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# Unsaturated genistein disaccharide glycoside as a novel agent affecting microtubules

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ABSTRACT

Genistein, due to its recognized chemopreventive and antitumor potential, is a molecule of interest as a lead compound in drug design. While multiple molecular targets for genistein have been identified, so far neither for this isoflavonoid nor for its natural or synthetic derivatives disruption of microtubules and mitotic spindles has been reported. Here we describe such properties of the synthetic glycosidic derivative of genistein significantly more cytotoxic than genistein, 7-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(6-*O*-acetyl-hex-2-ene-α-D-erythro-pyranosyl)genistein, shortly named G21. We found that G21 causes significant mitotic delay, frequent appearance of multipolar spindles, and alteration of the interphase microtubule array.



Epidemiological and experimental studies place genistein among chemopreventive agents and a complementary drugs in a treatment of cardiovascular diseases, postmenopausal syndromes, and cancer.[1,2] At lower concentrations, essentially not exceeding 20 μM, this isoflavonoid may act as a selective estrogen receptor modulator (SERM), with higher affinity to ERβ than ERα, while at higher doses genistein reveals antiproliferative activity. Although multiple molecular targets have been identified or suggested, the major mechanism by which genistein impedes growth of cancerous cells are thought to be: the inhibition of the activity of tyrosine kinases, topoisomerase II, and transcription factor NF-κB, as well as affecting activities which control cell cycle.[3]

These observations inspired several groups to synthesize various derivatives of genistein with the intention to either improve biochemical and pharmacokinetic characteristics of the parent drug or to obtain compounds containing essential elements of the parent substance but having novel properties and/or affecting novel molecular targets.[4–10] So far none of the novel derivatives revealed better inhibitory action on tyrosine kinase activity than genistein[11,12] while some approaches targeting genistein to tyrosine kinases through conjugation of the isoflavone with EGF or specific antibodies gave promising results.[13,14]

Much effort has been put into the design and synthesis of new genistein derivatives with improved anticancer activity that could affect proliferation of cancerous cells by mechanisms other than inhibition of tyrosine kinases and chemical glycosylation seems a viable option for such purpose.[15] The genistein derivatives, glycosides of 2,3-unsaturated sugars, were found to inhibit proliferation of multiple cancer cell lines at a concentration significantly (5–10 times) lower than a parent compound, what makes them attractive objects, both for pharmacological studies and as lead compounds for further modifications. Of multiple novel derivatives, the most promising obtained so far is: 7-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(6-*O*-acetyl-hex-2-ene-α-D-erythropyranosyl) genistein, shortly named G21.[16,17]

In the present report we describe the results of our investigation which demonstrate, that the synthetic genistein glycoside, G21 has the potential to affect microtubule dynamics, leading to malformation of mitotic spindles. To our knowledge, the G21 is the first derivative of genistein exhibiting features of a mitotic spindle poison. We also describe the simplified method of regio- and stereoselective synthesis of this compound.

This novel genistein derivative was earlier obtained in a multistep procedure based on palladium catalyzed exchange of unsaturated anomeric carbonate esters,[16,18] which resulted in the mixture of 7-, 4′- regio- and α,β-stereoisomers and meticulous chromatography was required in order to isolate the desired compound. Here, we describe a novel approach to synthesis of G21 which resulted in high regio- and stereoselectivity of glycosylation of genistein aglycon, in keeping with observation that usually the reactivity of the phenolic hydroxyl group at 7-OH is higher than that of other positions in flavonoids.[19–21] Both the anomeric leaving group and the method of its activation have been changed. As a part of the

