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Cellular Localisation of Antitumoral 6-Alkyl Thymoquinones Revealed by an Alkyne–Azide Click Reaction and the Streptavidin–Biotin System

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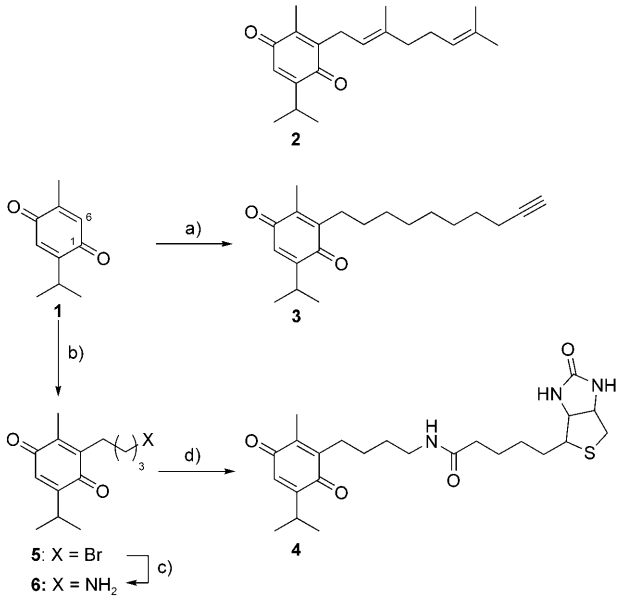
The subcellular distribution and accumulation of thymoquinone **1**, a natural anticancer agent, has hitherto been unknown. We prepared 6-(dec-9-ynyl)thymoquinone **3**, an alkyne-labelled derivative with anticancer activity similar to that of its parent compound **1**. Alkyne **3** was seen, after a Huisgen-type click reaction with 3-azido-7-hydroxycoumarin, to accumulate in distinct compartments of the nuclei of PtK_2 potoroo kidney cells, and in adjoining regions that were stained with an antibody specific for the Golgi apparatus. In contrast, a biotin-labelled thymoquinone **4** seemed to accumulate across the entire cell nucleus upon visualisation with streptavidin; but this was less easily traceable because of co-staining of other structures such as mitochondria. In conclusion, for small drug-like molecules, visualisation by alkyne–azide cycloaddition seems to be superior to conventional visualisation by the biotin–streptavidin system.

Introduction

The Cu^I-mediated 1,3-dipolar cycloaddition ("click reaction") between alkyne-labelled proteins and fluorogenic or radio-labelled azides (to give the corresponding 1,2,3-triazoles) has been occasionally employed for in vivo visualisation.[1–4] In comparison, applications of this reaction to the localisation of low-molecular compounds in cells and biological tissues are rare.[5] These molecules would benefit highly from an alkyne label that not only is biologically inert but also induces minimal structural alteration, compared to that with the more common biotin labels. A particularly small compound of hitherto unknown subcellular distribution is thymoquinone (TQ, **1,** Scheme 1), a constituent of the volatile oil of black seed (*Nigella sativa*). It shows antioxidative, anti-neoplastic and anti-inflammatory properties.[6,7] TQ induces apoptosis in cancer cells in both p53-dependent and p53-independent ways, and it was found to delay tumour growth by the induction of cell-cycle arrest in certain xenograft models.[8–12] In a preceding article we showed that some non-natural 6-alkylthymoquinones, such as the geranyl derivative **2**, differed little from TQ in their efficacy against various tumour cell lines, and in their mode of apoptosis induction: elevated levels of reactive oxygen species, a loss of the mitochondrial membrane potential and the activation of caspases.[13,14] In order to use alkyne–azide cycloaddition to localise such TQ derivatives in living cells, we prepared 6-(dec-9-ynyl)thymoquinone **3**; this features a C_{10} appendage and a degree of unsaturation, like analogue **2**. We expected **3** to exhibit anticancer effects and affinities to subcellular components, similar to those of 6-geranylthymoquinone **2** and of TQ. The actual visualisation was to be effected by a Cu^I-catalysed click reaction of the terminal alkyne **3** with 3-azido-7-hydroxycoumarin **7** to afford a strongly fluorescent 1,2,3-triazole **8** (Scheme 2). For comparison, we also prepared the C_{10}-teth-



