Anticancer activities of bis(pyrazol-1-ylthiocarbonyl)disulfides against HeLa cells

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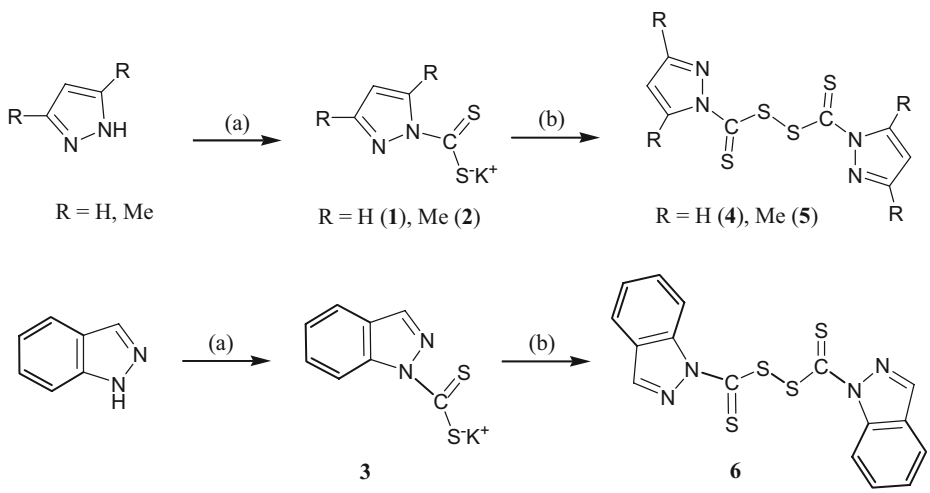
Oxidation of the pyrazol-1-yldithiocarbamate compounds {[3,5-$R_2C_3HN_2CS_2$]$^-$ (R = H, Me} and indazol-1-lydithiocarbamate by iodine produces the sulfur–sulfur coupling compounds {R'C(S)S-S(S)CR'} (R' = pyrazolyl, 3,5-dimethylpyrazolyl, indazolyl). All compounds were spectroscopically characterised, and, in some cases, structurally characterised. The X-ray structures reveal that these compounds contain a disulfide bridging the pyrazolylthiocarbonyl units. Two of the three disulfide compounds showed very good anticancer activities against HeLa cells at micromolar concentrations, with the most active compound active being 9.6 times more selective in its activity towards tumour cells than normal cells.

Keywords: dithiocarbamates, pyrazolyl, indazolyl, disulfide, anticancer, HeLa cells

Dithiocarbamates (dtcs) are of interest as therapeutic agents.[1] For instance, the well known chelating agent diethyldithiocarbamate ([Et_2NCS_2]$^-$), is a good anticancer agent,[2] and the sulfur–sulfur dimer of [Et_2NCS_2], tetraethylthiuram disulfide (disulfram), has profound anticancer activity[3] where extensive pharmacokinetics studies have proved that it has an excellent safety record as well.[4]

Several mechanisms of action of disulfram as anticancer agent have been proposed. These include DNA topoisomerase inhibition [5] angiogenesis reduction[6] and proteasome pathways inhibition.[7] These facts suggest induction of apoptosis, even though the actual mechanisms have not yet been fully established.[8-10] These mechanistic studies suggest that the potency of disulfide compounds is associated with the interactions of the S-S moiety with sulfhydryl groups of enzymes.[11,12] Two examples of disulfide closely related to disulfram, are diallyl disulfide[13] and 1-methyl-1-propyl-2-imidazolyl disulfide.[14] The former is found in garlic and has been shown to effectively inhibit the growth of human breast cancer cells *in vitro* and *in vivo* by apoptosis through inducing caspase-3.[13] The diallyl disulfide antitumor action shows no side effects. Experiments involving HeLa cells treated with 1-methyl-1-propyl-2-imidazolyl disulfide have shown thioredoxin reductase as a target for disulfide anticancer activity.[14] Therefore, there is no doubt that the extensive work on disulfram and related sulfur–sulfur containing compounds clearly point to some therapeutic properties associated with disulfide compounds. This current study reports the potential of pyrazolyl-based disulfide compounds as anticancer agents. Interestingly their dithiocarbamate precursors exhibit no anticancer activity.