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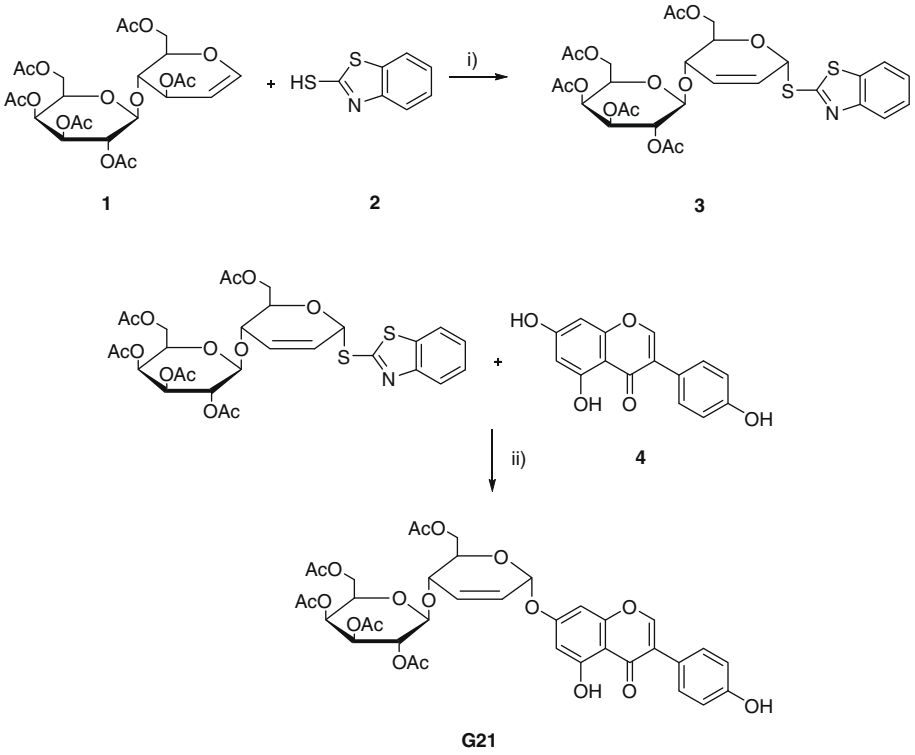



**Figure 1.** Scheme of the stereoselective synthesis of G21. Reagents: (i) $InCl_3$, $CH_2Cl_2$, yield 83%; (ii) $Cu(OTf)_2$, $CH_2Cl_2$, yield 76%.

program to develop novel, versatile methods for the stereoselective synthesis of glycosides derivatives of genistein we have become interested in relatively novel class of glycosyl donors, substituted thioimidoyl derivatives.[22] A number of such moieties have already been employed at the anomeric center in an attempt to achieve better stereo control of the glycosylation[23] and phosphorylation process,[24] although there was no precedence in their application to glycal chemistry.

Admittedly, in case of glycals, unfavorable regioselectivity in reaction of thiol group containing substrates could be expected on the ground of hard and soft acids and bases theory[25] but, since benzotiazol derivatives were not examined before, we took a chance of obtaining desired 1-thiolglycoside, even through isolation form a mixture. Here we describe the synthesis of novel *S*-benzothiazolyl derivatives of 2,3-unsaturated pyranoses and their application for stereoselective and regioselective glycosylation of genistein (Fig. 1). For this purpose lactal (**1**) was first reacted with 2-mercapto-benzothiazole (**2**) in the presence of a promoter in methylene chloride at room temperature to afford benzothiazolyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-6-*O*-acetyl-hex-2-ene-1-thio-α-D-erythropyranoside (**3**) in 83% yield. Having obtained required intermediate thioglycoside we turned our attention to the evaluation of its glycosylating properties. It was hypothesized that heavy metal salt-based promoter system would complex sulfur and nitrogen atoms in benzothiazole aglycon, improving the leaving group ability.[26] Thus, thioglycoside was coupled with genistein (**4**) in the presence of a thiophilic metal promoter in dry methylene chloride. The highest regio- and stereoselectivities for the synthesis of a title compound were obtained in the presence of AgOTf and $Cu(OTf)_2$. When MeOTf was used the methylation of genistein was observed. In reaction using NIS/TMSOTf as a promoter the mixture of glycosides was obtained (α/β: 3/1) and products of reaction of genistein (not identified) were formed. The scheme of the novel synthesis of G21 is shown in Figure 1.