Scheme 1. Reagents and conditions: a) undec-10-ynoic acid, $AgNO_3$, $(NH_4)_2S_2O_8$, CH_3CN/H_2O, 100 °C, 4 h, 26%; b) 5-bromovaleric acid, $AgNO_3$, $(NH_4)_2S_2O_8$, CH_3CN/H_2O, 100 °C, 16 h, 13%; c) 1. urotropine, $CHCl_3$, RT, quant., 2. EtOH, HCl, reflux, quant.; d) biotin, DCC, DMAP, HOBt, CH_2Cl_2/DMF, RT, 41%.

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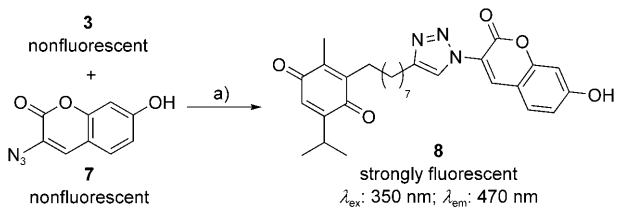

Scheme 2. Copper(I)-catalysed 1,3-dipolar azide–alkyne cycloaddition (click reaction) under cell-free conditions: a) $CuSO_4$, sodium ascorbate (Click-iT Cell Reaction Buffer Kit), 30 min, RT.

ered biotin conjugate **4** to be visualised via the streptavidin–biotin system (Scheme 1).[15]

Results and Discussion

Synthesis of labelled 6-alkylthymoquinones 3 and 4

Compound **3** was prepared by a known procedure from TQ, undec-10-ynoic acid and $Ag_2S_2O_8$ in aqueous acetonitrile (Scheme 1).[16,17] For the synthesis of the biotin conjugate **4** the same protocol was employed to first prepare the bromobutyl derivative **5** from TQ and ω-bromovaleric acid. This bromide was then converted to the corresponding amine **6** by a Delépine reaction with urotropine.[18,19] Finally, amide **4** was obtained from condensation of amine **6** with biotin in the presence of dicyclohexylcarbodiimide (DCC), 4-(*N*,*N*-dimethylamino)pyridine (DMAP) and 1-hydroxybenzotriazole (HOBt).[20]

Biological studies

The labelled TQ-derivatives **3** and **4** were first tested by using the MTT assay for anti-proliferative activity in cells of human 518A2 (melanoma), HL-60 (leukaemia), HT-29 (MRP 1-rich colon carcinoma), KB-V1^{VBL} (cervix carcinoma overexpressing P-gp) and MCF-7^{TOPO} (BCRP-rich breast adenocarcinoma).[21] As the measured activities, at least of acetylene **3**, lay in the ranges of those of TQ and the previously tested geranyl derivative **2**,[13] we assumed that the attachment of an alkyne label would not much alter the cellular uptake and distribution of conjugate **3** when compared to TQ and **2** (Table 1). This might not be true for the biotin conjugate **4**, given its more widely deviating IC_{50} values. It is worthy of note that neither **3** nor **4** inhibited the growth of non-malignant human foreskin fibroblasts at concentrations below 100 μM.

Table 1. Inhibitory concentrations IC_{50}[a] in μM of compounds **1**–**4** determined by the MTT assay when applied to various human cancer cells.