Iodine oxidation of the appropriate dithiocarbamate salts {pyrazol-1-yldithiocarbamate (**1**), 3,5-dimethylpyrazol-1-yldithiocarbamate (**2**)[15,16] or indazol-1-yldithiocarbamate (**3**)} produced one known (**5**)[17] and two new (**4** and **6**) bis (dithiocarbonyl)disulfides as yellow powders in moderate yields (Scheme 1). Except for **6**, compounds **4** and **5** were readily soluble in chlorinated solvents. NMR data are consistent with the formulations in Scheme 1 and the $^{13}C\{^1H\}$ NMR data showed typical C(C=S) peaks around 193 ppm compared to C(C=S) peaks of the starting dithiocarbamates which appeared between 218.6 and 222.2 ppm. IR spectra of compounds **4**–**6** showed bands assignable to the υ(C=S) and υ(C–S) vibrational modes in the region of 1334–1254 cm^{-1} and 869–901 cm^{-1}, respectively. The structures of **4** and **5** were confirmed by single crystal X-ray crystallography, and have structures similar to the closely related bis [(3,5-dimethylpyrazol-1-yl)ethyl]disulfide reported by Mills *et al*.[18] and bis[(3-hydroxymethyl-5-methylpyrazol-1-yl) thiocarbonyl]disulfide reported by El ldrissi *et al*.[19]



Scheme 1 Reagents and conditions: (a) KOH, CS_2, THF, rt, 20 min, 71–79%; (b) I_2, MeOH, rt, 15 min, 56–75%.

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Table 1 Crystal data and structure refinement for **4** and **5**

Parameter	**4**	**5**
Empirical formula	$C_8H_6N_4S_4$	$C_{12}H_{14}N_4S_4$
Formula weight	286.41	342.51
Temperature (K)	293(2)	100(2)
Wavelength (Å)	0.71073	0.71073
Crystal system	Triclinic	Monoclinic
Space group	$P\bar{1}$	I2/a
Unit cell dimensions		
a(Å)	5.43340(10)	10.6094(16)
b(Å)	31.3556(7)	9.4667(14)
c(Å)	7.2893(2)	15.156(2)
α	90°	90°
β	107.9860(10)°	101.760(2)°
γ	90°	90°
Volume (Å$^{3)}$	1181.17(5)	1490.2(4)
Z	4	4
Density (calculated) (Mg/m^3)	1.611	1.527
Absorption coefficient (mm^{-1})	0.780	0.632
F(000)	584	712
Final R indices (R[1])	0.0416	0.0262
Reflections collected	20623	13011
Completeness to theta	100.0%	98.5%
Goodness of fit on F^2	1.116	1.062
Largest diff. peak and hole (e Å^{-3})	0.664 and –0.617	0.483 and –0.251

Orange crystals suitable for single crystal X-ray analysis were obtained from slow evaporation of THF solutions of **4** and **5**. The crystal data are presented in Table 1, while molecular structures and selected bond distances and angles are shown in Figs 1 and 2. All the bond distances and angles of **4** and **5** are normal. In **4** and **5**, the two planar fragments –C(= S)- pyrazolyl ring are connected by a disulfide bridge. The C–S–S–C torsion angle is 83.67(6)° in **4** and is substantially larger at 101.70(7)° in **5**. In both compounds the S–C–N–N chains are in the E configuration. Compounds **4** and **5** are structurally similar to other disulfide compounds, *e.g.* bis[(3,5-dimethylpyrazol-1-yl)ethyl]disulfide {(S–S = 2.0396(6) Å, S–C = 1.8097(15)/1.8156(16) Å, C–S–S = 103.95(1)/105.73(6)°},[18] bis(2,4-imidazolidinedione-5-ethyl)disulfide {(S–S = 2.022(4) Å, S–S = 2.022(4) Å, S–C = 1.801(7)/1.817(7) Å, C–S–S = 103.1(3)/103.9(3)°}[20] and didenzoyldisulfide {(S–S = 2.020(1) Å, S–C = 1.805(2)/1.823(2) Å, C–S–S = 100.2(1)/101.7(1)°}.[21]

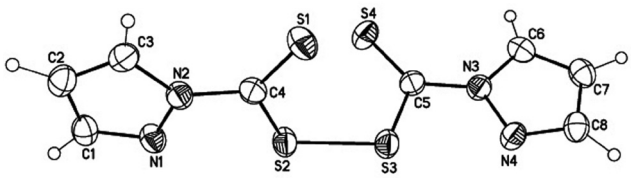

Fig. 1 A molecular drawing of **4** shown with 50% probability ellipsoids. The hydrogen atoms omitted for clarity. Selected bond distances [Å] and angles [°]: S(1)–C(4), 1.621(2); S(2)–C(4), 1.778(3); S(2)–S(3), 2.0194(10); N(1)–N(2), 1.370(3); N(2)–C(4), 1.386(3); C(4)–S(2)–S(3), 102.49(9); C(1)–N(1)–N(2); C(4)–N(2)–N(1), 104.1(2).

