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# METHODS AND APPLICATIONS OF ANTICANCER BIOASSAYS

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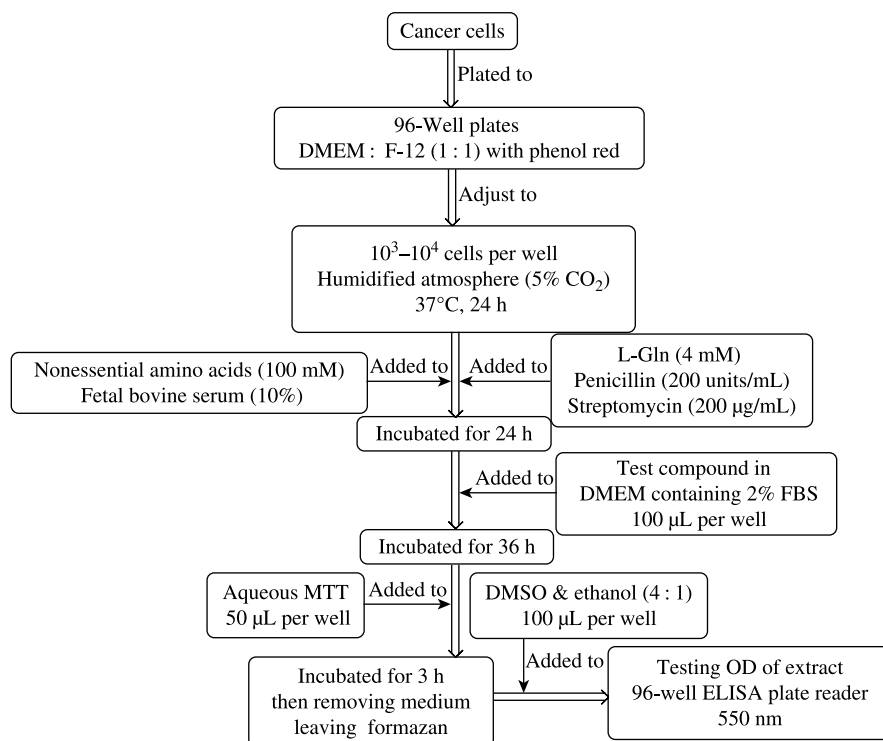
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Cancer is a major human disease and causes great suffering and financial loss worldwide. Although a great deal of progress has been made in cancer chemotherapy, the incidence and mortality rate of most forms of cancer remain very high. Continuous efforts have been made in establishing efficient diagnostic methods and developing safe drugs or drug candidates to prevent and treat all human cancers. Extensive interest has been attracted to exploring pathologic mechanism-based assays as *in vitro* and *in vivo* evaluations for anticancer agents. *In vitro* assays usually consist of cell culture systems with neoplastic cell lines from human or other animal tumors as targets. The capacity of test compounds inhibiting the growth or reducing the survival of cancer cells in culture media and the potency of test compounds inducing structural change of cancer cells in culture media are generally correlated with the *in vivo* potency of a cancer therapeutic agent. To directly observe anticancer capacity, test compounds are administered to animals. Thus, great achievements in chemotherapy in recent years have been due to development of anticancer assays. In this chapter, 22 models used in anticancer research are described: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for six carcinoma cells,<sup>[1–12]</sup> flow cytometric assay for cell apoptosis,<sup>[11]</sup> DNA fragmentation assay,<sup>[11]</sup> Bcl-XL/BH3 interaction assay,<sup>[13]</sup> dissociation enhanced lanthanide fluoro-immunoassay (DELFLIA),<sup>[14]</sup> Ishikawa cell and rat assay for detecting antiestrogens,<sup>[15–23]</sup> adenosine triphosphate (ATP) assay for eight cells,<sup>[24]</sup> alkaline phosphatase (AP) activity assay,<sup>[25,26]</sup> tumor

endothelial cell tube formation assay,<sup>[27]</sup> antiangiogenic assay,<sup>[28]</sup> *in vivo* hollow fiber assay,<sup>[29]</sup> VX2 rabbit lung assay,<sup>[30]</sup> insulin-like growth factor-I (IGF-I)-induced kinase receptor activation assay,<sup>[31]</sup> insulin-like gD- trkA-induced kinase receptor activation assay,<sup>[32]</sup> UV spectra-based calf thymus DNA intercalation assay,<sup>[33,34]</sup> fluorescence spectra-based calf thymus DNA intercalation assay,<sup>[33,34]</sup> P-glycoprotein pump (P-gp) in MCF-7R cells assay,<sup>[35]</sup> P-gp-related efflux carrier assay,<sup>[36]</sup> [<sup>3</sup>H]substrate transport inhibition assay,<sup>[36]</sup> lactate dehydrogenase release assay,<sup>[36]</sup> functional assay of mitochondrial P-glycoprotein (P-gp),<sup>[37]</sup> and resistance index value assay.<sup>[38]</sup>

### 1.1 MTT ASSAY FOR SIX CARCINOMA CELLS<sup>[1-12]</sup>

Human gastric cancer cells (SGC-7901, SC-M1, and BGC-823), breast adenocarcinoma (MCF-7 and MDA-MB-231), stomach adenocarcinoma (AGS), colon carcinoma cells (HCT-116), and lung (A549 and NCI-H460) and central nervous system carcinoma cells (SF-268) were plated at 37°C for 24 h on 96-well plates at a density of 10<sup>3</sup> to 10<sup>4</sup> cells per well with Dulbecco's Modified Eagle's Medium (DMEM): F-12 (1 : 1) with phenol red (Sigma, St. Louis, MO, USA) in a humidified atmosphere (5% CO<sub>2</sub>). To this medium, L-Gln (4 mM), penicillin (200 units/mL), streptomycin



**Figure 1.1** Flowchart of MTT assay for cancer cells.

(200  $\mu\text{g}/\text{mL}$ ), DMEM nonessential amino acids (100 mM), and 10% fetal bovine serum (FBS) were added and incubated for 24 h. Then the cells were treated with the test compound in a medium containing 2% FBS (100  $\mu\text{L}$  per well) and incubated for an additional 36 h. To each well, 50  $\mu\text{L}$  aqueous 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; 5 mg/mL) solution was added, and the mixture was incubated at 37°C for another 3 h. After removing the MTT solution, formazan was extracted from the cells in each well with 100  $\mu\text{L}$  of a 4/1 dimethyl sulfoxide (DMSO)/ethanol mixture. Optical density (OD) was measured around 550 nm with a 96-well enzyme-linked immunosorbent assay (ELISA) plate reader. All assays were repeated three times. Viability of the cells treated with test compound was determined as: % Cell viability = [(average OD of test compound)/(average OD of control)]  $\times$  100. A general procedure is summarized in Fig. 1.1.