Compound/cell line	1	2	3	4
518A2	28 ± 9[b]	23 ± 7[b]	24 ± 9	34 ± 16
HL-60	28 ± 6[b]	29 ± 12[b]	3.0 ± 0.8	>100
HT-29	47 ± 19[c]	n.m.[d]	49 ± 13	>100
KB-V1^{VBL}	32 ± 6[b]	16 ± 2[b]	26 ± 9	30 ± 17
MCF-7^{TOPO}	27 ± 6[b]	27 ± 2[b]	10 ± 2	50 ± 4

[a] Values are derived from concentration–response curves obtained by measuring the percentage absorbance of viable cells relative to untreated controls (100%) after 72 h exposure of 518A2, HL-60, HT-29, KB-V1^{VBL} and MCF-7^{TOPO} cells to the test compounds. Values represent means of four independent experiments ± standard deviations. [b] Values taken from ref. [13]. [c] Value taken from ref. [22]. [d] Not measured.

Next, the click reaction between the alkynylthymoquinone **3** and 3-azido-7-hydroxycoumarin **7** was carried out on a 5 μM scale under cell-free conditions in a commercial buffer (Click-iT Cell Reaction Buffer), in order to check its feasibility and to determine the optimum excitation and emission wavelengths for its intended visualisation within PtK_2 potoroo (*Potorous tridactylis)* kidney epithelial cells (Scheme 2).[23–25] PtK_2 cells lend themselves ideally to this experiment because of their flat shape. Panel A of Figure 1 shows the results of staining PtK_2 cells by incubation first with alkyne **3** (5 μM, 16 h) and then with azide **7** (5 μM) in Click-iT Cell Reaction Buffer for 30 min. The blue fluorescence of coupling product **8** is localised to distinct nuclear regions that might be rich in DNA (e.g., chromatin) or RNA (nucleoli), and in regions adjoining the nucleus. The latter were stained in control experiments with a dictyosome-specific antibody and a secondary antibody conjugated with Alexa Fluor 594 following the click reaction; so, they could be the Golgi apparatus or parts of the endoplasmic reticulum (Figure 1B). Figure 1C is an overlay of panel B and a brightfield image to better indicate the cell boundaries. The functions of the Golgi apparatus are as diverse as the glycosylation, sorting and secretion of proteins and metabolites of xenobiotics produced in the endoplasmic reticulum. For the identification of the true molecular targets of **3,** further experiments will be necessary.

In contrast, the biotin conjugate **4** seemed to accumulate across the nucleus of PtK_2 cells upon incubation at concentrations of 6.5 μM for 24 h (Figure 2). However, visualisation with streptavidin was less easily traceable because of co-staining of other structures such as mitochondria; this gave a blurred fluorescent background. Since biotin also plays a critical role in histone modification, the accumulation of **4** in PtK_2 nuclei cannot be interpreted as being solely a consequence of the thymoquinone moiety.[26] The actual visualisation was performed by incubation with a streptavidin–Alexa Fluor 488 conjugate (10 μg mL^{-1}), followed by mounting in ProLong Antifade Gold that contained DAPI (1 μg mL^{-1}) for staining the nuclei. Compared to controls (MeOH, Figure 2A), the cells exposed to **4** (Figure 2B) showed a superposition of the blue DAPI fluorescence and the green fluorescence of the streptavidin–biotin aggregate.

Conclusions

The thymoquinone derivative **3** bears a biologically inert terminal acetylene label that does not significantly alter its polarity and cytotoxicity compared to analogues without this label, such as the geranyl conjugate **2**. The distribution of compound **3** in living PtK_2 was visualised by a click reaction with 3-azido-7-hydroxycoumarin; this produced the fluorescent 1,2,3-triazol that accumulated in distinct regions within and adjoining the nuclei of PtK_2 cells. As no molecular target with a strong affinity to acetylene is known within these cellular compartments,

