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Preclinical report

Structure–activity studies on gossypol in tumor cell lines

Michael D Shelley,[1] Laura Hartley,[1] Paul W Groundwater[2] and Reginald G Fish[1]
[1]Research Laboratories, Velindre NHS Trust, Whitchurch, Cardiff, CF14 2TL, UK. [2]School of Health Sciences, University of Sunderland, Sunderland, SR2 3SD, UK.

Gossypol [(2,2′-binaphthalene)-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl] 1a is a naturally occurring compound extracted from the cotton plant and has been extensively studied as an oral male contraceptive. Its favorable toxicity profile, and the more recent demonstration of anti-tumor activity in animals and humans, prompted us to investigate the role of the aldehyde groups in a structure–activity study in cultured tumor cells. Four racemic compounds were evaluated: gossypol 1a, gossypolone 2, the bis Schiff's base of L-phenylalanine methyl ester with gossypol (bis Schiff's base) 1c and apogossypol 1b. The former two compounds both retain the aldehyde functional groups at positions 8 and 8′ of the molecule whilst in the latter two compounds the aldehydes are blocked or absent, respectively. In addition, the *l*- and *d*-isomers of gossypol 1a, the bis Schiff's base 1c and the half Schiff's base 1d (one aldehyde blocked) were tested. The cell lines studied included melanoma (SK-mel-19), cervix (Sihas), small cell lung (H69) and myelogenous leukemia (K562). Cytotoxicity was measured using the MTT and flow cytometric viability assays. Racemic gossypol 1a and gossypolone 2 induced similar dose-dependent decreases in cell viability in all the cell lines with IC_{50} values of 23–46 and 28–50 μM, respectively. In contrast, the racemic bis Schiff's base derivative of gossypol 1c and apogossypol 1b showed minimal activity in any cell line up to 50 μM. The *l*-enantiomer of gossypol 1a was significantly more active than the *d*-enantiomer (IC_{50} of 20 versus >50 μM, respectively). When one aldehyde of either enantiomer was blocked 1d cytoxicity was comparable to the *l*-enantiomer of gossypol. The data suggest that only one aldehyde group is required for the cytotoxicity of gossypol 1a, irrespective of the stereo-configuration [© 2000 Lippincott Williams & Wilkins.]

Key words: Cytotoxicity, gossypol, structure–activity.

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Correspondence to: MD Shelley, Research Laboratories, Velindre NHS Trust, Whitchurch, Cardiff, CF14 2TL, UK.
Tel: (+44) 1222 615888; Fax: (+44) 1222 521609;
E-mail: mike.shelley@velindre.tr.wales.nhs.uk

Introduction

Gossypol [(2, 2′-binaphthalene)-8, 8′-dicarboxaldehyde-1, 1′, 6, 6′, 7, 7′-hexahydroxy-5, 5′-diisopropyl-3, 3′-dimethyl] **1a** is a naturally occurring, yellow, lipid soluble compound found as a racemate in high concentrations in the pigment glands of the cotton plant (*Gossypium*). It has potent anti-fertility activity and has been extensively studied as a male oral contraceptive.[1] In addition, gossypol has been shown to have anti-viral,[2] anti-parasitic[3] and anti-inflammatory properties.[4] The racemate is also cytotoxic towards a number of tumor cell lines *in vitro*[5–8] at concentrations 2- to 7-fold less than those causing toxic effects in normal cells such as bone marrow[9] and epithelial cells.[7] In animal studies, gossypol has been shown to be active against murine breast carcinoma[10] and prostate tumors in rats.[11] Clinical studies have demonstrated that a well-tolerated oral dose of gossypol can induce tumor regression in chemotherapy-resistant adrenocortical carcinoma.[12]

Gossypol **1a** is a monoterpenoid, polyphenolic dialdehyde and this interesting structure may account for its observed diverse pharmacological properties. It is an amphiphilic molecule, the non-polar moiety resides in the naphthalene rings and the iso-propyl group, whereas the polar domains are the carbonyl and the hydroxyl groups. The hydrophobic rings can interact with phospholipids in biological membranes forming more rigid structures.[6,13] Gossypol can behave as an anti-oxidant[14] and the two aldehyde groups can interact with biologically important basic amino acids such as lysine, glutamine and phenylalanine forming Schiff's bases.[15,16] This latter property probably accounts for its ability to bind to proteins[17,18] and inhibit the activity of a number of enzymes, to variable extents.[19,20]