The advantages of this procedure in comparison with previously reported one[16,18] is the accessibility of the stable glycosylating donor, and high regio- and stereoselectivity of its action under mild activation conditions.[27]

The structure of obtained glycoside (G21) was confirmed by $^1$H NMR spectral data and by comparison with a known compound. The physical and spectral properties of α-glycoside were identical as in literature data.[16] The $^1$H NMR spectra of α-glycoside showed a characteristic signal at $\delta$ 5.72 ppm (ddd, 1H, $J_{1,2}$ = 0.8 Hz, $J_{1,3}$ = −1.9 Hz, $J_{1,4}$ = 0.7 Hz, H-1), $\delta$ 5.92 ppm (dd, 1H, $J_{3,4}$ = 2.76 Hz, H-3), $\delta$ 6.30 ppm (ddd, 1H, $J_{2,3}$ = 10.28 Hz, $J_{2,4}$ = −1.3 Hz, H-2). The $^1$H NMR spectrum of β-glycoside gave H-1, 5.83 ppm ($J_{1,2}$ = 1.65 Hz, $J_{1,3}$ = 1.25 Hz, $J_{1,4}$ = 1.2 Hz, $J_{1,5}$ = −0.4 Hz) H-2, 6.25 ppm (ddd, $J_{2,3}$ = 10.24 Hz, $J_{2,4}$ = −3.1 Hz), and H-3, 6.02 ppm (ddd, $J_{3,4}$ = 1.5 Hz). Relevant NMR spectra are available as Supplementary data.

Compound G21 was previously shown to inhibit proliferation of many cancer cell lines, however, the mechanism of its action was not studied in details.[16,17] For biological evaluation of G21 activity human prostate cancer cell line, DU 145 was chosen and grown under standard conditions.[28] These results, together with appropriate control experiments, as well as additional data on cytotoxicity of G21 against human colon carcinoma HCT 116 cells are available as Supplementary data.

In order to assign the mechanism of antiproliferative activity of G21 we investigated the influence of genistein and G21 on the cell cycle with flow cytometer.[29]

In subconfluent control cells we observed two main populations of cells with different DNA content: 60% of cells were in G1 phase of a cell cycle (2C DNA), and around 20% of cells were in G2/M phase of a cycle (4C DNA). As shown in Figure 2. the treatment of cells with G21 resulted in a significant accumulation of cells in G2/M phase of a cycle (4C DNA) as compared to cells treated with genistein.

In the next step we determined microscopically the fraction of mitotic cells.[30] Treatment of cells for 24 h with G21 led to the increase of the mitotic index from 6%, observed in control, to