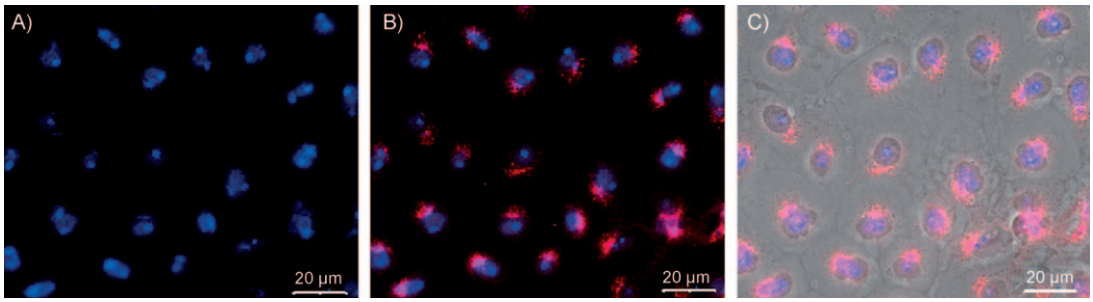

Figure 1. A) PtK_2 cells incubated first with **3** (5 μM, 16 h) then with azide **7** (5 μM, 30 min) in Click-iT Cell Reaction Buffer (blue stain); B) PtK_2 cells additionally stained with a Golgi-selective antibody and a secondary antibody conjugated with Alexa Fluor 594 (red stain); C) overlay of B) with bright field image.

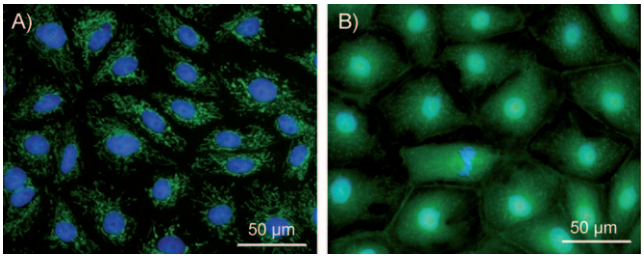

Figure 2. A) PtK_2 control cells (MeOH) with the addition of streptavidin–Alexa Fluor 488 (green) visualises biotin-containing mitochondria; nuclei are stained with DAPI (blue). B) PtK_2 cells incubated with **4** (6.5 μM, 24 h); localisation is visualised by streptavidin–Alexa Fluor 488 (green). Nuclei (chromosomes) are stained with DAPI.

we assume that the targeting effect can be attributed to the thymoquinone core itself. In contrast, the visualisation of the biotin-labelled thymoquinone **4** by streptavidin was less conclusive because of the ubiquitousness of biotin in cells. In conclusion, for small drug-like molecules, a visualisation based upon the Huisgen alkyne–azide cycloaddition reaction is more reliable than conventional visualisation of biotin-labelled compounds by interaction with streptavidin.

Experimental Section

Instrumentation and chemicals

Fluorescence microscopy: We used an Axioplan fluorescence microscope (Zeiss, Jena, Germany) equipped with an Axiocam camera, 20×, 40× and 63× objectives, and an XBO150W super pressure mercury lamp. Data analysis was performed with AxioVision 3.1 software. IR spectra were obtained on a Spectrum One FTIR spectrophotometer (PerkinElmer) equipped with an ATR sampling unit. NMR spectra were obtained from an Avance 300 spectrometer (Bruker) with chemical shifts (δ) given in ppm downfield from Me_4Si. EIMS was performed on a Varian MAT 311A, and microanalyses on a 2400 CHN analyser (PerkinElmer); corrected analyses (with ±0.2% for C, H, N) were obtained for **3** and **4**. Column chromatography (CC) used silica gel 60 (230–400 mesh). All reagent-grade chemicals were purchased from commercial sources, and solvents were dried and distilled before use. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was purchased from ABCR (Karlsruhe, Germany). 3-Azido-7-hydroxycoumarin **7** was prepared according to a published procedure.[24]