It was suggested over 70 years ago that the aldehyde groups in gossypol **1a** may be important for some of its

pharmacological actions[21] and more recently it was found that modification of the aldehyde groups reduces the cytotoxicity towards peripheral lymphocytes without altering gossypol's antiviral activity.[22] The role of the aldehyde groups in the observed antitumor activity is, however, unknown. In this paper, we describe the cytotoxicity of gossypol in four tumor cell lines, and compare and contrast its activity after blocking (by forming gossypol-Schiff's bases **1c**) or removing both aldehyde groups (forming apogossypol **1b**). An oxidation product (gossypolone **2**), which retains both aldehyde groups and is a suggested major metabolite in animals,[23] was also investigated. In addition we have synthesized novel stereoisomers in an attempt to probe into the stereo-specific mechanism(s) of cytotoxicity.

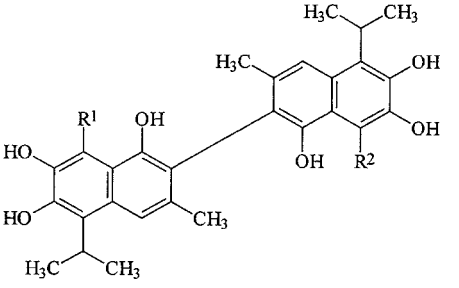
Methods

Test compounds

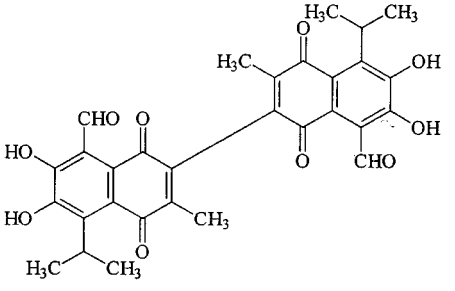
Racemic gossypol **1a** was obtained from the Institute of Bioorganic Chemistry, Academy of Sciences, Rebublik Uzbekistan (Figure 1). The ^{1}H-NMR spectrum indicated greater than 95% purity with peaks at δ 1.52 [12H, d, J=7 Hz, CH(CH_3)$_2$], 2.11 (6H, s, Ar-CH_3), 3.94 [2H, sept., J=7 Hz, CH(CH_3)$_2$], 5.82 (2H, s, C_1-OH), 6.544 (2H, s, C_6-OH), 7.82 (2H, s, C4-OH), 11.14 (2H, s, CHO), 15.18 (2H, s, C_7-OH). This is in agreement with previously published data for gossypol **1a**.[24] Gossypolone **2** and apogossypol **1b** were purchased from Sigma (Poole, UK). Reverse-phase high-pressure liquid chromatography (HPLC; Gilson, Cambridge, UK) on a Waters C_{18} column using a mobile phase of acetonitrile:0.1 M KH_2PO_4 (82:18, pH 3.2) with UV detection at 254 nm, indicated >95% purity.

Preparation of compounds

Racemic bis Schiff's bases ***1c***. All solvents and chemicals used in the preparation of the test compounds were purchased from Sigma. Racemic gossypol **1a** acetic acid 100 mg (1 equivalent) was dissolved in diethyl ether (10 ml) and washed twice with water (2 × 10 ml) to remove the acetic acid. The ether layer was dried ($MgSO_4$) prior to evaporation under reduced pressure. Dichloromethane (1 ml/100 mg gossypol), propan-2-ol (1 drop/100 mg gossypol) and L-phenylalanine methyl ester (5.1 equivalents) were added, and the reaction mixture was incubated at room temperature in the dark for 12 h and the reaction was monitored by HPLC. At the end of the incubation period the composition of this racemate



1 a R^1 = R^2 = CHO (Gossypol)
b R^1 = R^2 = H (Apogossypol)
c R^1 = R^2 = CH=NCH(CO_2CH_3)CH_2Ph (Bis Schiff's base)
d R^1 = H, R^2 = CH=NCH(CO_2CH_3)CH_2Ph (Half Schiff's base)



2 (Gossypolone)

Figure 1. The chemical structure of gossypol **1a** and related compounds.

was approximately 1:1 bis *l*-Schiff's base:bis *d*-Schiff's base and the reaction mixture was evaporated under reduced pressure to give the mixture of the bis Schiff's bases **1c** in 40% yield.

