Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 906–916
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Aldehyde dehydrogenase inhibitors: α,β-Acetylenic *N*-substituted aminothiolesters are reversible growth inhibitors of normal epithelial but irreversible apoptogens for cancer epithelial cells from human prostate in culture

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Received 19 January 2007; received in revised form 31 May 2007; accepted 8 June 2007
Available online 6 July 2007

Abstract

The pharmacomodulation of the N atom of α,β-acetylenic aminothiolesters or the replacement of the thiolester moiety by more electrophilic groups did not permit any clear rationale to be established for improving the selective growth-inhibitory activity of this family of compounds over that of the previously synthesized α,β-acetylenic aminothiolesters DIMATE and MATE [G. Quash, G. Fournet, J. Chantepie, J. Goré, C. Ardiet, D. Ardail, Y. Michal, U. Reichert, Biochem Pharmacol 64 (2002) 1279–92]. Hence DIMATE and MATE were investigated more thoroughly for selectivity and growth-inhibitory activity using human prostate epithelial *normal* cells (HPENC) on the one hand and human prostate epithelial *cancer* cells (DU145) on the other. Unequivocal evidence was obtained showing that both compounds were reversible growth inhibitors of HPENC but irreversible growth inhibitors of DU145. Growth-inhibition of DU145 was due to the induction of early apoptosis as revealed by the flow cytometric analytical profile of inhibitor-treated cells, of the decrease in the redox potential and increase in superoxide anion content of their mitochondria. Of the two intracellular enzymes: aldehyde dehydrogenases 1 and 3 (ALDH1 and ALDH3) targeted by DIMATE and MATE, ALDH3 was inhibited to the same extent by both compounds whereas ALDH1 was less susceptible to inhibition by MATE. As the induction of ALDH3 by xenobiotics is hormone-dependent, MATE, the more selective of the two inhibitors, is a useful tool not only for examining the role of the ALDH3 isoform in hormone-sensitive and resistant prostate cancer cells in culture but also for investigating if it can inhibit the growth of xenografts of prostate cancer in immunodeficient mice.

Keywords: Acetylenic aminothiolesters; ALDH1 and ALDH3 inhibitors; Human prostate epithelial normal cells; Human prostate cancer cells; Apoptosis

1. Introduction

We have previously shown that 4-amino-4-methyl-pent-2-ynthioic acid, *S*-methyl ester (ATE) was a competitive irreversible inhibitor of aldehyde dehydrogenase 1 (ALDH1) with an apparent K_i of 280 μM and an inducer of apoptosis (IC_{50} 4 μM) in mouse lymphoid cells BAF3 overexpressing the antiapoptotic gene bcl2 [1]. In two rat hepatoma cell lines in culture (JM2 and 7777) treated with DIMATE, the activities of both ALDH1 and ALDH3 were inhibited and there was a concomitant inhibitory effect on cell growth with IC_{50}s of 10–25 μM [2]. On the contrary, the morpholino derivative

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doi:10.1016/j.ejmech.2007.06.004

MATE was a very weak inhibitor of yeast ALDH1 (15% inhibition at 600 μM) [3] but retained good growth-inhibitory activity towards BAF3 bcl2 (IC_{50} 12 μM) [4].

On DU145 (cerebral metastases of human prostate cancer) cells in culture, the IC_{50}s of DIMATE and MATE were similar: 6–9 μM whereas on human embryonic lung normal fibroblasts (MRC5), the IC_{50} of DIMATE was 8 μM, (not different from that observed on DU145) but that of MATE (29 μM) showed about a three-fold increase. From these preliminary observations, MATE appeared to show some selectivity towards cancer compared to normal cells, but this interpretation was subject to caution because MRC5 cells are fibroblasts whereas DU145 are of epithelial origin; hence the difference in susceptibility to MATE may simply be a reflection of the differences in tissue origin. To try to improve the efficacy and selectivity of these thiolester compounds as inhibitors of the growth of human prostate cancer cells, additional derivatives were synthesized by pharmacomodulation of the N atom and changing the thiolester moiety. Screening for growth-inhibitory efficacy was done on the well-characterised cell line: DU145 and for selectivity on their true counterparts human prostate epithelial normal cells (HPENC). DU145 does not possess the androgen receptor (AR) and their growth is not stimulated by either androgens or antiandrogens [5]. They also overexpress the antiapoptotic gene bcl2 [6] involved in resistance to chemotherapy [7].