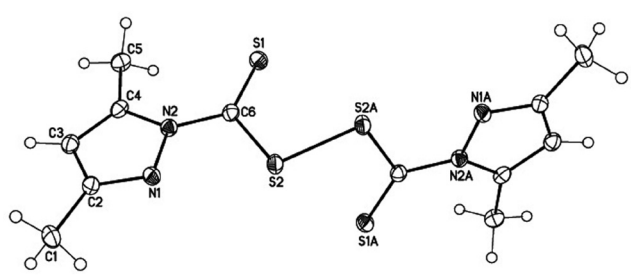

Fig. 2 A molecular drawing of **5** shown with 50% probability ellipsoids. The hydrogen atoms omitted for clarity. Selected bond distances [Å] and angles [°]: S(1)–C(6), 1.6294(11); S(2)–C(6), 1.7958(11); S(2)–S(2)#1, 2.0309(6); N(1)–N(2), 1.3884(12); N(2)–C(6), 1.3825(13); C(6)–S(2)–S(2)#1, 102.78(4); C(2)–N(1)–N(2); 105.28(9); C(6)–N(2)–N(1), 91.07(12).

Compounds **1**–**6** (Scheme 1) were screened for their antitumour activities against human cervix epithelial carcinoma (HeLa) cells and human lymphocytes (PBMCs). All data were acquired in triplicate and the final values recorded as averages. Table 2 lists the dose values that caused 50% inhibition of cell growth (IC_{50}).

In order to establish the activities of the disulfide compounds, it was important to investigate the activities of the primary dtcs (**1**, **2** and **3**) from which the disulfides were prepared. These experiments helped in determining whether the observed activities of the disulfides are due to the involvement of the S-S moiety in compounds **4**–**6**. Compounds **1**–**3** were found to be inactive against HeLa cells (Table 2). The disulfides **4** and **5** on the other hand showed moderate to very good antitumour activities against HeLa cells. Compound **4** had an IC_{50} value of 3.7 μM (Table 2) compared to cisplatin (1.1 μM), while that of **5** (17.8 μM) was *ca* 16 times less active than cisplatin. Compound **6** could not be tested due to its low solubility in DMSO.

Compound **4** was further tested against PBMCs, allowing us to establish its antitumour specificity against cancerous HeLa. The IC_{50} values registered for resting and stimulated lymphocytes were 34.0 μM and 36.4 μM, respectively (Table 3), as compared to its activity of 3.7 μM against HeLa cells.

$$TS = \frac{\text{Mean } IC_{50} \text{ of the normal cells (stimulated + resting lymphocytes)}}{\text{Mean } IC_{50} \text{ of the cancer cells}} \quad (1)$$

Table 2 Growth inhibition values of compounds **1-6** tested against HeLa cells

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	>50	**4**	3.7 ± 0.2
2	>50	**5**	17.8 ± 4.2
3	>50	**6**	ND
Cisplatin	1.1 ± 0.2		

IC_{50} is the concentration of drug required to inhibit cell growth by 50%. ND, Not done.

Table 3 Tumour specificity of **4**

Compound	HeLa IC_{50} (μM)	Lymph (resting) IC_{50} (μM)	Lymph (stimulated) IC_{50} (μM)	Tumour Specificity
4	3.7	34.0	36.4	9.6

IC_{50} is the concentration of drug required to inhibit cell growth by 50%. Lymph, lymphocytes

These results were used to calculate the tumour specificity factor (TS) as shown in equation (1).

The TS for **4** was found to be 9.6 (Table 3) indicating that that cancerous HeLa cells were approximately ten times more sensitive to the cytotoxic action of **4** compared to normal cells. Certainly this selectivity is superior to that of disulfram, which is reported to be 2.9 by Wickström and coworkers using the same cell-lines.[3] Compound **4** is structurally similar to disulfram which has anticancer activity against many cell lines, such as melanoma CRL1585 (IC_{50} = 2.5 μM), prostate adenocarcinoma CRL1435 (IC_{50} = 2.5 μM),[22] and cervical adenocarcinoma HeLa (~ 45 μM, $p<0.05$).[3] Even though there are no clearly established structural activity relationships for disulfram, the anticancer activity of disulfram is believed to be due to the presence of the S–S moiety that allows for the formation of diethyldithiocarbamate metabolites that react with sulfhydryl groups of several enzymes.[11,12] Compound **4** could thus be acting via a similar mechanism in its activity against HeLa cells; where it reduces *in vivo* to pyrazolyldithiocabarmate and then reacts with enzymes and DNA residues to trigger apoptosis.