Bis l-*Schiff's base and Bis* d-*Schiff's base.* In order to test the individual bis Schiff's bases **1c**, an aliquot of the racemic mixture prepared above was adsorbed onto silica and separated using flash chromatography with a gradient mobile phase of hexane:ethyl acetate (100:0 to 42:58). Using this procedure the bis *l*-Schiff's base eluted first, followed by the bis *d*-Schiff's base. Fractions containing at least 95% of each base were pooled and rotary evaporated to dryness.

Half l-*Schiff's base and half* d-*Schiff's base* ***1d***. The bis *l*-Schiff's base **1c** and bis *d*-Schiff's base (50 mg) were hydrolysed separately with concentrated sulfuric acid:acetic acid:ether (2.5:1:1) at 0°C in the dark. Using HPLC to monitor the hydrolysis and ^{1}H-NMR analysis of the products, it was apparent that after 4 h 70% of the respective bis Schiff's base had been converted to the half Schiff's base. The hydrolysis was stopped at this point, and water (60 ml) and ether (60 ml) added to remove the acid and extract the half Schiffs base **1d**, which was then filtered and purified by preparative HPLC (Waters preparative C_{18} Microbondapak column 19×300 mm with 10 μm particle size).

Enantiomers of gossypol ***1a***. Using the same procedure as above, but hydrolysing the separate *l*- and *d*-bis Schiff s bases **1c** for longer (12 h), resulted in complete removal of the L-phenylalanine methyl ester groups from both aldehydes. After washing and extraction with 10 volumes of ether, the individual *l*- and *d*-gossypol **1a** enantiomers were regenerated.

All compounds were greater than 95% pure as indicated by analytical HPLC, optical rotation and ^{1}H-NMR. The compounds were stored desiccated at −20°C and were stable for at least 6 months under these conditions. Stock solutions (10 mM) of the test compounds were made up immediately before use in dimethyl sulfoxide and further diluted with cell medium.

Cell lines and cytotoxicity assays

Four established tumor cell lines were used: SK-mel-19 (melanoma), Sihas (cervix), H69 (small cell lung) and K562 (myelogenous leukaemia). The SK-mel-19 and Sihas lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Life Technologies, Paisley, UK), and the H69 and K562 cell lines were grown in RPMI plus 10% FCS. All cells were cultured at 37°C in humidified 5% CO_2 in air (pH 7.4). Exponentially growing cell cultures were exposed to the medium containing the test compounds in the concentration range 0–50 μM for 24 h. Controls contained diluent only and the effect of the compounds was evaluated using two viability assays.

MTT assay. Cells (5×10^3) were grown for 48 h in 96-well flat-bottomed plates (Becton Dickinson, Oxford, UK) and incubated in humidified 5% CO_2 at 37°C. After drug exposure 20 μl of 5 mg/ml MTT in phosphate-buffered saline (Sigma) was added to each well for 4 h. After incubation, 100 μl of a lysing buffer consisting of 20% w/v sodium lauryl, 50% v/v *N*,*N*-dimethyl formamide sulfate (Sigma) and distilled water were added. The plates were incubated overnight at 37°C to solubilize the formazan and the color formation was measured at 570 nm using an ELISA plate reader. The percentage viability was determined by dividing the optical density of the treated cells by that of the controls and multiplying by 100.[8]

Flow cytometry assay. After drug exposure, duplicate cultures (5×10^4 cells) were taken and the adherent lines were harvested by trypsinization (0.05% trypsin, 0.02% EDTA in Gibco A). The cells were centrifuged at 250 *g* for 5 min and resuspended in 1 ml of fresh medium. An equal number of 6 μm polythene beads (Polysciences, Northampton, UK), fluorescein diacetate (50 μl of 100 ng/ml acetone solution) and propidium iodide (50 μl of 100 μg/ml water) were added. Analysis of viability was performed on a FACScan (Becton Dickinson) using Lysys II software. Fluorochromes were exited at 488 nm with an air-cooled argon laser (15 mW) and standard filters were used to collect green fluorescence (FDA, 530/30 nm) or red fluorescence (PI, 625/35 nm). Signals were triggered by forward light scatter (5000 for each sample), and log amplification of red and green fluorescence signals was used for data collection. Viable cells were identified as those having high green fluorescence and low red. Surviving cells were expressed as a percentage of the control.[25]

Statistics

All experiments were carried out at least 4 times for each assay. The data represents the mean (±SEM) and experiments were compared using analysis of variance. Probability (p) values of 0.05 or less were considered to be significant. Linear regression analysis was used to compare methods of assay.