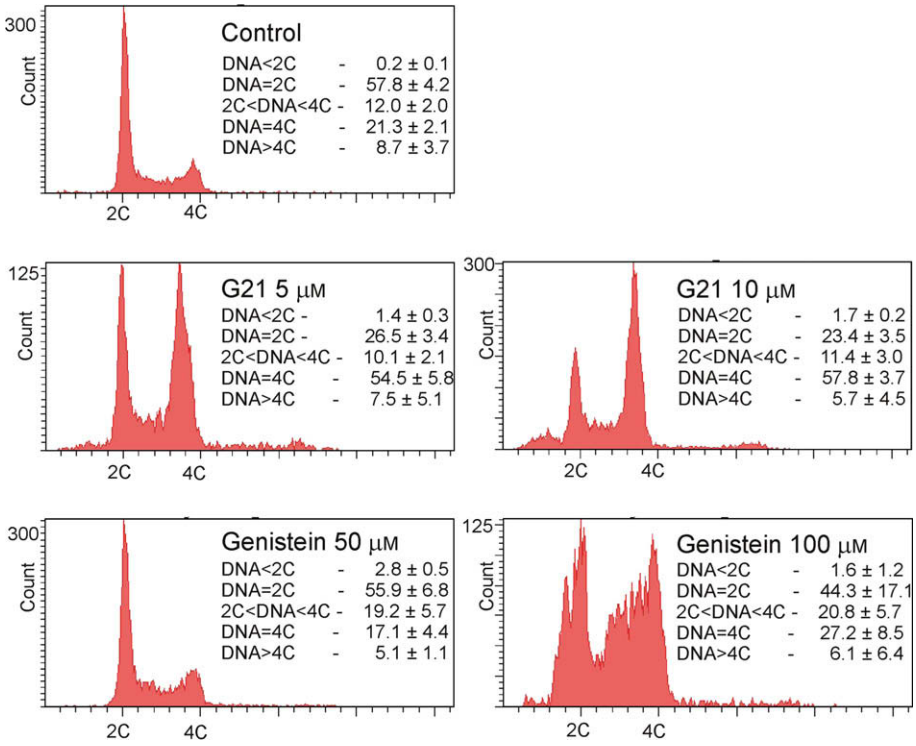



**Figure 2.** Flow cytometry analysis of cell cycle and DNA ploidy of DU 145 cells treated with genistein and G21. The 2C DNA corresponds to cells at G1 phase of a cell cycle, and the 4C DNA corresponds to cells at G2/M phase. Cells were collected after 24 h incubation with drugs at concentration indicated in the picture. Histograms are representative for the treatment. Numerical data on the histograms represents mean number of cells with indicated DNA content ± standard deviation, calculated from three independent experiments.

almost 30%. (Table 1). In contrast to significant increase of the mitotic index values after G21 treatment, the mitotic index after the dose of genistein which caused visible accumulation of 4C DNA cells was lower than in untreated control. Whereas genistein blocked cell cycle in G2 phase, G21 almost did not influence this phase, blocking the cells in mitosis.

In the next step we decided to check if the mitotic block is related to changes in mitotic spindle structure in immunofluorescently stained specimens.[31] Analysis of mitotic spindle aberrations after G21 treatment is presented in Figure 3.

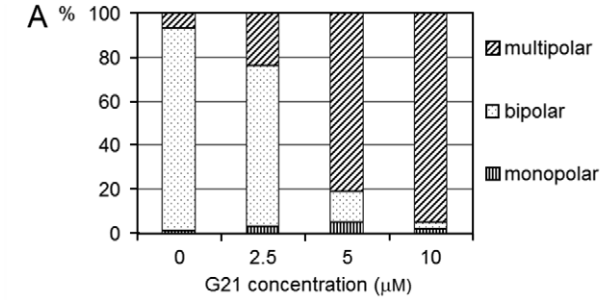
In control cells most of mitotic spindles had two regular poles, and genistein did not influence their structures even at 100 μM concentration (not shown).

Multipolar or monopolar spindles occurred very rarely. On a contrary, in cells treated with G21, aberrant, mainly multipolar mitotic spindles were observed. The fraction of cells containing aberrant mitotic spindle increased in a G21 dose-dependent manner, and already at 5 μM most of mitosis were aberrant (Fig. 3B and C). Multipolar spindles were also observed after treatment of cells with equitoxic to G21 doses of known drugs altering microtubule dynamics, such as vinblastine and paclitaxel (not shown), and as described in literature.[32,33] Since both, microtubule destabilizing agents, that is, vinblastine, and assembly promoting ones, that is, paclitaxel, may cause multipolar spindle formation,[32,33] it is not possible to assign if a drug inhibits or stimulates microtubules assembly. However, the type of changes of interphase microtubules array indicates whether a given chemical stabilizes or destabilizes microtubules. The results of a study of interphase microtubule array, performed on cells treated with G21, two reference drugs which affect microtubule dynamics: vinblastine, inhibiting microtubules assembly and paclitaxel—microtubules assembly stimulating drug, and with genistein, which is known not to interact with microtubules[34] are presented in Figure 4.

**Table 1**
Mitotic index counted upon microscopic examination

| Control | Genistein | | G21 | |
|---|---|---|---|---|
| | 50 μM | 100 μM | 5 μM | 10 μM |
| 5.9 ± 1.2 | 6 ± 2.7 | 2.2 ± 0.7 | 29.4 ± 0.1 | 28.1 ± 0.6 |

Means and standard deviations are calculated from three independent experiments.



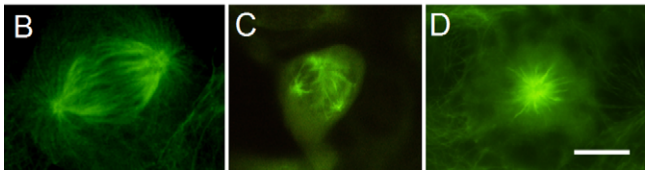




**Figure 3.** Types of mitotic spindles in cells treated with G21. (A) Influence of growing concentration of G21 on the frequency of abnormal spindles. (B) Normal bipolar spindle. (C) Multipolar spindle. (D) Monopolar spindle. In control cells normal, bipolar spindles dominate. After treatment with G21 multipolarity is frequently observed. Scale bar—20 μm.