In summary, isolation of new bis(dithiocarbonyl)disulfides (**4**–**6**) is indicative of how compounds **1**–**3** can readily undergo oxidation. The presence of the disulfide moiety was confirmed from the solid state structures of **4** and **5**. And whereas the salts that formed the disulfides, **1**–**3,** show no inhibition to the growth of HeLa cells, the disulfides, **4** and **5** are active against cancerous cells, with **4** being the most active and fairly selective. Given the fact that the respective monomeric dithiocarbamates (dtcs) were inactive, it is possible that the S-S motif is responsible for the active metabolites. The selectivity of **4** towards HeLa cells over normal cells is superior compared to that of disulfram using the same cells,[3] a finding which should encourage further investigation of compounds containing sulfur–sulfur moieties as anticancer agents.

Experimental

IR spectra were recorded as pure solids on a Bruker Tensor27 spectrophotometer fitted with an ATR-IR probe. ^{1}H NMR and ^{13}C NMR spectra were recorded ^{1}H and ^{13}C{^{1}H} NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer (300 MHz) in D_2O and $CDCl_3$ at room temperature. Elemental analysis was performed on a Fisons elemental analyser at the University of Cape Town, South Africa. Literature compounds, pyrazol-1-yldithiocarbamate (**1**) and 3,5-dimethylpyrazol-1-yldithiocarbamate (**2**), were prepared according to literature methods.[15,16] As for the known bis(3,5-dimethylpyrazol-1-yldithiocarbonyl)disulfide (**5**), a different synthetic procedure from the one reported in the literature[17] was employed. All experiments were performed under dry, deoxygenated nitrogen atmosphere using Schlenk techniques. Solvents were dried by conventional methods.

Preparation of potassium indazol-1-yldithiocarbamate (**3**)
To a solution of indazole (1.0 g, 8.46 mmol) in THF (20 mL) was added finely powdered KOH (0.48 g, 8.46 mmol). The mixture was left to stir for 2 min and CS_2 (0.46 g, 8.46 mmol, 0.36 mL) added dropwise while stirring the solution. The solution turned from a yellow to orange and a copious amount of precipitate formed. After 20 min, the yellow product was isolated by filtration using a G4 frit, washed with diethylether and dried *in vacuo*. Yield 79%; ^{1}H NMR (300 MHz, D_2O) δ 8.75 (d, 1H, $^4J_{HH}$ = 8.7 Hz, H), 7.89 (s, 1H, H), 7.49 (d, 1H, $^3J_{HH}$ = 8.7 Hz, H), 7.24 (t, 1H, $^4J_{HH}$ = 15.0 Hz, H), 7.01 (t, 1H, $^4J_{HH}$ = 15 Hz, H). ^{13}C{^{1}H} NMR (300 MHz, D_2O) δ 219.2 (C(C=S)), 138.6 (C(5C-pz), 129.0 (C(3C-pz)), 126.9 (C(7C–Ph)), 123.6 (C(4C–Ph), 121.4 (C(8C, 9C–Ph)), 117.2 (C(6C–Ph)). IR (cm^{-1}) $\upsilon_{C=N}$ = 1608, υ_{C-S} = 1297, $\upsilon_{C=S}$ = 852; Anal. Calcd for $C_8H_7KN_2S_2$: C, 41.00; H, 3.01; N, 11.95; S, 27.36. Found: C, 41.42; H, 2.97; N, 11.51; S, 26.97%.

General procedure for preparation of **4**–**6**
To solutions of the dtcs in methanol were added iodine pellets in a 2:1 ratio (dtcs:iodine). The resultant solution was stirred at room temperature during which yellow precipitates were formed. The reactions were left to run for 15 min. The products were isolated as yellow solids by filtration and washed with excess methanol to give pure products. The spectroscopic and microanalysis data of the compounds are as follows.

Bis(pyrazol-1-yldithiocarbonyl)disulfide (**4**): Yellow powder 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (s, 2H, $^3J_{HH}$ = 3.6 Hz, 5-pz), 7.91 (s, 2H, $^3J_{HH}$ = 3.3 Hz, 3-pz), 6.56 (s, 2H, $^3J_{HH}$ = 3.9 Hz, 4-pz). ^{13}C{^{1}H} NMR (300 MHz, $CDCl_3$) δ 192.3 (C(C=S), 145.6 (C(5, 5'-pz)), 131.5 (C(3, 3'-pz)), 111.6 (C(4, 4'-pz)). IR (cm^{-1}) $\upsilon_{C=N}$ = 1533, υ_{C-S} = 1254, $\upsilon_{C=S}$ = 869; Anal. Calcd for $C_8H_6N_4S_4$: C, 33.55; H, 2.11; N, 19.56; S, 44.78. Found: C, 33.73; H, 2.64; N, 18.95; S, 44.32%.