To try to make this study as pertinent as possible to the *in vivo* situation, two additional cell lines were used. The MOP cell line was chosen as representative of cells that persist in a low androgen environment after patient castration. Indeed, these cells were isolated after continuous culturing of LNCaP (Lymph Node metastasis of Carcinoma of the Prostate) cells in a steroid deprived medium for over one year. Their isolation and growth characteristics in response to androgens and antiandrogens have previously been described in detail [8] and contrary to the parent cell line LNCaP, their growth in culture, on adding androgens is partially inhibited [8]. The other cell line was derived from MOP cells grafted subcutaneously in female Swiss nude mice. Tumours were allowed to grow to approximately 300 mm^3 before the start of androgen treatment. With weekly androgen administration, tumour regression was almost complete after 3 weeks but then tumours reappeared at 5–6 weeks in spite of continuous androgen treatment. Cultures of these tumours gave rise to the ME cell line. Both cell lines possess the androgen receptor with the same mutation which is present in the parental cell line LNCaP [8], but from the differences in their response to androgens and antiandrogens in culture they are representative of the heterogeneity observed in a population of human prostate cancer cells *in vivo*. The determination of the activity of our α,β-acetylenic *N*-substituted aminothiol esters on such a spectrum of cells in culture was considered to be essential before attempting any experimentation on mice *in vivo*.

Compounds less active than DIMATE or MATE were not retained for further investigations. Those with increased growth-inhibitory activity compared to that of DIMATE and MATE were screened for selectivity on HPENC. When there was some evidence for selectivity towards DU145 compared to HPENC, investigations were pursued: to determine whether growth was inhibited reversibly or irreversibly, to identify the ALDH isoform implicated therein, to assess if apoptosis had taken place and if so, to measure some of the biochemical changes associated with apoptosis such as the mitochondrial redox potential and superoxide anion levels.

2. Materials and methods

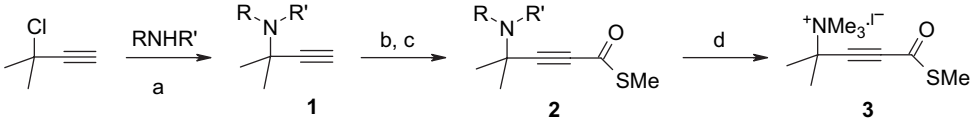
2.1. Cell cultures

DU145, MOP and ME were all grown in RPMI 1640 supplemented with 10% foetal calf serum (FCS) in the case of DU145, and 5% charcoal-treated FCS in the case of MOP and ME. Human prostate epithelial normal cells (HPENC), it's specialized medium PrEGM™ and medium supplement SingleQuots® were obtained from BioWhittaker. FCS (10%) was an additional supplement for growing HPENC.

All cells were maintained at 37 °C in a humid atmosphere of air/CO_2 (95/5). Cells were harvested by trypsinisation, washed once in phosphate buffered saline (PBS) by centrifugation at 300g for 5 min and finally suspended in PBS at the appropriate density 2×10^4 per well in 48-well plates and 1×10^5 per well in 24-well plates. Compounds were added 4 h after seeding and growth was determined at the intervals indicated by measuring protein content by the Lowry method [9] or by cell-count using a Coulter counter (Coulter Electronics, Luton, UK).

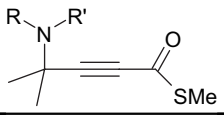
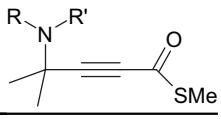
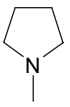
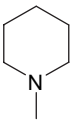
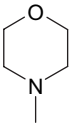
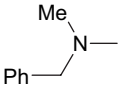
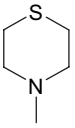
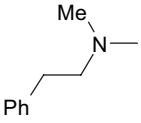
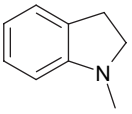
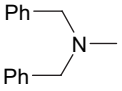
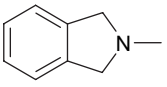
2.2. Chemistry

The protocol used for the aminothiolesters' synthesis is depicted in Scheme 1. It consists of a nucleophilic addition of an amino-acetylide on carbon oxysulfide followed by treatment with methyl iodide as described before [3]. Required propargyl amines were prepared by standard methods from 3-chloro-3-methyl-1-butyne [10]. Yields and structures of compounds **2a–l** are given in Table 1. The preparation of thioamide **4** and it's iminium salt **5** was carried out using Hartke's procedure [11] from corresponding 1-alkyne and commercial *N*,*N*-dimethylthiocarbamoyl chloride as depicted



Scheme 1. Aminothiolester synthesis: (a) Cu, CuCl, NEt_3, Et_2O see Ref. [10]; (b) BuLi, THF, −70–0 °C; (c) COS, −70–0 °C then MeI; (d) MeI, EtOAc, rt.