## 1.2 FLOW CYTOMETRIC ASSAY FOR CELL APOPTOSIS<sup>[11]</sup>

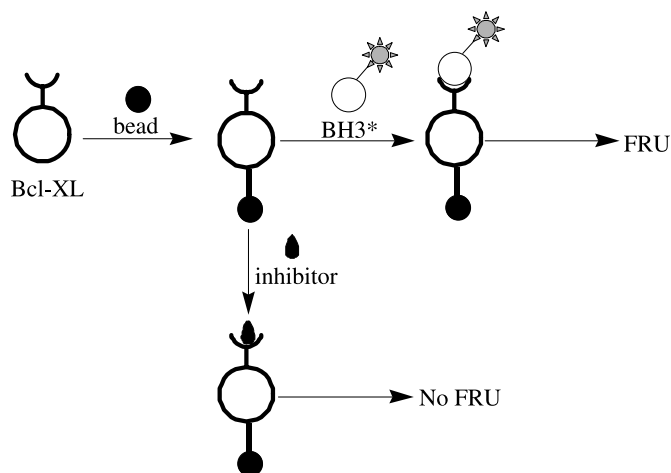
Trypsinized cancer cells ( $1 \times 10^6$  cells/mL) were successively washed twice in ice-cold Hanks solution, fixed in 75% ice-cold ethanol for at least 1 h, washed twice with phosphate-buffered saline (PBS), stained in 100  $\mu\text{g}/\text{mL}$  propidium diiodide (PI, Sigma, St. Louis, MO, USA), 10  $\mu\text{g}/\text{mL}$  Rnase in PBS, and incubated at 37°C for 30 min. After excitation of the fluorescent dye, fluorescence emitted from the propidium–DNA complex was quantitatively analyzed on the FACSCalibur system (BD Biosciences, San Jose, CA, USA).

## 1.3 DNA FRAGMENTATION ASSAY<sup>[11]</sup>

To identify DNA ladder using DNA fragmentation assay, the cancer cells ( $3 \times 10^4$  cells/mL) were plated into a 60-mm culture dish. The cells were treated with 50  $\mu\text{M}$  and 100  $\mu\text{M}$  quercetin, from which DNA samples can be extracted at 12, 24, and 48 h. Cells of different treatment groups were collected, washed once with ice-cold PBS, centrifuged, and the supernatants were removed carefully. The cells were dispersed in 50  $\mu\text{L}$  lysis buffer [10 mM Tris pH 7.4, 100 mM NaCl, 25 mM ethylenediamine tetraacetic acid (EDTA), and 1% sarkosyl] and incubated at 50°C for 3 h. Mixing 4  $\mu\text{L}$  DNA sample dye with bromophenol blue buffer, the solution was electrophoresed on 1.2% agarose gel at 50 V for 3 h until bromophenol blue dye reaching the foreland of the gel, and the DNA was visualized under UV light.

## 1.4 Bcl-XL/BH3 INTERACTION ASSAY<sup>[13]</sup>

In the presence of 50  $\mu\text{L}$  test compound, His-tagged Basal cell lymphoma-extra large protein (His-Bcl-XL, 3  $\mu\text{M}$ ) was preincubated with 50  $\mu\text{L}$   $\text{Ni}^{2+}$ -sepharose magnetic beads. The total assay volume was 100  $\mu\text{L}$  at 5% DMSO in buffer B (1 mM  $\text{MgCl}_2$ , 100 mM KCl, 10 mM Tris-HCl, pH 7.9) containing protease inhibitors and

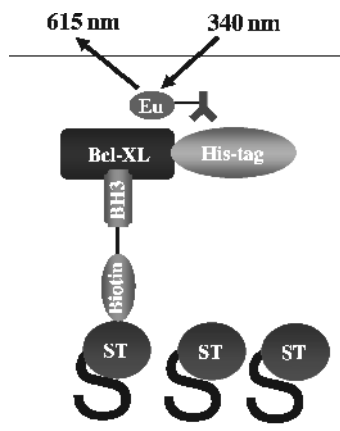


**Figure 1.2** Principle of Bcl-XL/BH3 interaction assay.

was mixed for 1 h at room temperature. Subsequently, 50  $\mu\text{L}$  fluoresceinated GQVGRQLAIGADINR (BH3\*; 6  $\mu\text{M}$ ) was added, followed by further incubation for 1 h in the dark. Reagents were removed, and the beads were washed with 150  $\mu\text{L}$  5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 2 mM EDTA, and Proteases Inhibitors Mix (Sigma, St. Louis, MO, USA). Then the protein was eluted from beads with 0.5 M imidazole. Fluorescence was directly determined by measuring the relative fluorescence units (RFU) at 485-nm excitation and 530-nm emission (detector: Fusion; Packard Bio-Science, Perkin-Elmer, Inc., Boston, MA, USA). The assay was performed in 96 wells, blocked with bovine serum albumin (BSA) 3%. The protein:protein displacement was evaluated with the equation  $\text{Displacement} = [1 - (\text{RFUs} - \text{RFUb}) / (\text{RFUc} - \text{RFUb})] \times 100$ , where RFUs, RFUb, and RFUc refer to the RFU in the presence of the tested samples, of the average of four wells without BH3\*, and of the average of eight wells without added sample. Intra- and interexperiment variability was evaluated as standard deviation (SD) and variability coefficient (CV). The principle of this assay is represented in Fig. 1.2.

### 1.5 DISSOCIATION-ENHANCED LANTHANIDE FLUORO-IMMUNOASSAY (DELFI<sup>a</sup>)<sup>[14]</sup>

To each well of 96-well plates coated with streptavidin (Perkin-Elmer Inc., Boston, MA, USA), 100  $\mu\text{L}$  of a variable concentration of biotin-labeled BH3 peptide, biotin-(CH<sub>2</sub>)<sub>6</sub>-GGGQVGRQLAIGDDINR, was added (concentration, 2 to 40 nM). After 1 h (15 min to 1 h) incubation and three washing steps, the unbound biotin-BH3 peptide was removed. To each well, 89  $\mu\text{L}$  anti-His Europium (Eu)-antibody conjugate (7.2 nM), 1  $\mu\text{L}$  DMSO containing test compound, and 10  $\mu\text{L}$  His6-Bcl-XL (or any other antiapoptotic His6-Bcl-2 family protein; concentration, 1–150 nM)



**Figure 1.3** Representation for detecting BH3–Bcl-2 interactions with DELFIA.

were added. After 1 h incubation, the well was washed five times to remove the unbound protein (and so the Eu-antibody if displaced by test compound), then 200  $\mu$ L enhancement solution was added. After 30 min incubation, the fluorescence was measured (excitation wavelength, 340 nm; emission wavelength, 615 nm). To give the relaxation properties of Eu, the measurements were made in time-resolved mode; controls included unlabeled peptide and blanks receiving no compounds; the DELFIA could also be obtained by using glutathione s-transferase (GST)-fusion Bcl-2 proteins and anti-GST Eu-antibody (Perkin-Elmer Inc., Boston, MA, USA); Z0 factor measurements were obtained by repeating the experiments (positive and negative controls) multiple times; and the assay buffer from Perkin-Elmer was used in each step. The representation of detecting BH3–Bcl-2 interactions with DELFIA is given in Fig. 1.3.