Results

The results of the cytotoxicity experiments with the MTT assay for the racemates of gossypol **1a**, gossypolone **2**, apogossypol **1b** and the bis Schiff's base **1c** are illustrated in Figure 2. All cell lines exhibited a similar cytotoxicity profile although there was some difference in sensitivity. The bis Schiff's base **1c** derivative of gossypol **1a**, where both aldehydes are blocked, showed little activity in any of the four cell lines studied and only induced a mean reduction in viability of 17% at 50 μM. A similar lack of activity was observed with apogossypol **1b**, inducing a mean reduction in cell viability of 15% at the same molar concentration. There was no statistical difference in cytotoxicity between these two compounds in the four cell lines. L-Phenylalanine methyl ester was tested as a control and was found to be inactive in all cell lines at concentrations up to 50 μM.

Both racemic gossypol **1a** and gossypolone **2** induced a dose-dependent decrease in cell viability in all the cell lines. There was no statistical difference between these two compounds at any concentration, although there were some differences in cell sensitivity. Both agents had similar activity in the K562, H69 and Sihas cell lines with IC_{50} values (the concentration required to reduce cell viability by 50%) ranging from 35 to 46 and 38 to 50 μM for gossypol **1a** and

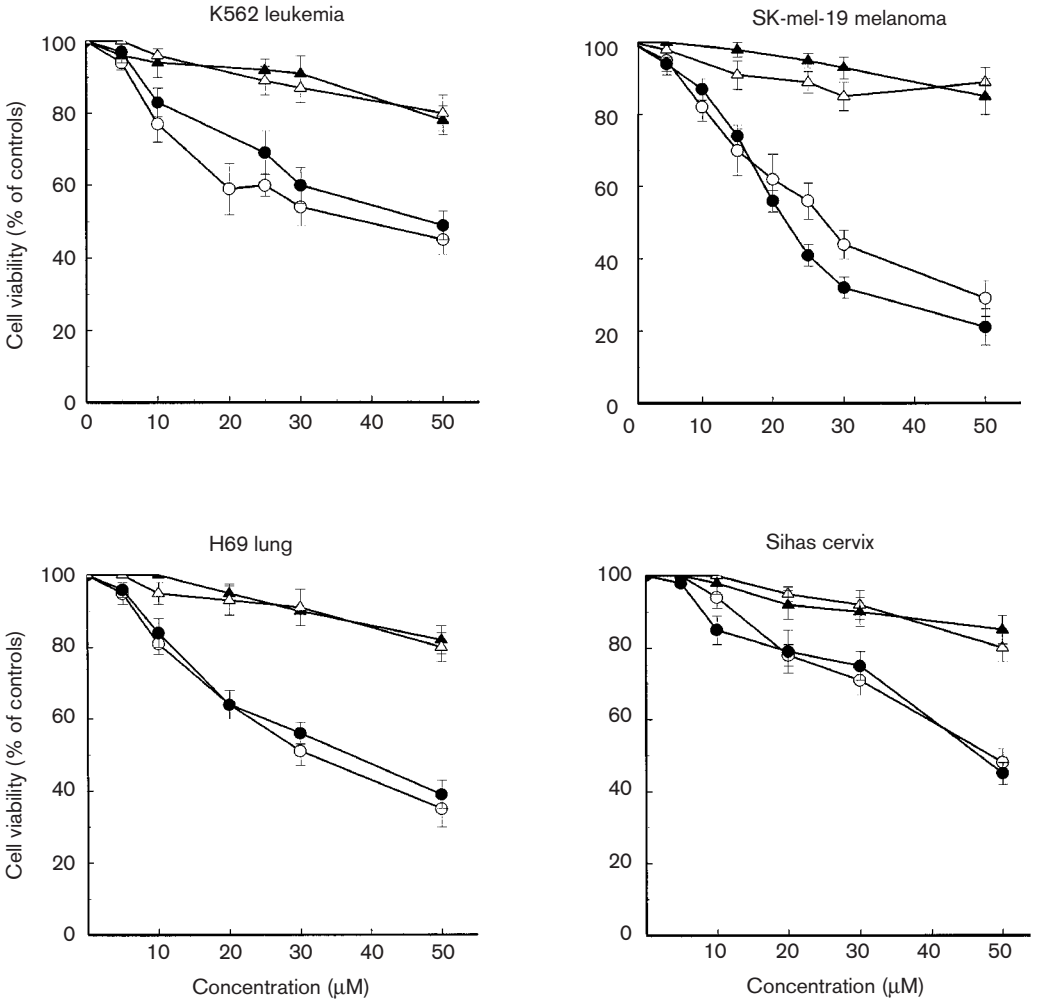


Figure 2. Cell viability (MTT assay) of four tumor cell lines after exposure to racemic gossypol **1a** (○), racemic gossypolone **2** (●), racemic apogossypol **1b** (▲) and the racemic bis Schiff's base **1c** (△).

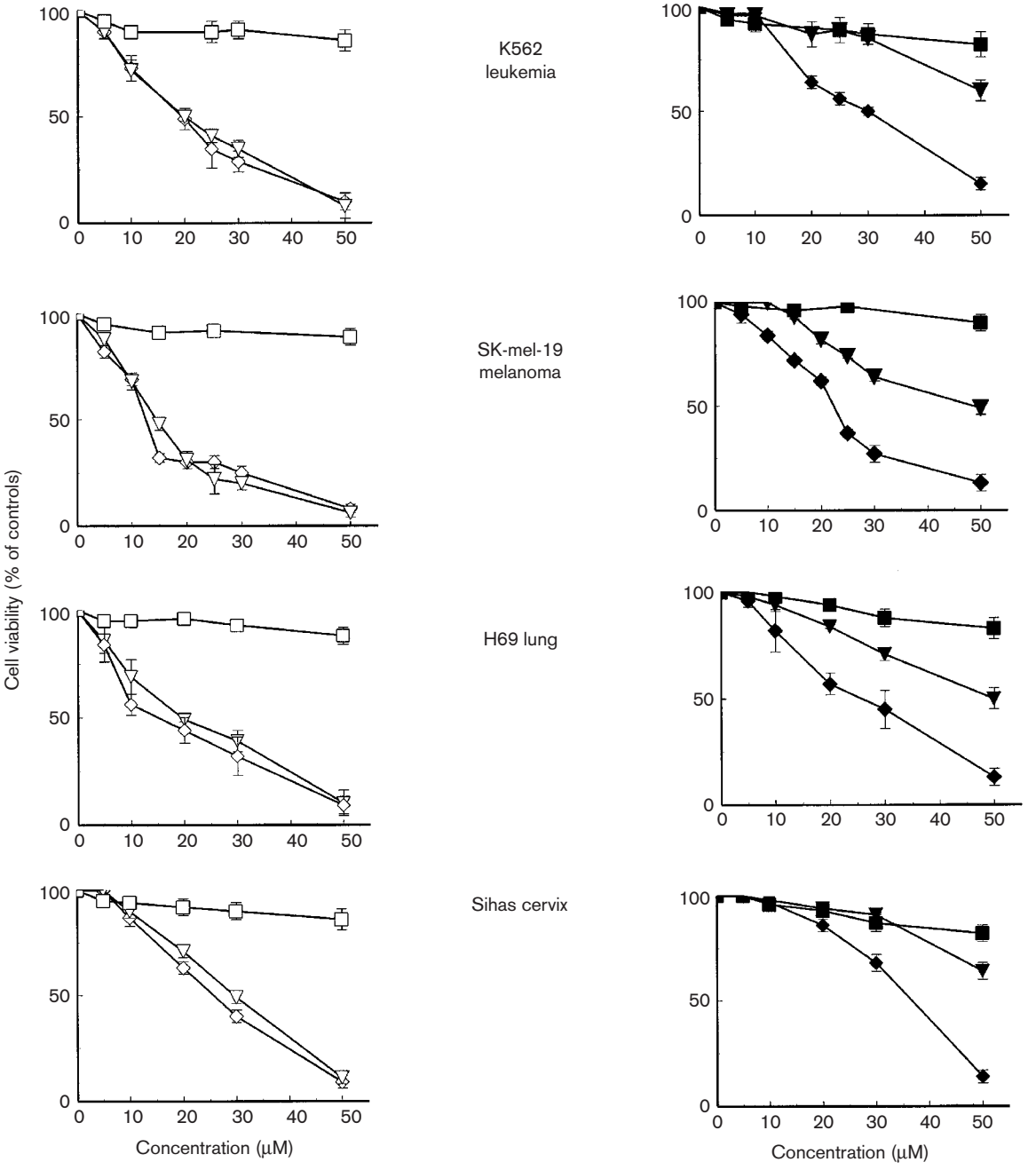


Figure 3. The cytotoxic effects of gossypol enantiomers **1a** (▽, ▼), the individual bis Schiff's bases **1c** (□, ■) and the individual half Schiff's bases **1d** (◇, ◆) in the four tumor cell lines studied. The left-hand graphs represent the *l*-isomers of gossypol and derivatives, and the right-hand graphs represent the respective *d*-isomeric forms.