Table 1
Aminothiolester synthesis

			
	**2a** (83%) – Ref. [3] DIMATE		**2g** (85%)
	**2b** (75%)		**2h** (88%)
	**2c** (72%)		**2i** (93%) – Ref. [3] MATE
	**2d** (59%)		**2j** (97%)
	**2e** (59%)		**2k** (56%)
	**2f** (31%)		**2l** (39%)

in Scheme 2 (yield: 58%). The iminium salt was obtained by reaction of **4** with methyl iodide (yield: 86%).

2.2.1. General remarks

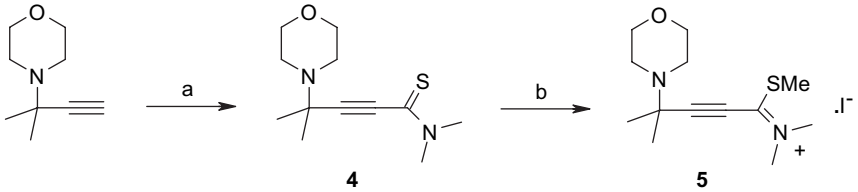
For general remarks see Ref. [3], chemistry section.

*2.2.2. 4-(Dipropylamino)-4-methyl-2-pentynethioic acid, S-methyl ester (**2b**)*

Prepared as described for DIMATE **2a** [3] by using the 2-methyl-*N*,*N*-dipropyl-3-butyn-2-amine [10]. Scale: 3 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 95/5), yield: 72% – colorless oil – IR (neat): $\nu = 2200$, 1460, 1650, 1080, 870 – ^{1}H NMR ($CDCl_3$): $\delta = 0.86$ (s, 6H), 1.42 (s, 6H), 1.47 (m, 4H), 2.38 (s, 3H), 2.52 (m, 4H).

*2.2.3. 4-Methyl 4-(methylphenylamino)-2-pentynethioic acid, S-methyl ester (**2c**)*

Prepared as described for DIMATE **2a** [3] by using the *N*-(1,1-dimethyl-2-propynyl)-*N*-methyl- benzenamine [10]. Scale: 6 mmol, purification by chromatography on silica gel (petroleum ether/Et_2O = 94/6), yield: 72% – orange oil – IR (neat): $\nu = 3080$, 3060, 3020, 2200, 1640, 1580, 930, 860, 790, 770, 700 – ^{1}H NMR ($CDCl_3$): $\delta = 1.43$ (s, 6H), 2.41 (s, 3H), 2.86 (s, 3H), 7.13–7.22 (m, 1H), 7.25–7.33 (m, 3H) – ^{13}C NMR ($CDCl_3$): $\delta = 12.5$, 29.1, 39.4, 55.0, 81.8, 96.9, 125.7, 127.6, 128.5, 150.3, 176.4 – LREIMS: 247 (M^{+}•, 4), 204 (100), 200 (69), 148 (64), 113 (47), 107 (81), 77 (58) – Anal.: calc. for $C_{14}H_{17}NOS$: %C = 67.98, %H = 6.93, %S = 12.96; found: %C = 68.37, %H = 6.93, %S = 13.11.



Scheme 2. Synthesis of compounds **4** and **5**: (a) *N*,*N*-dimethylthiocarbamoyl chloride, Et_3N, PPh_3, CuI, $PdCl_2(PPh_3)_2$, rt; (b) MeI, CH_2Cl_2, 0 °C to rt.

*2.2.4. 4-[Methyl (2-benzyl)amino]-4-methyl-2-pentynethioic acid, S-methyl ester (**2d**)*

Prepared as described for DIMATE **2a** [3] by using the *N*-(1,1-dimethyl-2-propynyl)-*N*-methyl-benzylamine [12]. Scale: 5 mmol, purification by chromatography on silica gel (petroleum ether/Et2O = 95/5), yield: 90% − yellow oil − IR (neat): $\nu = 3080$, 3060, 3020, 2200, 1640, 1600 − ^{1}H NMR ($CDCl_3$): $\delta = 1.55$ (s, 6H), 2.20 (s, 3H), 2.43 (s, 3H), 3.63 (s, 2H), 7.20–7.50 (m, 5H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.5$, 28.0, 36.2, 54.9, 56.6, 81.2, 96.2, 126.9, 128.3, 128.6 (2C), 140.0, 176.5 − Anal.: calc. for $C_{15}H_{19}NOS$: %C = 68.93, %H = 7.33, %S = 12.27; found: %C = 68.70, %H = 7.30, %S = 12.53.