## 1.6 ISHIKAWA CELL AND RAT ASSAY FOR DETECTING ANTIESTROGENS<sup>[15–23]</sup>

Ishikawa cells (50,000 cells/well, in triplicate, Tsukuba University, Japan) were grown in 96-well plates in estrogen-free medium (phenol red free, with charcoal-stripped calf serum) in the presence of (stimulatory assay) test compounds as well as antiestrogens at concentrations that were varied over several log orders. For the antiestrogen assay, a range of concentrations of samples was added concurrently with 1 nM antiestrogens. After growth for 3 days, to determine alkaline phosphatase (AP) activity, the cells were frozen, defrosted, and incubated with the chromogenic substrate *p*-nitrophenylphosphate at room temperature. The hydrolysis product, *p*-nitrophenol, was measured kinetically at 405 nm.

The specificity of the antiestrogenic activity was determined using the estrogen receptor (ER) subtypes, ER $\alpha$  and ER $\beta$ , in ER element (ERE)-transfected human

choriocarcinoma JAR cells transfected with plasmids containing a consensus ERE fused to a firefly luciferase reporter gene and separately with the expression vectors for either human ER $\alpha$  or human ER $\beta$ . JAR cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.5% nonessential amino acids, and 1% PEST (100 U penicillin/mL and 100  $\mu$ g streptomycin/mL). After seeding in 6-well plates for 24 h, cells were transfected using the Mirus Trans IT reagent in a serum- and antibiotic-free mixture of phenol red-free OptiMEM with 0.1–0.4  $\mu$ g pC  $\times$  N2 human ER $\alpha$  or pC  $\times$  N2 h-ER $\beta$  and 0.75  $\mu$ g 3 $\times$  ERE-TATA-Luc reporter constructed by introducing an HpaI/BglII fragment containing 3 $\times$  ERE-TATA into SmaI/BglII of the pGL3-Luc basic vector.<sup>[13–15]</sup> Medium was replaced with a phenol red-free RPMI containing 10% dextran-coated charcoal-treated calf serum, 0.5% nonessential amino acids, and no PEST. Antiestrogens were added 24 h later. Cells were incubated at 37°C in 5% CO<sub>2</sub> for 12 h and then harvested in 10 mM Tris-HCl/10 mM EDTA/150 mM NaCl and centrifuged at 4000  $\times$  g for 4 min. The supernatant was removed, and cell pellets were lysed in Lysis Buffer 2. Using the GenGlow system (Promega, Madison, WI), luciferase activity was measured.

To detect *in vivo* estrogenic/antiestrogenic activity, ER $\alpha$  was determined by Western blotting. After the AP assay, the Ishikawa cells were washed three times with PBS and lysed using 1% Nonidet P-40 and 0.1% sodium dodecyl sulfate in the presence of protease inhibitors. Proteins (25  $\mu$ g/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on ice using 10% polyacrylamide gel and transferred to nitrocellulose membranes stained with Ponceau red before the antibody incubation to ensure proper transfer. After blocking the membranes with 5% powdered milk in water, immunoblotting was performed. The blots were incubated overnight at 4°C with ER $\alpha$  monoclonal antibody clone 6F11. Using peroxidase-labeled horse anti-mouse secondary antibody and Chemiluminescence Reagent Plus (PerkinElmer, Wellesley, MA), ER $\alpha$  was detected. Using a digital imaging analysis system, the intensity of the signal was analyzed.  $\beta$ -Actin was used as an internal control to normalize the amount of protein loaded in the gels.

The uterotopic assay was performed with stimulation in immature rats. Female Sprague–Dawley rats (22 days old) were injected subcutaneously daily for 3 days with antiestrogens. Control animals received vehicle (0.1 mL sesame oil). Then, animals were killed, and uteri removed, dissected, blotted, and weighed. To determine whether antiestrogens had tissue-selective effects in cholesterol, uteri, and bone, ovariectomized female Sprague–Dawley rats (250 g) were injected with antiestrogens subcutaneously for 35 days. Then the animals were killed by exsanguination under ether anesthesia. The total cholesterol concentration of the serum was determined by a commercial chromogenic assay. The uteri were dissected, weighed, fixed in formalin, and embedded in paraffin to prepare for 5- $\mu$ m sections. Using the Openlab image analysis system (Improvision, Lexington, MA), endometrial luminal epithelium and glandular cell height were measured. The tibia were dissected free of extraneous tissue and then histomorphometrically analyzed. The bones were fixed in 70% ethanol, dehydrated in graded ethanol, and cleared in toluene. The specimens were then infiltrated with increasing concentrations of methylmethacrylate (MMA) and

embedded in MMA. After polymerization, MMA blocks were cut to size, sanded, and polished to the appropriate level to prepare for 4- to 5- $\mu\text{m}$  sections. The sections were mounted on gelatin-coated slides and stained with toluidine blue and measured using the computerized Osteomeasure analysis system (Osteometrics, Atlanta, GA).

### 1.7 ATP ASSAY FOR EIGHT CELLS<sup>[24]</sup>

The primary cryopreserved human hepatocytes (lot HH227 in Li's Universal Medium), human renal proximal tubule cells (RPTCs in REGM), human small airway epithelial cells (SAECs in SAGM), human aortic endothelial cells (HAECs in EGM-2), normal human astrocytes (AGM), breast cancer cells (MCF-7 and MDA-MB-468 in MEME), and human colon tumor cell line (HCT-116 in MEME) in 25-cm<sup>2</sup> flasks were subcultured into 75-cm<sup>2</sup> flasks after approximately 4 days (75% confluent). Before trypsinizing and plating, the cells were cultured for another 7 days. The cells were plated into 96-well plates ( $1 \times 10^4$  to  $1 \times 10^5$  cells per well) with 100  $\mu\text{L}$  medium and incubated at 37°C and a highly humidified atmosphere of 95% air and 5% carbon dioxide for 24 h before the treatment with the tested compound. On the treatment day, the media were removed from all wells and replaced with 100  $\mu\text{L}$  medium containing various concentrations of the tested compound. The treatment was performed in triplicate for an incubation period of 24–96 h. After the treatment period, the cells in individual wells were processed to determine viability. After removal of the treatment media, each of the individual wells was washed twice with isotonic PBS (pH 7.4), and then 50  $\mu\text{L}$  mammalian cell lysis solution was added. The cell lysate was combined with the substrate solutions of the ATPlite system (Perkin-Elmer Inc., Boston, MA) and analyzed for chemiluminescence on a Wallac Victor 1420 Multilabel Counter (Wallac, Turku, Finland). Results were expressed as relative viability and were obtained according to the equation:  $\text{Relative viability} = (\text{ATP}_{\text{treatment}} / \text{ATP}_{\text{control}}) \times 100\%$ .