gossypolone **2**, respectively. The SK-mel-19 cell line was the most sensitive, with corresponding IC_{50} values of 24 and 28 μM.

Figure 3 represents the MTT cytotoxicity results for the *l*- and *d*-isomers of gossypol **1a**, and the half **1d** and bis Schiff's bases **1c** of gossypol. The *l*- and *d*-isomers of gossypol **1a** showed contrasting activity, the *l*-gossypol **1a** being significantly more active ($p < 0.05$) than the *d*-gossypol **1a** in all cell lines. The mean ($\pm$SD) IC_{50} value for *l*-gossypol **1a** was 20 ± 5 μM and that for the *d*-gossypol **1a** was 50 μM in two cell lines (in the K562 and Sihas cell lines, 50% cell kill was not reached at a concentration of 50 μM *d*-gossypol **1a**).

Neither the *l*- or *d*-isomer of the bis Schiff's base **1c**

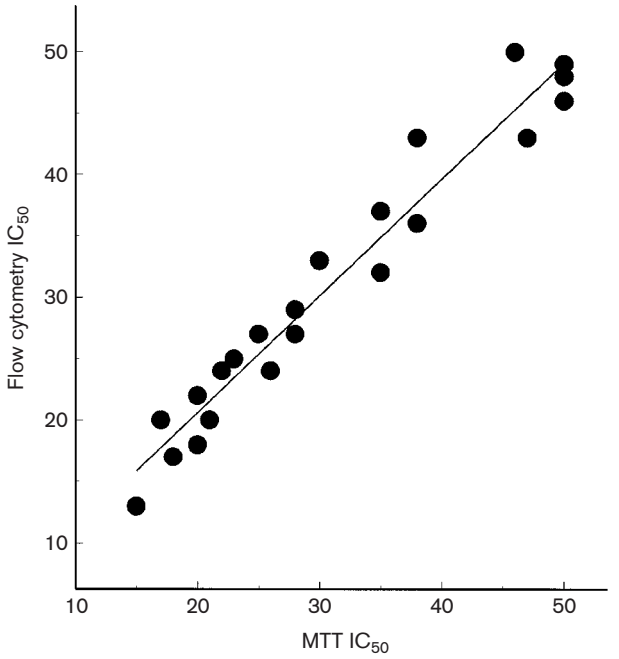


Figure 4. The correlation between the IC_{50} values determined from the MTT and flow cytometric viability assays.

was active in any cell line with a mean ($\pm$SD) percent cell kill at 50 μM of 10 ± 3 and 15 ± 5, respectively. In contrast, both the *l*- and *d*-half Schiff's bases **1d** induced a potent dose-dependent cell kill comparable to that for *l*-gossypol **1a**. The mean ($\pm$SD) IC_{50} values were 22 ± 6 and 26 ± 5 for the *l*- and *d*-half Schiff's bases **1d**, respectively.

The MTT data were confirmed by evaluating the test compounds using the flow cytometric assay for cell viability. Both assays gave comparable results and Figure 4 shows the correlation between the mean IC_{50} values for the two assays. Linear regression analysis indicated a strong positive correlation with an r^2 of 0.96.

Discussion

Gossypol **1a** has a number of sites available for chemical modification to investigate structure–activity relationships. These include six phenolic hydroxy groups and two aldehyde groups. Gossypol **1a** can interact with amines to form Schiff's bases, and in the present study we looked at the cytotoxic activity of a number of analogs of racemic gossypol **1a** and its enantiomers to determine the role of the aldehyde groups and their stereo-configuration.