*2.2.5. 4-[Methyl (2-phenylethyl)amino]-4-methyl-2-pentynethioic acid, S-methyl ester (**2e**)*

Required *N*-(1,1-dimethyl-2-propynyl)-*N*-methyl-phenethylamine was prepared from 3-chloro-3-methyl-1-butyne according to Ref. [10], yield − 55% on 4 mmol scale, pale yellow solid − Fus. 39–41 °C − IR (KBr): $\nu = 3180$, 3080, 3060, 3020, 2080, 1600, 745, 700 − ^{1}H NMR ($CDCl_3$): $\delta = 1.40$ (s, 6H), 2.25 (s, 1H), 2.39 (s, 3H), 2.65–2.90 (m, 4H), 7.23–7.30 (m, 5H). The 4-[methyl (2-phenylethyl) amino]-4-methyl-2-pentynethioic acid, *S*-methyl ester was prepared as described for DIMATE **2a** [3] by using the *N*-(1,1-dimethyl-2-propynyl)-*N*-methyl-phenethylamine. Scale: 2 mmol, purification by chromatography on silica gel (petroleum ether/Et_2O = 95/5), yield: 90% − colorless oil − IR (neat): $\nu = 3080$, 3060, 3020, 2200, 1640, 1600, 800, 750, 700 − ^{1}H NMR ($CDCl_3$): $\delta = 1.37$ (s, 6H), 2.34 (s, 3H), 2.65 (m, 4H), 2.85 (m, 4H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.5$, 27.9, 35.6, 36.7, 54.6, 54.9, 80.1, 96.5, 126.1, 128.4, 128.9, 140.4, 176.5 − HRCIMS: calc. for $C_{16}H_{21}NOS$ $[M+H]^+$ 276.1422, found 276.1424.

*2.2.6. 4-Methyl-4-[bis(phenylmethyl)amino]-2-pentynethioic acid, S-methyl ester (**2f**)*

Prepared as described for DIMATE **2a** [3] by using the *N*-(1,1-dimethyl-2-propynyl)-*N*-(phenylmethyl)-benzenemethanamine [13]. Scale: 1.0 mmol, yield: 31% (not optimized) as a yellow oil − IR (neat): $\nu = 3080$, 3060, 3020, 2200, 1640, 1600, 955, 920, 820, 760, 740, 720, 700 − ^{1}H NMR ($CDCl_3$): $\delta = 1.45$ (s, 6H), 2.45 (s, 3H), 3.82 (s, 4H), 7.32–7.43 (m, 10H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.6$, 29.4, 55.7, 56.5, 80.9, 97.0, 126.7, 128.1, 128.3, 141.2, 176.7 − LREIMS: 337 ($M^{+\bullet}$, 3), 322 (76), 294 (17), 91 (100) − Anal.: calc. for $C_{21}H_{23}NOS$: %C = 74.74, %H = 6.87, %O = 4.74; found: %C = 74.83, %H = 6.94, %O = 4.91.

*2.2.7. 4-Methyl-4-(1-pyrrolidinyl)-2-pentynethioic acid, S-methyl ester (**2g**)*

Prepared as described for DIMATE **2a** [3] by using the 1-(1,1-dimethyl-2-propynyl)-pyrrolidine [14]. Scale: 3 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 70/30), yield: 85% − slightly yellow oil − IR (neat): $\nu = 2200$, 1650, 1090, 920, 790 − ^{1}H NMR ($CDCl_3$): $\delta = 1.45$ (s, 6H), 1.81 (4H), 2.39 (s, 3H), 2.72 (m, 4H) − Anal.: calc. for $C_{11}H_{17}NOS$: %C = 62.52, %H = 8.11; found: %C = 62.15, %H = 8.11.

*2.2.8. 4-Methyl-4-(1-piperidinyl)-2-pentynethioic acid, S-methyl ester (**2h**)*

Prepared as described for DIMATE **2a** [3] by using the 1-(1,1dimethyl-2-propynyl)-piperidine [15]. Scale: 7 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 80/20), yield: 88% − yellow oil − IR (neat): $\nu = 2200$, 1650, 950, 900, 850, 790, 730 − ^{1}H NMR ($CDCl_3$): $\delta = 1.32$ (s, 8H), 1.50 (4H, m), 2.28 (s, 3H), 2.47 (m, 4H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.2$, 24.0, 26.2, 27.1, 47.9, 54.4, 81.1, 96.1, 176.0 − Anal.: calc. for $C_{12}H_{19}NOS$: %C = 63.96, %H = 8.50; found: %C = 63.85, %H = 8.57.