### 1.8 AP ACTIVITY ASSAY<sup>[25,26]</sup>

AP is a glycoprotein-structured metallophosphatase and presents in many tissues of all living beings from bacteria to mammals. AP may catalyze the hydrolysis of various monophosphate esters at alkaline pH. Based on the conversion of *p*-nitrophenylphosphate (*p*-NPP) to *p*-nitrophenol, the colorimetric determination of the resulting colored product was performed. In the assay, the cells (mouse myeloma cell lines P3, FO, SP2; erythroleukemia cell K562 and B cell hybridoma 1G2,  $1.5 \times 10^5$  to  $2.5 \times 10^5$  cells/well) were cultured in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C in 96-well plates or 25-cm<sup>2</sup> Falcon tissue culture flasks containing DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 2 mmol/mL glutamine for 4 days. Then cells were washed twice with PBS. Cell viability was assessed by exclusion of trypan blue dye (0.4%). The plates or tubes were centrifuged for 5 min at 3000 rpm. The supernatant was discarded,

and the pellet was resuspended in 100  $\mu\text{L}$  substrate buffer (10 mM diethanolamine, 0.5 mM  $\text{MgCl}_2$  pH 10.5) containing 1 mg/mL *p*-NPP and incubated at room temperature for 20 min. The reaction was stopped with 50 mL 2 N NaOH. OD at 405 nm was determined using a Titertek Uniscan II ELISA Reader. According to the standard curve, AP activity was identified. For this standard curve, various concentrations of commercially provided AP from 0 to 400 ng/mL in a volume of 100  $\mu\text{L}$  were added to flat-bottom 96-well plates, with 100  $\mu\text{L}$  *p*-NPP (2 mg/mL) at room temperature incubated for 20 min. AP activity (OD) was determined spectrophotometrically at 405 nm. Each concentration of AP was measured in triplicate.

### 1.9 TUMOR ENDOTHELIAL CELL TUBE FORMATION ASSAY<sup>[27]</sup>

Murine tumor endothelial cells (3B11) were cultured in a humidified incubator with 5%  $\text{CO}_2$ , DMEM supplemented with 10% FBS, and  $1 \times$  penicillin/streptomycin. Two days before the confluence of 3B11 cells reaching 80% to 95%, the cells ( $6 \times 10^5$ ) were cultured in a T75 flask. One day before performing this assay, a Matrigel matrix was incubated on ice overnight. On the day this assay was performed, 0.15 mL Matrigel matrix (basement membrane, BD Biosciences, Bedford, MA) was transferred onto a 48-well plate (or 0.25 mL to 24-well plate or 0.3 mL to 4-well chamber slides). The plates or slides were incubated at  $37^\circ\text{C}$  and in 5%  $\text{CO}_2$  for 30 min, during which 3B11 cells in T75 flasks were washed with PBS, digested with 1X trypsin solution for about 3 min, and suspended in 8 mL DMEM complete medium. The cells were counted and diluted to  $4 \times 10^5/\text{mL}$  in DMEM complete medium. The diluted testing compound (120  $\mu\text{L}$ ) was transferred to a 1.5-mL tube and mixed with equal volume of endothelial cells. To each well containing the Matrigel matrix, this mixture (200  $\mu\text{L}$ ) was added. The plates or slides were incubated at  $37^\circ\text{C}$  and in 5%  $\text{CO}_2$  for 4–16 h depending on the tube size. Under a phase-contrast inverted microscope at  $5 \times$  objective magnification (or  $2.5 \times$  and  $10 \times$  objectives) using computer-controlled ProMax software (Bio-TEC Instruments Inc., Winooski, VT), endothelial cell tube formation could be continuously monitored and imaged without fixation and staining.

For quantitative analysis, after carefully removing the cell culture medium in the well, the tubes were fixed for 30 min in 1 mL 3.6% formaldehyde at room temperature, washed with PBS three times, permeated via incubation in 1 mL 0.1% Triton X-100–PBS for 5 min, washed with PBS another two times, and blocked in 1 mL 1% BSA–PBS for 30 min at room temperature, stained with rhodamine phalloidin (200  $\mu\text{L}$ ) in 1% BSA–PBS for 60 min, washed twice with PBS again, and imaged by 2D wide-field fluorescence or 3D confocal fluorescence microscopy.

Two-dimensional wide-field fluorescence image-based quantitative analysis was performed on a Leica DC350F CCD camera attached to an inverted Leica (Wetzlar, Germany) DMIL microscope and captured using ProMax software. Images were then converted to TIFF files of high resolution for analysis. Rhodamine phalloidin was used for both fluorescently labeling of the actin cytoskeleton, which is abundant in the endothelial cell cytoplasm, and monitoring endothelial cell cytoplasm tube

formation under various conditions. The spatial location of the branched and clustered endothelial cells in the Matrigel were defined by use of the fluorescent rhodamine signal. The analysis was two-dimensional due to the single wide-field fluorescent images. To account for the 2D feature of the images, area calculations were important. The threshold tool Image Pro Plus software (Media Cybernetics Inc., Bethesda, MD) was used to trace the fluorescent signal in each 2D image. In these 8-bit images, a signal with fluorescent intensity between 50 and 256 was considered a real signal. This threshold was pseudo-colored in green by the software and displayed as a layer over the 8-bit gray-scale original image that showed the spatial area of interest. Once traced, the area colored in green was outlined with the software, and then these outlined regions were used to calculate 2D parameters including area (as a uniform height), perimeter, and average length of each connected vessel.

Three-dimensional images were performed on a Rainbow Radiance 2100 Laser Scanning Confocal system attached to a Nikon TE2000-U inverted microscope (BioRad-Carl Zeiss Inc., Thornwood, NY), optical image slices (8-bit) were obtained with a  $10\times$  objective to capture as much area as possible ( $3\text{-}\mu\text{m}$ -interval step slices). Using Laser Sharp 2000 software (BitPlane Inc., Saint Paul, MN), the images were imported into the Imaris software and the macro "filament tracker" used to generate a 3D solid mask of the original fluorescence signal. As mentioned above, the rhodamine phalloidin fluorescent signal of the 3D image was also used to define the spatial location of the clusters and branches of endothelial cells in the Matrigel. In these 8-bit images, a threshold between 70 and 256 was considered a true signal. The filament tracker tool in Imaris automatically traced the threshold signal and created a 3D cylindrical outline of the endothelial cell network. With the outline of the filament track, the software can extract parameters including average vessel length, area, volume, number of free unassociated termination points, and number of branch points. In addition, the "spot tracking" tool in Imaris automatically located the 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained nuclear fluorescence signal and marked the center of each nucleus with a solid sphere. The Imaris software calculated the parameters including the number of nuclei per branch, per tube, or per field and their fluorescent intensities and locations and displayed them in Excel (Microsoft, Redmond, WA) for statistics and graphing.

## 1.10 ANTIANGIOGENIC ASSAY<sup>[28]</sup>

Human RPE cells (ARPE-19) were cultured ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ) to confluence in  $56.7\text{-cm}^2$  culture dishes with DMEM/F-12 growth medium (15 mL; ATCC) supplemented with 10% heat-inactivated FBS and penicillin (100 units/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ). In hypoxia experiments, the medium was replaced with 15 mL DMEM/F-12 growth medium supplemented with penicillin (100 units/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) only, and the cells were incubated in a hypoxic incubator ( $37^{\circ}\text{C}$ , 3%  $\text{O}_2$ ) for 24 h. The culture medium was removed and centrifuged to remove any cellular debris, and the supernatant was frozen at  $-80^{\circ}\text{C}$  until further use. Conditioned medium from two separate experiments was pooled together

before freezing at  $-80^{\circ}\text{C}$ . All experiments were carried out using cells within the first five passages. Observation of the cells by light microscopy after more than 36 h of hypoxia demonstrated that they were viable and healthy.

