (Sigma–Aldrich, Germany) (90 μl), previously prepared in TNT solution [20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100] was added to the lysates and incubated overnight at 4 °C. The immunoprecipitates (separated by centrifugation 15,000*g*, 2 min, 4 °C) were washed twice in NET buffer and twice in PBS with 1% aprotinin and 1 mmol/L PMSF. Proteins were eluted with the SDS sample loading buffer before the immunoblot analyses with specific antibodies against p34$^{cdc2}$ or p21 (Santa Cruz).

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