Syntheses

*6-(Dec-9-ynyl)thymoqinone (**3**):* A mixture of thymoquinone **1** (100 mg, 0.61 mmol), 10-undecynoic acid (89 mg, 0.49 mmol), $AgNO_3$ (13 mg, 0.08 mmol) and CH_3CN/H_2O (1:1, 15 mL) was stirred and heated at 100 °C while a solution of $(NH_4)_2S_2O_8$ (139 mg, 0.61 mmol) in H_2O (0.61 mL) was slowly added. The resulting mixture was refluxed for a further 4 h, then cooled, diluted with H_2O, and extracted with Et_2O. The organic phases were washed with brine and dried over Na_2SO_4. Volatile solvents were removed under vacuum, and the residue thus obtained was purified by CC (EtOAc/cyclohexane, 1:4). Yield: 39 mg (26%) as yellow oil; $R_f = 0.59$ (cyclohexane/toluene, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.08$ (d, $J =$ 6.9 Hz, 6H), 1.2–1.4 (m, 10H), 1.49 (m, 2H), 1.91 (t, $J = 2.6$ Hz, 1H), 1.98 (s, 3H), 2.15 (dt, $J = 7.1$, 2.6 Hz, 2H), 2.45 (t, $J = 7.6$ Hz, 2H), 3.02 (dsept, $J = 6.9$, 1.2 Hz, 1H), 6.44 (d, $J = 1.2$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 11.7$, 18.3, 21.4, 26.7, 28.4, 28.6, 28.7, 28.9, 29.2, 29.7, 29.8, 29.9, 68.1, 84.7, 129.9, 134.2, 145.3, 154.6, 187.0, 188.5; IR (ATR): $\nu_{max} = 3290$, 2928, 2856, 1641, 1612, 1463, 1377, 1306, 1244, 893 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 300 (37) $[M]^+$, 257 (10), 180 (19), 150 (100), 121 (53), 67 (52).

*6-(4-Bromobutyl)thymoquinone (**5**):* A mixture of thymoquinone **1** (200 mg, 1.22 mmol), ω-bromovaleric acid (180 mg, 0.97 mmol), $AgNO_3$ (20 mg, 1.22 mmol) and CH_3CN/H_2O 1:1 (15 mL) was heated at 100 °C while a solution of $(NH_4)_2S_2O_8$ (280 mg, 1.22 mmol) in H_2O (0.61 mL) was slowly added. The resulting mixture was refluxed overnight, then cooled, diluted with H_2O, and extracted with Et_2O. The organic phases were washed with brine and dried over Na_2SO_4. Volatile solvents were removed under vacuum, and the remainder was purified by CC (EtOAc/cyclohexane, 1:4). Yield: 47 mg (13%) as a yellow oil; $R_f = 0.41$ (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.08$ (d, $J = 6.9$ Hz, 6H), 1.46 (tt, $J = 7.9$, 8.2 Hz, 2H), 1.59 (m, 2H), 1.99 (s, 3H), 2.49 (t, $J = 7.9$ Hz, 2H), 3.00 (dsept, $J = 6.9$, 1.2 Hz, 1H), 3.65 (t, $J = 6.4$, 2H), 6.44 (d, $J = 1.2$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 11.7$, 21.4, 24.8, 25.8, 26.3, 26.7, 32.5, 130.0, 141.1, 144.9, 154.6, 187.1, 188.4; IR (ATR): $\nu_{max} = 2958$, 2934, 2870, 1707, 1645, 1614, 1459, 1377, 1308, 1246, 1060, 1025, 892, 799, 709 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 236 (100), 203 (68), 175 (100), 147 (68).