In short interfering RNA (siRNA) experiments, ONTARGETplus SMARTpool hypoxia-inducible factor (HIF)-1 $\alpha$  siRNA or vascular endothelial growth factor (VEGF) siRNA was used with fluorescent siGLO RISC-free siRNA as a negative control. Cells with 90% confluence were washed with OptiMEM I reduced-serum medium and then incubated ( $37^{\circ}\text{C}$ , overnight) in reduced-serum medium (15 mL) containing 600 pmol siRNA and 30  $\mu\text{L}$  Lipofectamine 2000. The transfection solution was aspirated. The cells were washed with culture medium, given 36 h to recover from transfection, and then hypoxic experiments were performed. The amount of siRNA and transfection agent used was optimized; under the conditions used, 100% transfection was observed under fluorescent microscopy, and little to no cell toxicity was observed under light microscopy. With Western blot techniques, it was confirmed that HIF-1 $\alpha$  and VEGF levels were barely detectable up to 72 h after siRNA transfection.

Conditioned medium was assayed for angiogenic factors [VEGF, angiogenin, placental growth factor (PIGF), erythropoietin (EPO), leptin, tumor growth factor (TGF)- $\beta$ 1, basic fibroblast growth factor (bFGF), interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1] using Quantikine human immunoassay kits and for antiangiogenic factor pigment epithelium-derived factor (PEDF) using Chemikine pigment epithelium-derived factor sandwich ELISA kit according to the manufacturers' instructions and in triplicate.

### 1.11 *IN VIVO* HOLLOW FIBER ASSAY<sup>[29]</sup>

Cell confluent monolayers were collected by centrifugation and then resuspended in conditioned medium at a density of  $10^6$  or  $5 \times 10^5$  cells/mL. Fibers were filled with the cells and incubated in 6-well plates at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere for 24 h. Female athymic NCr *nu/nu* mouse (5–6 weeks old) hosted up to six fibers, which were implanted into two physiologic compartments. For intraperitoneal implants, the mouse was given a small incision through the skin and musculature of the dorsal abdominal wall. After inserting the fiber samples into the peritoneal cavity in a craniocaudal direction, the incision was closed with skin staples. For subcutaneous implants, the mouse was given a small skin incision at the nape of the neck for inserting an 11-gauge tumor implant trocar containing the hollow fiber samples, which was inserted caudally through the subcutaneous tissues. The fibers were deposited during withdrawal of the trocar. The incision was closed with skin staples.

Cell growth was assessed with fibers containing  $5 \times 10^5$ ,  $10^6$ , or  $5 \times 10^6$  cells/mL. For treatment protocols, to increase solubility the test compounds were coprecipitated with polyvinylpyrrolidone (PVP, MW 360,000) and then dissolved in PBS. Mice were randomized into PBS vehicle control groups (6 mice/group) and test compound treatment groups (3 mice/group for each dose tested). From days 3 to 6 after implantation, test compounds were administered by intraperitoneal injection once daily, and body weights of mice were recorded daily. On day 7, mice were anesthetized

by intraperitoneal injection of a mixture of ketamine (13.3 mg/100 g body weight) and xylazine (1.3 mg/kg body weight) and the fibers were retrieved. The fibers were placed into 6-well plates, each well containing 2 mL fresh and prewarmed culture medium (with 20% calf serum or FBS) and equilibrated for 30 min at 37°C. Using MTT assay, the survival of the cell mass in the intact hollow fibers was defined. Briefly, to each dish 1 mL of prewarmed culture medium (with 20% calf serum or FBS) containing 1 mg MTT was added, and the dishes were incubated at 37°C for 4 h. The culture media were removed by aspiration. To each well, 2 mL normal saline containing 2.5% protamine sulfate solution was added. The plates were stored at 4°C for 24 h, and protamine sulfate solution was removed by aspiration and replaced by 2 mL fresh protamine sulfate solution. The plates were stored at 4°C for at least 2–4 h. The fibers were transferred to 24-well plates, cut in half, and allowed to dry overnight. The residual formazan was extracted with DMSO (250  $\mu$ L/well) for 4 h at room temperature on a rotation platform. Aliquots (150  $\mu$ L) of DMSO containing formazan were transferred to individual wells of 96-well plates and assessed for optical density at a wavelength of 540 nm. The effect of the treatment regimen was determined by the net growth percentage of the cells relative to changes in body weight.

### 1.12 VX2 RABBIT LUNG ASSAY<sup>[30]</sup>

After the addition of VX2 cells isolated from freshly excised lung tumors of a donor rabbit (ca. 750,000 live VX2 cells/ $\mu$ L) to fresh rabbit plasma, thrombin was added. Before it clotted, using a syringe this plasma mixture was quickly drawn into 5-cm lengths of PE-90 tubing. A single 5-cm newly coagulated clot (volume ca. 30  $\mu$ L, containing ca. 25,000 VX2 cells) was recovered in 0.5 mL saline and injected intravenously (ear) into a recipient rabbit. At 28 d after seeding, the rabbit was anesthetized by intravenously administered 35 mg/kg sodium pentobarbital and exsanguinated through a cannula (PE-190 tubing) placed in a carotid artery. From lung tumors, VX2 cells were harvested and used to seed the lungs of another rabbit. Twenty-four hours later, the rabbits were given test compounds. At 28 d after seeding, each rabbit with VX2 tumor was anesthetized, exsanguinated through a cannula (PE-190 tubing) placed in a carotid artery, and positioned on its back. At the sternum, the chest cavity was opened, and by syringe any obvious fluid within the interpleural space was drawn off with minimal contamination by blood. The fluid was weighed and centrifuged at  $1200 \times g$  for 5 min. From a clear and pale yellow supernatant the pellet [equivalent to  $(9.5 \pm 3.2) \times 10^6$  live VX2 cells/mL of effusion] was separated. The supernatant (pleural effusion) was snap-frozen (liquid N<sub>2</sub>) and stored at  $-40^\circ\text{C}$ . The lungs and only their tumors larger than 0.02 g were excised and individually weighed.

### 1.13 INSULIN-LIKE GROWTH FACTOR-I-INDUCED KINASE RECEPTOR ACTIVATION ASSAY<sup>[31]</sup>

Human breast adenocarcinoma (MCF-7) cells were cultured in 150-cm<sup>2</sup> tissue culture flasks,  $1.5 \times 10^6$ /flask for 4–7 days. For the assay, cells ( $2 \times 10^5$ /well) were cultured

in flat-bottom microtiter plates in 100  $\mu\text{L}$  F-12/DMEM 50:50 at 37°C in 5%  $\text{CO}_2$  for 24 h. The medium was supplemented with 10% FBS, 25 mM HEPES, and 2 mM L-Gln. After removal of the supernatants, the plates were lightly blotted on paper towels. To each well, medium containing either test compounds or the recombinant insulin-like growth factor I (IGF-I) standards were added, kept for 15 min, decanted and blotted. To each well, 100  $\mu\text{L}$  lysis buffer containing 0.5% Triton X-100, 2 mM sodium orthovanadate (to prevent dephosphorylation of the receptors), and a cocktail of protease inhibitors was added to generate crude lysates that were transferred to an ELISA plate that had been coated overnight with an insulin-like growth factor-I receptor (IGF-IR)-specific antibody (3B7, 5.0  $\mu\text{g}/\text{mL}$ ) and “blocked” with 0.5% BSA. With incubation the IGF-IR in the crude lysates was effectively banded and thus directly purified in the ELISA well. The plate was extensively washed to remove unbound material, and with biotinylated anti-phosphotyrosine monoclonal antibody (4G10) and horse radish peroxidase (HRP)-conjugated dextran-streptavidin the degree of anti-phosphotyrosine binding was visualized with the development of a tetramethyl benzidine (TMB) substrate. The absorbance at 450 nm was read with a reference wavelength of 650 nm (A450).