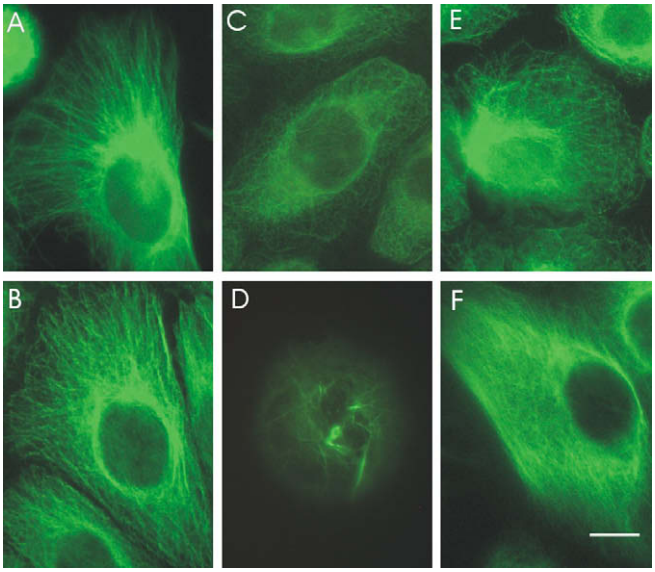



**Figure 4.** Immunofluorescent staining of β-tubulin in interphase DU 145 cells. Cells were treated with a vehiculum (control) or indicated drugs for 24 h. (A) Control; (B) 100 μM genistein; (C) 5 μM G21; (D) 25 μM G21; (E) 1 nM vinblastine; (F) 2 nM paclitaxel, (note that concentration in C, E, F are equitoxic and correspond to 50% cytotoxicity). G21, vinblastine and paclitaxel cause different perturbations of microtubule array described in details in the text. Genistein does not induce any changes in microtubule array. Scale bar—10 μm.

Genistein—as expected—caused no alteration of microtubule array even at 100 μM concentration. At the concentration of G21 ~$IC_{50}$ (5 μM) the microtubule network was markedly loosened at the cell periphery (Fig. 4C) and microtubules acquired characteristic, curly appearance, resembling the pattern described for vinblastine used at ~$IC_{50}$ concentration (Fig. 4E). This pattern was essentially different from the one observed for cells treated with paclitaxel (Fig. 4F), where microtubule network was noticeably denser than in control cells or the ones treated with genistein, G21 and vinblastine. It has to be noted that at high concentration of G21 (25 μM) microtubules disappeared almost completely (Fig. 4D). The ability of G21 to influence the structure of microtubule network, and to induce mitotic spindle aberration was not limited to DU 145 cells, and comparable effects were also observed in other cell lines under study (not shown).

In summary, we have found a new method for the regio- and stereoselective synthesis of the glycosyl genistein derivative, G21. Extensive biological studies have shown that this compound acts as a microtubule destabilizing agent. This ability has not been reported previously for any isoflavonoid derivative.

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## Supplementary data

Effect of G21, lactal, genistein and mixture of these compounds on the proliferation of human prostate cancer cells DU 145 and human colon carcinoma cells HCT 116. NMR spectra of G21. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.089.