*6-(4-Aminobutyl)thymoquinone (**6**):* Bromide **5** (40 mg, 0.13 mmol) was dissolved in dry $CHCl_3$ and treated with urotropine (19 mg, 0.13 mmol). The resulting mixture was stirred at room temperature for 8 h, treated with conc. aqueous HCl (2 mL), then stirred and heated for a further 4 h. It was cooled to ambient temperature, diluted with H_2O, and extracted with EtOAc. The organic phases were washed with brine and dried over Na_2SO_4. Volatile solvents were removed under vacuum, and the residue was purified by CC (EtOAc/cyclohexane, 1:1). Yield: 30 mg (quant.) as a yellow oil; $R_f =$

0.38 (EtOAc/cyclohexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.08$ (d, $J = 6.9$ Hz, 6H), 1.6 (m, 4H), 1.99 (s, 3H), 2.49 (t, $J = 7.9$ Hz, 2H), 3.01 (dsept, $J = 6.9$, 1.2 Hz, 1H), 3.66 (t, $J = 6.4$ Hz, 2H), 6.45 (d, $J = 1.2$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 11.8$, 21.4, 24.9, 25.8, 26.3, 26.7, 32.5, 130.0, 140.2, 144.9, 154.6, 187.3, 188.4; IR (ATR): $\nu_{max} = 3378$, 2961, 2935, 2873, 1709, 1647, 1612, 1459, 1378, 1308, 1246, 1060, 1025, 892, 799, 708 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 236 (100) $[M+1]^+$, 221 (24), 203 (67), 175 (85), 147 (52), 135 (30), 105 (35), 91 (46), 53 (43), 41 (36).

N-*[4-(5-isopropyl-2-methyl-3,6-dioxocyclohexa-1,4-dienyl)butyl]biotinamide (**4**):* A mixture of biotin (52 mg, 0.21 mmol), HOBt (10 mg, 0.06 mmol), dry DMF (20 mL) and DCC (50 mg, 0.23 mmol) was stirred at room temperature for 3 h, then treated with amine **6** (50 mg, 0.21 mmol) and catalytic quantities of DMAP. Stirring was continued overnight, then volatile solvents were removed under vacuum, and the residue thus obtained was purified by CC (CH_2Cl_2/MeOH, 12:1). Yield: 40 mg (41%); yellow oil; $R_f = 0.31$ (CH_2Cl_2/MeOH, 12:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.85$ (m, 2H), 1.09 (d, $J = 6.9$ Hz, 6H), 1.1–1.7 (m, 8H), 2.00 (s, 3H), 2.02 (m, 2H), 2.2–2.4 (m, 4H), 2.91 (m, 1H), 3.13 (dsept, $J = 6.9$, 1.2 Hz, 1H), 4.07 (t, $J = 6.4$ Hz, 2H), 4.32 (m, 2H), 6.46 (d, $J = 1.2$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 11.8$, 21.4, 24.9, 25.6, 26.7, 27.9, 28.2, 28.8, 30.9, 32.5, 33.9, 40.5, 55.3, 60.1, 61.7, 130.0, 137.6, 144.7, 154.6, 163.4, 172.4, 187.0, 188.3; IR (ATR): $\nu_{max} = 3326$, 2927, 2851, 2111, 1719, 1629, 1571, 1511, 1448, 1344, 1310, 1185, 1045, 891, 759 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 464 (43) $[M]^+$, 227 (100), 218 (47), 166 (17), 97 (38).