#### 1.14 INSULIN-LIKE gD.trkA-INDUCED KINASE RECEPTOR ACTIVATION ASSAY<sup>[32]</sup>

gD.trk-transfected CHO cells were seeded ( $5 \times 10^4$ /well) in 96-well plates containing 100  $\mu\text{L}$  medium and cultured at 37°C in 5%  $\text{CO}_2$  for 16 h. The well supernatants were decanted, and the plates were blotted on a paper towel. To each well, 50  $\mu\text{L}$  test compounds or the recombinant purified rhNGF, rhNT4/5, rhBDNF, or rhNT3 standards diluted in stimulation medium were added. The cells were stimulated at 37°C for 20 min, and the well supernatants were once again blotted on a paper towel. To each well, 100  $\mu\text{L}$  lysis buffer was added to lyse the cells and solubilize the receptors. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES, 0.5% Triton X-100, 0.01% thimerosal, 30 U/mL aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 2 mM sodium orthovanadate. The plate was then agitated gently on a plate shaker for 60 min at room temperature.

#### 1.15 UV SPECTRA-BASED CALF THYMUS DNA INTERCALATION ASSAY<sup>[33,34]</sup>

Spectra were recorded on a UV-3100 spectrophotometer with a 1-cm quartz cuvette. In the absorbance titrations, the concentration of the test compound was kept constant while varying the concentration of calf thymus DNA. To take the influence from the absorbance of DNA into account, an equal amount of DNA was added into the sample cell and the reference cell. All absorbance measurements were made at 25°C and 286 nm. The absorption spectrum was recorded after each addition of DNA until no further decrease in absorbance was observed. Before measurements,

all solutions were allowed to equilibrate for 5 min. By the equation  $[\text{DNA}]/(\epsilon_A - \epsilon_F) = [\text{DNA}]/(\epsilon_B - \epsilon_F) + 1/K_b/(\epsilon_A - \epsilon_F)$ , the binding constant ( $K_b$ ) may be calculated, where  $\epsilon_A$ ,  $\epsilon_B$ , and  $\epsilon_F$  correspond with the apparent extinction coefficient of test compound, the extinction coefficient for free test compound, and the extinction coefficient for test compound in the fully bound form, respectively. Using the plot of  $[\text{DNA}]/(\epsilon_A - \epsilon_F)$  versus  $[\text{DNA}]$ ,  $K_b$  was defined by the ratio of the slope to the y intercept, and its value reflected the intercalation strength.

### 1.16 FLUORESCENCE SPECTRA-BASED CALF THYMUS DNA INTERCALATION ASSAY<sup>[33,34]</sup>

Spectra were recorded on a Perkin-Elmer LS50B luminescence spectrometer with a 1-cm quartz cuvette and the temperature maintained with a CS501 Superthermostat. In the fluorescence titrations, the concentration of the test compound was kept constant while varying the concentration of calf thymus DNA. The experiments were carried out in buffer. The concentration of test compound typically was 13.8 mM, DNA concentration varying from 0 to about 0.5 mM until no further decrease in the fluorescence intensity for the test compound was found. Throughout the course of the titration, the solution system was continuously stirred. Before measurements, all solutions were equilibrated for 5 min. For fluorescence spectra, the samples were excited at 290 nm, and a slit width of 5 nm was used for the excitation and emission beams, and from 200 to 700 nm the emission spectra were recorded. According to the equation  $I_0/I = 1 + K_{vs}[\text{DNA}]$ , the fluorescence quenching data were plotted, where  $I_0$  and  $I$  are the fluorescence intensities in the absence and presence of DNA, respectively, and  $K_{vs}$  is the Stern–Volmer quenching constant, which measures the efficiency of quenching by DNA. The data from fluorescence titrations were also useful for determining the binding constant of test compound with DNA. The concentration of the free test compound was calculated according to the equation  $C_F = C_T(I/I_0 - P)/(1 - P)$ , where  $C_T$  is the initial concentration of the test compound,  $C_F$  is the concentration of the free test compound, and  $P$  is the percentage of the observed quantum yield of the fluorescence of the totally bound test compound.  $P$ -value was obtained from the plot of  $I/I_0$  versus  $1/[\text{DNA}]$  as the limiting fluorescence yield given by y intercept. The amount of bound test compound can be calculated from  $C_T - C_F$ . According to the equation  $r/C_F = K_i(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$ , the plot of  $r/C_F$  versus  $r$  was constructed, where  $r$  equals  $C_T - C_F/[\text{DNA}]$ ,  $K_i$  is the binding constant, and  $n$  is the binding site size in base pairs. With Origin graph software (7.0, OriginLab.) on an Ascend computer, fitting the binding data into the equation  $r/C_F = K_i(1 - nr)\{(1 - nr)/[1 - (n - 1)r]\}^{n-1}$ , the values of  $K_i$  and  $n$  can be extracted.

### 1.17 P-GLYCOPROTEIN PUMP IN MCF-7R CELLS ASSAY<sup>[35]</sup>

To assess the inhibitory effect of test compounds on the P-gp in MCF-7R cells (human breast adenocarcinoma cells, resistant to Adriamycin or doxorubicin) maintained at

37°C in humidified atmospheric air containing 5% CO<sub>2</sub> in nutrient mixture (F10/HAM) containing L-glutamine, with 10% fetal calf serum, 1% nonessential amino acids, 60 µg/mL tylosin, and 1% antibiotic/antimycotic solution, the cells were seeded in 96-well plates at  $3 \times 10^4$  cells in 200 µL medium per well. The plates were incubated at 37°C for 24 h in humidified atmospheric air containing 5% CO<sub>2</sub>. The medium was replaced with fresh medium containing 0.3 µM rhodamine 6G together with test compounds and thoroughly mixed. Each 50 µL of the mixture was diluted with 450 µL fresh medium containing 0.3 µM rhodamine 6G. After incubation (in the presence of rhodamine 6G) of test compound, reserpine (positive control), or rhodamine 6G alone (negative control), the plates were further incubated at 37°C for 3 h. The cells were washed twice with 200 µL ice-cold PBS and trypsinized with 100 µL phenol red-free trypsin solution for 15 min, which was transferred onto empty wells. To the cells was added 100 µL of 4% SDS in PBS, and the plates were shaken for 2 h to dissolve the cells and release rhodamine 6G, which was determined by measuring the dye fluorescence on a microplate fluorescence reader at excitation and emission wavelengths of  $530 \pm 25$  nm and  $590 \pm 35$  nm, respectively.

