Supplementary Materials

Innovative TiO_2/ZnO-Organoselenium Composites with Diselenide Linkages for Enhanced Anticancer Efficacy

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1. Chemistry

Synthesis of 4-selenocyanatoaniline (2) and diaryl diselenide A[1-3]

Selenium dioxide (30 mmol, 3300 mg) was added to malononitrile (15 mmol, 1000 mg) in 10 mL DMSO, and the mixture was stirred for 20 min at room temperature. Next, the mixture was filtered off to get rid of any formed black selenium, and aniline (12.5 mmol) was then added, and the reaction mixture was further stirred for 15 minutes. Water was then added, and the obtained precipitate was filtered and the obtained solid was washed several times with distilled water and dried under vacuum.

4-Selenocyanatoaniline (**2**) was obtained as a yellow solid (88 % yield). Mp: 73–74 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, J = 8.4 Hz, 2H, Ar-H), 6.64 (d, J = 8.4 Hz, 2H, Ar-H), 3.95 (s, 2H, NH_2).

Diaryl diselenide **A** was synthesized by dissolving 4-selenocyanatoaniline (**2**) (2 mmol) in ethanol (20 ml) containing NaOH (500 gm). The reaction was stirred for 2 h and the formation of diaryl diselenide **A** was followed by TLC. The reaction was quenched by adding ice into the reaction mixture. The yellow formed precipitate was filtered and washed several times with water and dried under vacuum.

Diaryl diselenide **A** (**2**) was obtained as pale-yellow crystals (82 % yield. Mp: 78–80 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.3 (m, 4H, Ar-H), 6.5 (m, 4H, Ar-H), 3.7 ppm (s, 4H, NH_2).

Synthesis of methyl 2-amino-5-selenocyanatobenzoate (6) and diaryl diselenide B[4, 5]

The methyl 2-amino-5-selenocyanatobenzoate (**6**) was synthesized from the reaction of methyl 2-aminobenzoate with TSD prepared in situ from $CH_2(CN)_2$ and SeO_2 in 96% yields. Briefly, SeO_2 (30 mmol, 3300 mg) was added to $CH_2(CN)_2$ (15 mmol, 1000 mg) in 10 mL DMSO, and the mixture was stirred for 20 min at RT. Next, the mixture was filtered off to get rid of any formed

black selenium, and methyl 2-aminobenzoate (12.5 mmol, 1800 mg) was then added, and the reaction mixture was stirred for a further 2 hrs. Finally, adding 10 gm of ice terminated the reaction, and the formed precipitate was filtered, washed several times with H_2O and Na_2CO_3 solution, dried, and recrystallized from petroleum ether.

Diaryl diselenide B was synthesized from methyl 2-amino-5-selenocyanatobnzoate (4 mmol, 1000 mg) and sodium hydroxide (4 mmol, 160 mg) in EtOH (20 mL). It appeared as a single compound on TLC and was isolated as a yellow solid; yield: 1692.43 mg (92%); MP = 138–139 °C; Rf = 0.5(petroleum ether/ethyl acetate 4:3). IR(KBr): $\lambda_{max}.cm^{-1}$: 3455, 3344, 2931, 1684. ^{1}HNMR (400 MHz, DMSO) δ7.70(s,2H, Ar-H), 7.44(d, *J* = 8.6 Hz, 2H, Ar-H), 7.00(s, 4H,2NH_2), 6.77(d, *J* = 8.7 Hz, 2H, Ar-H), 3.74(s, 6H,2OCH_3). ^{13}CNMR (101 MHz, DMSO-d_6) δ167, 151, 140, 138, 117, 113, 108, 51. MS (EI, 70 ev) *m*/*z* (%) = 460.15(M+H, 20.76), 459.15(M, 5.20) or 230(24.42), 119(9.45), 91(100.0, base peak), 65(8.88).

2. Materials and Methods

SI1. Growth inhibition % against a panel of cancer and normal cell lines at 100 µg/mL

The antitumor activities of the examined diaryl diselenides (**A** and **B**) and their composites (**A(ZT)** and **B(ZT)**) against fifteen human cancer cell lines namely; HNO97, HN9, MCF7, MDA-MB-468, PC3, HEPG2 & HEP2, A431, A549 & A730, H1299, FaDu, HCT116, CACO2, and HELA, obtained from the ATCC (American Tissue Culture Collection), were evaluated by sulphorhodamine-B (SRB) assay [6]. Briefly, cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with the aforementioned compounds. Next, cells were treated with 100 µg/mL for the diaryl diselenides (**A** and **B**) and their composites (**A(ZT)** and **B(ZT)**) candidates.

For each concentration, three wells were used and incubation was continued for 48 h. DMSO was used as a control vehicle (1 % v/v). At the end of incubation, cells were fixed with 20% trichloroacetic acid, and stained with 0.4% SRB dye. The optical density (O.D.) of each well was measured spectrophotometrically at 570 nm using an ELISA microplate reader (TECAN sunrise™, Germany). The mean survival fraction at each drug concentration was calculated as follows: O.D. of the treated cells/O.D. of the control cells. The IC_{50} (concentration that produces 50% of cell growth inhibition) value of each drug was calculated using sigmoidal dose-response curve-fitting models (Graph Pad Prizm software, version 8).