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27. *Procedure for synthesis of benzothiazoyl glycosyl donor*: A solution of the per-*O*-acetyl-lactal (1 mM), 2-mercapto-benzothiazol (1 mM) in $CH_2Cl_2$ (2 mL) and freshly activated molecular sieves (3 Å, 0.2 g) was stirred under argon for 10 min, $InCl_3$ (0.15 mM) was added. The reaction mixture was then stirred for 60 min under reflux. Upon completion (TLC), the reaction mixture was diluted with $CH_2Cl_2$ (10 mL), the solid was filtered-off and the residue was washed with $CH_2Cl_2$ (5 mL). The combined filtrate was washed with 5% aq $NaHCO_3$ (3 × 10 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (chloroform) to allow the thioglycoside as colorless oil (600 mg, yield 83%). The structure was confirmed by $^1H$ NMR ($CDCl_3$) $\delta$ (ppm) 1.98 (s, 3H), 2.02 (s, 6H), 2.07 (s, 3H), 2,18 (s,3H), 3.90–4.34 (m, 7H), 4.62 (d, 1H, $J$ = 8 Hz), 5.05 (dd, 1H, $J_1$ = 10.2 Hz, $J_2$ = 3.4 Hz), 5.24 (dd, 1H, $J_2$ = 7.6 Hz), 5.42 (d, 1H, $J$ = 2.8 Hz), 5.72 (d, 1H, $J$ = 2.6 Hz), 5.94 (ddd, 1H, $J_1$ = 10.2 Hz, $J_2 = J_3$ = 2.5 Hz), 6.31(ddd, 1H, $J_2 = J_3$ = 0.8 Hz), 6.54 (d, 1H, $J$ = 2.2 Hz), 6.62 (d,1H, $J$ = 2.2 Hz), 6.95 (dd, 2H, $J_1$ = 6.6 Hz, $J_2$ = 2.2 Hz), 7.53 (dd, 2H), 8.01 (d, 1H), 8.19 (d, 1H). *Procedure for synthesis of G21*: A mixture of the glycosyl donor (Fig. 1, Formula **3**) (0.1 mM) and genistein (Fig. 1, Formula **4**) (0.15 mM) in $CH_2Cl_2$ (2 mL) was sonificated for 30 min, freshly activated molecular sieves (3 Å, 0.2 g), $Cu(OTf)_2$ (0.15 mM) was added and reaction mixture was stirred under argon for 1 h at room temp. Upon completion, the reaction mixture was diluted with $CH_2Cl_2$ (10 mL), the solid was filtered-off and the residue was washed with $CH_2Cl_2$ (10 mL). The combined filtrate was washed with aq $NaHCO_3$ and water (3 × 10 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by column chromatography on silica gel (chloroform) to allow the genistein glycoside as amorphous solid (630 mg, yield 76%).
28. *Cell culture conditions*: Cells were grown at 37 °C, in humidified atmosphere enriched with $CO_2$ (5%) in RPMI 1640 medium (Sigma–Aldrich, Germany) supplemented with non-inactivated fetal bovine serum (FBS) (10%; GIBCO Invitrogen, UK) and gentamicin sulfate (40 μg $mL^{-1}$; Sigma–Aldrich, Germany).
29. *Flow cytometry*: Floating cells were collected and added to adherent cells, harvested by trypsinization. Cells were washed with PBS and then fixed in ice-cold ethanol (70%) for 30 min, treated with RNase (100 μg $mL^{-1}$) and stained

with propidium iodide (PI) (100 μg $mL^{-1}$). The DNA content was analyzed using Becton Dickinson FACSCanto cytometer (BD Company, USA).

30. *Mitotic index determination*: Cells were grown in 3 cm culture dishes and collected after 24 h treatment. Floating cells were pooled with adherent cells after trypsinisation, washed with PBS, cytospinned, fixed with paraformaldehyde (4%) in PBS for 60 min, stained with DAPI (3 μM; Sigma–Aldrich, Germany), and mounted in DAKO® Fluorescent Mounting Medium (Dako, USA). Slides were examined under Nikon ECLIPSE E800 microscope using 40× objective. The mitotic cells were counted per 500 cells of the specimen.
31. *Microtubule array analysis*: Cells growing on 8-well chamber slides (Lab-Tek Permanox® slides, Nalgen Nunc International, Rochester, NY, USA) after treatment with genistein, G21, vinblastine or paclitaxel. Microtubules were immunostained as described by Huang, Y. T.; Huang, D. M.; Guh, J. H.; Chen, I. L.; Tzeng C. C.; Teng, C. M. *J. Biol. Chem*. **2005**, *280*, 2771. Cells were examined under an Nikon ECLIPSE E800 microscope using an oil immersion objective 100×. The type of mitotic spindle (bipolar = normal, multipolar or monopolar) was counted in 100 of mitotic cells per treatment. Experiments were repeated three times.
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