For fluorescence imaging, MCF-7R cells were seeded on Lab-Tek chamber slides at  $5 \times 10^4$  cells per chamber and incubated at 37°C for 24 h in humidified atmospheric air containing 5% CO<sub>2</sub>. The medium was replaced with fresh medium containing 0.3 µM rhodamine 6G with or without 50 µM reserpine, with 100 µg/mL test compounds in separate chambers of the slide. The cells were incubated again at 37°C for 3 h and imaged using fluorescence microscopy with a 510- to 560-nm band-pass excitation filter and a 590-nm long-pass emission filter set using a light-sensitive charge-coupled device digital camera.

### 1.18 P-GLYCOPROTEIN PUMP-RELATED EFFLUX CARRIERS ASSAY<sup>[36]</sup>

The inhibition by test compounds of P-gp-related efflux carriers was measured with the Caco-2 system via intestinal absorption of test compounds, in which P-gp was overexpressed. Caco-2 cells were harvested with trypsin–EDTA and seeded onto the Caco-2 assay system (MultiScreen) at a density of  $1 \times 10^4$  cells/well. In the first 6 days, the culture medium was replaced at intervals of 48 h, thereafter the culture medium was replaced at intervals of 24 h. After 21 days in culture, the Caco-2 monolayer was used for transport assay. The transepithelial electrical resistance (TER) of the monolayers was measured daily before and after the assay on an epithelial volt-ohmmeter. After a 21-day culture, the TER values were generally greater than 1000 Ω. Apical to basolateral ( $P_{app}$  A-B) and basolateral to apical ( $P_{app}$  B-A) permeability of various concentrations of test compounds (1–100 µM) were measured at 120 min. For this, the test compounds were dissolved in Hanks balanced salt solution (HBSS; pH 7.4) and sterile filtered. After 21 days of Caco-2 cell growth, the medium was removed from the filter wells and receiver plate. The filter well and receiver plate were filled with 75 µL and 250 µL fresh HBSS buffer, respectively. This procedure was repeated twice. The plates

were incubated at 37°C for 30 min, then the HBSS buffer was removed, and the solutions of test compounds were added to the filter well (75  $\mu\text{L}$ ). HBSS without test compounds was added to the receiver plate (250  $\mu\text{L}$ ). The plates were incubated at 37°C for 120 min. After incubation, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer and stored in a freezer ( $-20^\circ\text{C}$ ) pending analysis. The concentrations of test compounds were analyzed using UV-vis spectroscopy. The apparent permeability ( $P_{\text{app}}$ , nm/s) was obtained by the equation  $P_{\text{app}} = (V_A/\text{area} \times \text{time}) \times ([\text{Compd.}]_{\text{acceptor}}/[\text{Compd.}]_{\text{initial}})$ , where  $V_A$  is the volume ( $\mu\text{L}$ ) in the acceptor well, area is the surface area of the membrane (0.11  $\text{cm}^2$  of the well), time is the total transport time (7200 s),  $[\text{Compd.}]_{\text{acceptor}}$  is the concentration of tested compounds measured by UV spectroscopy, and  $[\text{Compd.}]_{\text{initial}}$  is the initial concentration of tested compounds ( $1 \times 10^{-4}$  M) in the apical or basolateral wells.

### 1.19 [ $^3\text{H}$ ]SUBSTRATE TRANSPORT INHIBITION ASSAY<sup>[36]</sup>

Caco-2 cells ( $1 \times 10^4$  cells/well) were seeded onto MultiScreen Plates for 21 days, and the cell monolayer exhibited a TER  $>800 \Omega \times \text{cm}^2$ . For assaying test compounds to basolateral compartment with and without P-gp inhibitors (from 200 nM to 400  $\mu\text{M}$ ), 20 nM [ $^3\text{H}$ ]vinblastine or [ $^3\text{H}$ ]mitoxantrone was added at 37°C for 120 min, and its appearance in the apical compartment was monitored. At 120 min (the end of the assay), 20  $\mu\text{L}$  sample was taken from the basolateral compartment to determine the concentration of radioligand on an LS6500 Beckman Counter. For each compound, [ $^3\text{H}$ ]vinblastine or [ $^3\text{H}$ ]mitoxantrone transport inhibition was calculated as radioactivity difference between radioligand with and without test compound. These differences were expressed as inhibition percentage at single concentration of test compound.

### 1.20 LACTATE DEHYDROGENASE RELEASE ASSAY<sup>[36]</sup>

Cell release of lactate dehydrogenase (LDH) representing cell death was performed with the CytoTox-One kit (Promega Corp., Madison, WI, USA), which was calculated relative to the LDH release from total lysis of cells in the untreated control. It was assumed that test compound-treated wells and the control wells contained the same total number of cells (dead plus live cells) at the end of the assay, and the cytotoxic effect of test compound was unaffected by any underestimation of cytotoxicity that could occur due to decreased total number of cells in the treated samples compared with the untreated control. Cells were seeded into 96-well plates for optical performance in the fluorescent cell-based assay in 100  $\mu\text{L}$  complete medium with or without various concentrations of test compound. The plate was incubated for 24 h in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C, and then 100  $\mu\text{L}$  substrate mix in assay buffer was added. Ten microliters of lysis solution from the CytoTox-One kit was added to untreated wells in order to estimate total LDH. Plates were protected

from light for 10 min at room temperature, and 50  $\mu$ L stop solution from the CytoTox-One kit was added to all wells. The fluorescence was recorded on a LS55 Luminescence Spectrometer (PerkinElmer) at 560-nm excitation wavelength and 590-nm emission wavelength. The cytotoxicity percentage was estimated by the equation (LDH in medium of treated cells – culture medium background)/(total LDH in untreated cells – culture medium background)  $\times$  100.

### 1.21 FUNCTIONAL ASSAY OF MITOCHONDRIAL P-gp<sup>[37]</sup>

To evaluate mitochondrial autofluorescence as well as the uptake and efflux of doxorubicin into and out of organelles, whole isolated mitochondria (50  $\mu$ g in hypertonic buffer) from doxorubicin-sensitive and resistant K562 cells were divided in test tubes either without any other drug or in the presence of specific monoclonal antibodies (4  $\mu$ g UIC2 or 1.5  $\mu$ g F4) or in the presence of inhibitor (5  $\mu$ M cyclosporin A or 10  $\mu$ M dexverapamil or 50  $\mu$ M quinine) as positive control or test compounds (in various concentrations to measure IC<sub>50</sub>). The tubes were preincubated at 20°C for 1 h (monoclonal antibodies) or 30 min (positive control and test compounds). To the tubes, doxorubicin (10  $\mu$ M final) was added, and the tubes were incubated at 20°C avoiding light exposure for another 1 h and kept on ice for a few minutes until flow cytometric analysis. In order to estimate doxorubicin efflux from mitochondria, 2 mL hypertonic buffer was added to the tubes containing test compounds, the tubes were centrifuged for 5 min at 450  $\times$  *g* and 4°C, the residue was washed once more with 2 mL buffer and diluted in 500  $\mu$ L buffer at 20°C. All tubes were incubated for 1 h at 20°C. After elimination of debris and aggregates, doxorubicin fluorescence (for uptake and retention evaluation) in each tube was measured on 10,000 isolated mitochondria with a flow rate of 500 events/s on a FACSVantage (Becton-Dickinson, Grenoble, France) flow cytometer.