SI2. Cytotoxicity evaluation against MDA-MB-468, FaDu, HCT116, and HELA cancer cell lines

The antitumor activities of the the diaryl diselenides (**A** and **B**) and their composites (**A(ZT)** and **B(ZT)**) against MDA-MB-468, FaDu, HCT116, and HELA cells, were evaluated by sulphorhodamine-B (SRB) assay [6]. Briefly, cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with the aforementioned compounds. Next, cells were treated with different concentrations of 62.5, 12.5, 25, and 50 µg/mL for the diaryl diselenides (**A** and **B**) and their composites (**A(ZT)** and **B(ZT)**) candidates.

For each concentration, three wells were used and incubation was continued for 48 h. DMSO was used as a control vehicle (1 % *v/v*). At the end of incubation, cells were fixed with 20% trichloroacetic acid, and stained with 0.4% SRB dye. The optical density (O.D.) of each well was measured spectrophotometrically at 570 nm using an ELISA microplate reader (TECAN sunrise™, Germany). The mean survival fraction at each drug concentration was calculated as

follows: O.D. of the treated cells/O.D. of the control cells. The IC_{50} (concentration that produces 50% of cell growth inhibition) value of each drug was calculated using sigmoidal dose-response curve-fitting models (Graph Pad Prizm software, version 8).

SI3. Protein expression for composite B(ZT) of the apoptosis and inflammation-related genes (Enzyme-linked Immunosorbent assay)

The microplate provided in this kit has been pre-coated with an antibody specific to P53 (Catalog No # SEH009Hu), BAX (Catalog No # E91343Mu), caspases 3 (Catalog No # SEA626Hu), 6 (Catalog No # E92340Hu), 8 (Catalog No # MBS452285), and 9 (Catalog No # ab119508), MMP2 (Catalog No # MBS9135926), MMP9 (Catalog No # MBS175780), BCL-2 (Catalog No # SEA778Ra), COX-2 (Cat No # MBS264304), IL-6 (Catalog No # SEA079Ra), and IL-1β (Catalog No # MBS175901). Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to P53, BAX, caspases 3, 6, 8, and 9, MMP2, MMP9, BCL-2, COX-2, IL-6, and IL-1β. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added, only those wells that contain P53, BAX, caspases 3, 6, 8, and 9, MMP2, MMP9, BCL-2, COX-2, IL-6, and IL-1β, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of P53, BAX, caspases 3, 6, 8, and 9, MMP2, MMP9, BCL-2, COX-2, IL-6, and IL-1β in the samples is then determined by comparing the O.D. of the samples to the standard curve. Average the duplicate readings for each standard, control, and sample, and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D.

and concentration for each standard and draw a best-fit curve through the points on the graph or create a standard curve on log-log graph paper with P53, BAX, caspases 3, 6, 8, and 9, MMP2, MMP9, BCL-2, COX-2, IL-6, and IL-1β concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, Curve Expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Procedure

1. Determine wells for diluted standard, blank, and sample (**B(ZT)**). Prepare 7 wells for standard, 1 well for blank. Add 100 μL each of dilutions of standard, blank, and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 h at 37 ºC.

2. Remove the liquid from each well, don't wash.

3. Add 100 μL of Detection Reagent A working solution to each well, cover the wells with the plate sealer, and incubate for 1 h at 37 ºC.

4. Aspirate the solution and wash with 350 μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it sit for 1~2 min. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100 μL of Detection Reagent B working solution to each well, cover the wells with the plate sealer, and incubate for 30 min at 37 ºC.

6. Repeat the aspiration/wash process for a total of 5 times as conducted in step 4.

7. Add 90 µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10-20 min at 37 ºC (Don't exceed 30 min). Protect from light. The liquid will turn blue with the addition of a Substrate Solution.

8. Add 50 µL of Stop Solution to each well. The liquid will turn yellow with the addition of the stop solution. Mix the liquid by tapping the side of the plate. If the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately.

3. Biological Data

Table S1. IC_{50} µg/mL of A, B, A(ZT), and B(ZT) on MDA-MB-468, FaDu, HCT116, and HELA cancer cells.

	MDA-MB-468	**FaDu**	**HCT116**	**HELA**
A	6 ± 1	12 ± 2	20.3 ± 1.27	5.33 ± 0.41
B	7.5 ± 0.62	21 ± 1.7	22.16 ± 1.46	5.53 ± 0.8
A(ZT)	7 ± 1	9 ± 1	12 ± 1	4 ± 00
B(ZT)	8.5 ± 1.5	5 ± 0.55	12.5 ± 0.5	4.4 ± .45
DOX	6.18 ± 0.11	13 ± 1.25	5.7 ± .4	4.8 ± 0.440

The data represent means of at least three independent experiments (n = 3) ± SD.

References

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