Methods

Cell lines and culture conditions: Human HL-60 leukaemia cells were obtained from the German Collection of Biological Material (DSMZ), Braunschweig; human melanoma cells 518A2 from the Department of Oncology and Hematology at Martin Luther University, Halle; KB-V1^{VBL} and MCF-7^{TOPO} cells from the Institute of Pharmacy at the University Regensburg; and the HT-29 colon carcinoma cells and human foreskin fibroblasts (HF) from the University Hospital, Erlangen. HL-60 and HT-29 cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), penicillin G (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), amphotericin B (0.25 μg mL^{-1}) and gentamycin (250 μg mL^{-1}; Gibco, Egenstein, Germany). 518A2, HF and KB-V1^{VBL} cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% FCS, penicillin G (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}), amphotericin B (0.25 μg mL^{-1}) and gentamycin (250 μg mL^{-1}). MCF-7^{TOPO} cells were grown in Eagle's minimum essential medium (EMEM, Sigma) supplemented with $NaHCO_3$ (2.2 g L^{-1}), sodium pyruvate (110 mg L^{-1}) and 5% FCS. PtK_2 cells were obtained from the American Type Culture Collection (ATCC) and cultivated in DMEM medium supplemented with 10% FCS. All cells were maintained in a moisture-saturated 5% CO_2 atmosphere at 37 °C in 75 mL culture flasks (Nunc, Wiesbaden, Germany). They were serially passaged following protease treatment with 0.05% trypsin/0.02% EDTA (PAA laboratories, Cölbe, Germany) or by using cell scrapers. Mycoplasma contamination was routinely monitored; only mycoplasma-free cultures were used.

Determination of tumour cell growth (MTT assay): HL-60 cells (5×10^5 mL^{-1}), 518A2, HT-29, KB-V1^{VBL} and MCF-7^{TOPO} cells (5×10^4 mL^{-1} each) and HF fibroblasts were seeded in 96-well tissue culture plates and cultured for 24 h. Incubation (5% CO_2, 95% humidity, 37 °C) of the cells following treatment with the test compounds was continued for 72 h. Blank and solvent controls were treated identically. MTT in phosphate buffered saline was added to a final concentration of 0.05% (HL-60, 518A2, HF) or 0.1% (HT-29, KB-V1^{VBL}, MCF-7^{TOPO}). After 2 h the precipitate of formazan crystals was re-dissolved in a 10% solution of sodium dodecylsulfate in DMSO (containing 0.6% acetic acid in the case of the HL-60 cells). For adherent cells (518A2, HT-29, KB-V1^{VBL} and MCF-7^{TOPO}), microplates were swiftly turned to discard the medium before adding the solvent mixture. The microplates were gently shaken in the dark for 30 min, and absorbance at 570 and 630 nm (background) was measured with an ELISA plate reader. All experiments were carried out in quadruplicate and the percentage of viable cells was calculated as the mean ± SD relative to the controls (100%).

Visualisation via click reaction: PtK_2 cells were grown in DMEM medium (750 μL) in 4-well plates (Nunc) on glass coverslips and incubated overnight with the alkyne **3** (5 μM). The medium was removed and the cells were washed twice with phosphate buffered saline. The cells were then fixed with 3.7% formalin (15 min), permeabilised with 0.1% triton X-100 (5 min) and incubated (30 min, RT) with 5 μM 3-azido-7-hydroxycoumarine **7** in Click-iT Cell Reaction Buffer (Molecular Probes, OR, USA) according to the manufacturer's procedure. For staining of the Golgi apparatus the cells were incubated with a primary mouse anti-Golgi mAb (1:100, Sigma) for 1 h, then with a secondary Alexa Fluor 594 goat anti-mouse IgG Ab (1:500, Molecular Probes) for 45 min, and finally mounted in ProLong Antifade Gold (Molecular Probes) that contained DAPI (1 μg mL^{-1}) for staining the nuclei.

Visualisation by the biotin–streptavidin system: PtK_2 cells were grown in DMEM medium (750 μL) in 4-well plates (Nunc) on glass coverslips, and incubated with the biotin-labelled thymoquinone **4** (6.5 μM) for 24 h. Cells were fixed with 3.7% formalin for 15 min, permeabilised with 0.1% Triton X-100 for 5 min. They were then incubated with the streptavidin–Alexa Fluor 488 conjugate (10 μg mL^{-1}, Invitrogen), and mounted in ProLong Antifade Gold (Molecular Probes) that included DAPI (1 μg mL^{-1}) to stain the nuclei.

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Keywords: alkynes · biotin · click chemistry · fluorescent probes · thymoquinone

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