### 1.22 RESISTANCE INDEX VALUE ASSAY<sup>[38]</sup>

(1) Determining doxorubicin sensitivity of MES-SA and MES-SA/Dx5 cells: Doxorubicin-sensitive MES-SA cells and doxorubicin-resistant MES-SA/Dx5 cells (during the exponential phase of growth with final concentration at  $1 \times 10^5$ /mL) in McCoy's 5 A medium containing 10% (v/v) fetal calf serum, penicillin (100  $\mu$ g/mL), and streptomycin (100  $\mu$ g/mL) were seeded in 96-well plates (100  $\mu$ L/well) coated with poly-L-lysine. The cultures were propagated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 4 h. To the plates, 25  $\mu$ L solution (final concentrations, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04  $\mu$ M) of doxorubicin in the growth medium was added and the cells were propagated for 48 h. For the control well, 25  $\mu$ L growth medium was added and the cells were also propagated for 48 h. To the blank well, which contains only the growth medium without cells, 25  $\mu$ L growth medium was added and was also propagated for 48 h. The medium was removed, the residue was washed with PBS and replaced by fresh medium, to

each well 25  $\mu\text{L}$  MTT (5 mg/mL) was added, and the plates were incubated for 4 h. After removing the growth medium, the residue was dried in the air, the residues were dissolved in 10  $\mu\text{L}$  DMSO, and the absorption values of light of the formed purple solutions were recorded on a Bio-Rad 450 microplate reader (Bio-Rad, Ontario, Canada). Survival % =  $(D_{Dx} - D_{\text{blank}})/(D_{\text{control}} - D_{\text{blank}})$ , where  $D_{Dx}$  represented the light absorption values of medium of cells plus doxorubicin and growth medium,  $D_{\text{control}}$  represented the light absorption values of medium of cells plus growth medium, and  $D_{\text{blank}}$  represented the light absorption values of growth medium alone. The survival and concentrations of doxorubicin were plotted to define the  $\text{IC}_{50}$  of doxorubicin against both MES-SA cells and MES-SA/Dx5 cells. Accordingly, the resistance index value was given by the equation ( $\text{IC}_{50}$  against MES-SA/Dx5)/( $\text{IC}_{50}$  against MES-SA).

(2) Determining the effect of test compounds on the sensitivities of doxorubicin-resistant MES-SA/Dx5 cells: MES-SA/Dx5 cells (during the exponential phase of growth with final concentration of  $1 \times 10^5/\text{mL}$  in McCoy's 5 A medium containing 10% (v/v) fetal calf serum, penicillin (100  $\mu\text{g}/\text{mL}$ ), and streptomycin ( $\mu\text{g}/\text{mL}$ ) were seeded in 96-well plates (100  $\mu\text{L}/\text{well}$ ) coated with poly-L-lysine. The cultures were propagated at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$  for 4 h. To the plates, 25  $\mu\text{L}$  solution (final concentrations, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04  $\mu\text{M}$ ) of doxorubicin in the growth medium was added and the cells were propagated for 48 h. To the plates, the solution (final concentration, 1.00  $\mu\text{M}$ ) of test compounds in the growth medium was added and the cells were propagated for 48 h. For the control well, 25  $\mu\text{L}$  growth medium was added, and the cells were also propagated for 48 h. To the blank well, which contained only the growth medium without cells, 25  $\mu\text{L}$  growth medium was added and propagated also for 48 h. The medium was removed, the residue was washed with PBS and replaced by fresh medium, to each well 25  $\mu\text{L}$  MTT (5 mg/mL) was added, and the plates were incubated for 4 h. After removing the growth medium, the residue was dried in the air, dissolved in 10  $\mu\text{L}$  DMSO, and the absorption values of light of the formed purple solutions were recorded on a Bio-Rad 450 microplate reader. Survival % =  $(D_{Dx} - D_{\text{blank}})/(D_{\text{control}} - D_{\text{blank}})$ , where  $D_{Dx}$  represented the light absorption values of cells plus doxorubicin plus growth medium plus test compounds,  $D_{\text{control}}$  represented the light absorption values of cells plus growth medium, and  $D_{\text{blank}}$  represented the light absorption values of growth medium alone. The survival and concentrations of doxorubicin were plotted to define the  $\text{IC}_{50}$  of doxorubicin against MES-SA/Dx5 cells. Accordingly, the resistance index value was given by the equation ( $\text{IC}_{50}$  of doxorubicin alone against MES-SA/Dx5)/( $\text{IC}_{50}$  of doxorubicin with test compounds against MESSA/Dx5).

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14. M.F. Rega, J.C. Reed, M. Pellecchia. Robust lanthanide-based assays for the detection of anti-apoptotic Bcl-2-family protein antagonists. *Bioorg Chem* 35 (2007) 113–120. *Note:* In time-resolved fluorescence resonance energy transfer (TR-FRET) assay, 29  $\mu$ L detection buffer, 5  $\mu$ L variable concentration of biotin-labeled BH3 peptide, biotin-(CH<sub>2</sub>)<sub>6</sub>-GGGQVGRQLAIIGDDINR (concentration, 2 to 5 nM), 5  $\mu$ L antiapoptotic Bcl-2 protein (1 to 200 nM), and 1  $\mu$ L DMSO containing test compound were mixed in each well of 96 wells (or 384) black plates (Perkin-Elmer). After 30 min (15 min to 1 h) incubation, to each well 5  $\mu$ L Eu-antibody conjugate (3 to 25 nM) and 5  $\mu$ L APC-streptavidin (1 to 4 nM) were added. The detection buffer was used. Fluorescence measurements were performed after 10 min or 2 h incubations at 340-nm excitation wavelength and 665-nm emission wavelength, controls included unlabeled peptide and blanks receiving no compounds, Z0 factor measurements were obtained by repeating the experiments (positive and negative controls) multiple times, and TR-FRET, as the DELFIA, could also be obtained using GST-fusion Bcl-2 proteins and anti-GST Eu-antibody.

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stirred for 5 min in an orbital shaker. To all wells, 50  $\mu\text{L}$  substrate solution was added, and the plate was stirred for 5 min in an orbital shaker. The plate was dark adapted for 10 min, and the luminescence was measured on Victor3. (b) Determination of cell growth was performed using MTT assay at 24 and 48 h. On day 1, 10,000 cells/well were plated in 96-well plates in a volume of 200  $\mu\text{L}$ , and on day 2, the various compounds alone or in combination were added. In all assays, the solvents (ethanol, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with test compounds, 0.5 mg/mL MTT was added to each well and, after 1 h incubation at 37°C, the supernatant was removed. The formazan crystals were dissolved in 100  $\mu\text{L}$  DMSO, and the absorbance values at 570 and 630 nm were determined on the SpectraCount microplate reader. (c) Effect of antiproliferative drug combination: In MCF7/Adr, test compounds and verapamil were used at 2  $\mu\text{M}$  and 20  $\mu\text{M}$ ; doxorubicin at 5  $\mu\text{M}$  ( $\text{IC}_{50}$  after 3 days test compounds exposure when the P-gp was not overexpressed); verapamil was used as reference compound. The schedule of test compound administration was the P-gp inhibitors plus doxorubicin for 2 days followed, after two wash steps with complete medium, by doxorubicin for 1 day. The analysis of cell growth inhibition was performed using the MTT assay.

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