Bis(3,5-dimethylpyrazol-1-yldithiocarbonyl)disulfide (**5**): Yellow powder 73% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.23, 6.16 (s, 2H, 4-pz), 2.83, 2.70 (s, 6H, 5-pz), 2.40, 2.33 (s, 6H, 3-pz). ^{13}C{^{1}H} NMR (300 MHz, $CDCl_3$) δ 193.2 (C(C=S)), 152.8 (C(5-pz)), 146.6 (C(3-pz), 113.8 (C(4-pz)), 17.1 (C($\underline{C}H_3$, 5-pz), 13.8 (C($\underline{C}H_3$, 3-pz); IR (ATR, cm^{-1}) $\upsilon_{C=N}$ = 1533, υ_{C-S} = 1261, $\upsilon_{C=S}$ = 879; Anal. Calcd for $C_{12}H_{14}N_4S_4$: C 42.08; H 4.12; N 16.36; 37.45. Found: C, 41.98; H, 4.01; N, 15.98; S, 37.12%.

Bis(indazol-1-yldithiocarbonyl)disulfide (**6**): Yellow powder 56% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.06 (d, 2H, $^4J_{HH}$ = 9.0 Hz, H), 8.35 (s, 1H, H), 7.85 (d, 1H, $^3J_{HH}$ = 8.4 Hz, H), 7.64 (1H, $^3J_{HH}$ = 15.0 Hz, H), 7.47 (t, 1H, $^4J_{HH}$ = 15 Hz, H). ^{13}C{^{1}H} NMR (300 MHz, $CDCl_3$) δ 191.8 (C(C=S)), 141.1 (C(5C-pz), 130.9 (C(3C-pz)), 131.2 (C(7C-Ph)), 126.2 (C(4C-Ph), 121.8 (C(8C, 9C-Ph)), 116.8 (C(6C-Ph)); IR (ATR, cm^{-1}) $\upsilon_{C=N}$ = 1605, υ_{C-S} = 1334, $\upsilon_{C=S}$ = 901; Anal. Calcd for $C_{16}H_{10}N_4S_4$: C 49.72; H 2.61; N 14.49; 33.18. Found: C, 49.82; H, 2.46; N, 13.90; S, 33.16%.

Biological experiments
Reagents and instrumentation. All commercial reagents were used as received. Phosphate Buffered Saline (PBS), Eagle's RPMI-1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit, phytohemagglutinin-protein form (PHA-P) and the 96-well flat-bottomed culture plates were all purchased from BD Biosciences Ltd. Compounds **1**–**6** were screened for their antitumour activities. Human cervix epithelial carcinoma (HeLa) cells and human lymphocytes (PBMCs), from preservative free heparinised peripheral blood, were obtained from the Department of Pharmacology and Pretoria Medical Hospital, University of Pretoria, South Africa. The absorbance values were recorded on a Whittaker Microplate Reader 2001 spectrophotometer at 570 nm and the reference wavelength of 630 nm.

Cell culture and drug treatment. HeLa cells were cultured in Eagle's medium with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1.0 mM sodium pyruvate and 5% bovine fetal calf serum, at 37 °C in an atmosphere of 5% CO_2. Cells were plated in 96-well sterile plates at a density of 1×10^4 cells/well in 100 μL of medium, and incubated for 1 h. Subsequently, compounds were added with concentrations from 0 to 50 μM. Cytotoxicity was determined by using MTT to stain treated HeLa cells after 7 days according to literature methods.[23] Cell survival was evaluated by measuring absorbance at 570 nm, using a Whittaker Microplate Reader 2001. The IC_{50} values were calculated with the Graphpad programme. All experiments were performed in triplicate. The inhibition of the growth of normal cells by **1**–**6** was also measured by employing PBMCs by using the procedure described above for HeLa cells, except for the fact that the treated PBMCs were incubated for 3 days. The lymphocytes were divided into two namely; (i) normal cells that were stimulated using PHA-P (stimulated lymphocytes) and (ii) un-stimulated normal cells (resting lymphocytes).

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Crystallographic data for structural analysis have been deposited with the Cambridge Crystallographic Data Centre, CCDC-702839 and 699573 for **4** and **5**, respectively. Copies of this data can be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: + 44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk). Additional supplementary material are NMR data and X-ray crystallography analysis method.

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