We have demonstrated the dose-dependent cytotoxicity of racemic gossypol **1a** in four different tumor cell lines using two viability assays. The extent of cytotoxicity *in vitro* may depend on the cell lines used and the method of assay; however, our results are comparable to those reported by other workers.[5,8] We have also shown that the oxidative metabolite racemic gossypolone is as active as the parent racemate. There are no published cytotoxicity data available for the gossypol derivatives, apogossypol **1b** and the racemic bis L-phenylalanine methyl ester Schiff's base **1c**, but both these compounds were relatively inactive in killing the tumor cell lines used in this study. One of the structural differences between these active and inactive compounds is that the former have two aldehyde groups at positions 8 and 8′ of the molecule. This suggests that cytotoxicity may be induced by the reaction of the aldehyde groups with molecules essential for cellular function.

The development of gossypol derivatives, including Schiff's bases and evaluation of their respective activity, has been extensively investigated with regard to gossypol's anti-fertility action. Despite an intensive search, none have been found to have anti-fertility activity comparable to gossypol **1a** *in vivo*.[26] In the anti-cancer field, there is only one report on the anti-tumor activity of gossypol **1a** analogs. In this study[27] four bis Schiff's bases of racemic gossypol were synthesized and evaluated for anti-proliferate activity in two malignant and one immortalized breast cell line. These derivatives possessed either ethyl, propyl, isopropyl or butylamine substituents. Only the isopropyl derivative showed activity comparable to gossypol **1a** in two of the three cell lines. The other three derivatives had negligible inhibitory activity at doses up to 25 μM, which is similar to our present study using the L-phenylalanine methyl ester Schiff's base **1c** of gossypol.

The two enantiomers of gossypol **1a** have markedly different cytotoxic activity. The *l*-enantiomer showed significant activity (IC_{50} 20 M) whereas the *d*-enantiomer only reached 50% cell kill in two of the cell lines at 50 μM. Therefore, the stereospecific configuration of the gossypol **1a** molecule appears to play a significant role in the killing of tumor cells *in vitro*. Based on its amphiphilic property, it would be expected that gossypol could intercalate into phospholipid bilayers and it is known that racemic gossypol increases the microviscosity of phospholipid membranes of viable adrenocortical cells *in vitro*.[6] Chiral interaction with the phosphate of the lipid membranes may account for the observed quantitative differential effects of the two gossypol **1a** enantiomers.

The cytotoxicity profiles of the Schiff's base isomers, compared to the parent gossypol enantiomers, indicated that when both the aldehydes are blocked,

forming the individual *l*- and *d*-bis Schiff's bases **1c**, the cytotoxic activity is abolished. This is to be expected since the racemic bis Schiff's base showed minimal activity. However, by blocking just one aldehyde of the gossypol enantiomers, forming the individual *l*- and *d*-half Schiff's bases **1d**, the activity was restored and comparable to that of the *l*-gossypol **1a** enantiomer. This suggests that only one aldehyde group is necessary for cytotoxic action and is not limited to one isomer form. It is intriguing to note that the potency of the *d*-enantiomer of gossypol **1a** can be more than doubled by blocking one aldehyde. This suggests that the role of the aldehyde in inducing cell kill may be more crucial than the stereo-configuration of the molecule.

It is probable that more than one mechanism of action is operating within this group of analogs. There is clearly a stereo-specific mechanism of action between the two enantiomers of gossypol **1a**, both of which contain two aldehydes. This suggests that the spatial configuration of the aldehydes is important. The blocking of one aldehyde introduces an increased non-stereo-specific activity to the enantiomer molecules, which is completely negated when both aldehydes are blocked. At present it is unclear what are the precise physicochemical properties of these molecules that determines their cellular activity, and further chemical and molecular characterization is needed to answer this question. For gossypolone **2**, the presence of the quinone moities may alter the cytotoxicity of the aldehyde groups as well as exerting a cytotoxic effect in their own right via semi-quinone formation.

Anti-cancer agents that interact with several essential cellular targets reduce the potential for developing tumor cell resistance. The cytotoxic potency of racemic gossypol **1a** is known to be the same in tumor cell lines made resistant to standard anti-neoplastic agents compared with the corresponding drug-sensitive cell lines,[7] suggesting that this compound can overcome some of the known mechanisms of tumor resistance. Further investigations on cytotoxic drug-resistant cell lines are clearly warranted. In addition, the fact that only one aldehyde seems to be important in cytotoxicity opens avenues for tagging the molecule with, for example, fluorescent probes to map intracellular distribution or interactions with specific antigens to localize the drug.

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