*2.2.9. 4-Methyl-4-(4-thiomorpholinyl)-2-pentynethioic acid, S-methyl ester (**2j**)*

Required 4-(1,1-dimethyl-2-propynyl)-thiomorpholine was prepared from 3-chloro-3-methyl-1-butyne according to Ref. [10], (yield 20% on 8 mmol scale) − yellow solid − Fus. 36–38 °C − IR (KBr): $\nu = 3300$, 3150, 2815, 2805, 2080 − ^{1}H NMR ($CDCl_3$): $\delta = 1.38$ (s, 6H), 2.30 (s, 1H), 2.73 (m, 4H), 2.90 (m, 4H) − ^{13}C NMR ($CDCl_3$): $\delta = 27.3$, 28.6, 49.1, 54.3, 71.2, 86.2. The 2-pentynethioic acid, 4-methyl-4-(4-thiomorpholinyl)-2-pentynethioic acid, *S*-methyl ester was prepared as described for DIMATE **2a** [3] by using the 4-(1,1-dimethyl-2-propynyl)-thiomorpholine. Scale: 1 mmol, purification by chromatography on silica gel (petroleum ether/Et_2O = 70/30), yield: 97% − white crystals − Fus. 42–44 °C − IR (KBr): $\nu = 2200$, 1640 − ^{1}H NMR ($CDCl_3$): $\delta = 1.37$ (s, 6H), 2.34 (s, 3H), 2.65 (m, 4H), 2.85 (m, 4H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.3$, 26.8, 28.5, 49.3, 54.7, 80.1, 95.4, 176.1 − LREIMS: 243 ($M^{+\bullet}$), 228 (100) 200 (29), 144 (26). Anal.: calc. for $C_{11}H_{17}NOS_2$: %C = 54.28, %H = 7.04, %S = 26.35; found: %C = 54.23, %H = 6.95, %S = 26.97.

*2.2.10. 4-(2,3-Dihydro-1H-indol-1-yl)-4-methyl-2-pentynethioic acid, S-methyl ester (**2k**)*

Prepared as described for compound **2g** by using the 1-(1,1-dimethyl-2-propynyl)-2,3-dihydro-1*H*-indole [16]. Scale: 1 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 98/2), yield: 39% − yellow oil − ^{1}H NMR ($CDCl_3$): $\delta = 1.66$ (s, 6H), 2.37 (s, 3H), 2.93 (t, $J = 8.05$, 2H), 3.40 (t, $J = 8.05$, 2H), 6.71–6.79 (m, 1H), 7.04–7.12 (m, 3H) − ^{13}C NMR ($CDCl_3$): $\delta = 12.5$, 26.5, 28.1, 49.5, 51.4, 80.1, 96.3, 111.3, 118.8, 124.5, 127.0 (4C), 131.5, 149.5, 176.6. Slow decomposition at room temperature.

*2.2.11. 4-(1,3-Dihydro-2H-isoindol-2-yl)-4-methyl-2-pentynethioic acid, S-methyl ester (**2l**)*

Prepared as described for compound **2g** by using the 2-(1,1-dimethyl-2-propynyl)-2,3-dihydro-1*H*-isoindole [17]. Scale: 0.8 mmol, purification by chromatography on silica gel (petroleum ether/ethyl acetate = 75/25), yield: 56% − beige solid − Fus. 64 °C decomposition − IR (KBr): $\nu = 3075$, 3040, 3020, 2890, 2200, 1640, 920, 800, 765, 750, 650, 610 − ^{1}H NMR

($CDCl_3$): 1.55 (s, 6H), 2.35 (s, 3H), 4.12 (s, 4H), 7.21 (m, 4H) – HRCIMS: calc. for $C_{15}H_{18}NOS$ $[M+H]^+$ 260.1109, found 260.1106.

2.2.12. N,N,4-Trimethyl-4-morpholin-4-ylpent-2-ynethioamide (4)

Obtained from corresponding amine following known procedure [11] as a yellow solid. Scale: 3 mmol, purification by chromatography on silica gel (CH_2Cl_2/MeOH = 96/4), yield: 58% – Fus. 102 °C – IR (KBr): $\nu = 2955$, 2205, 1510, 1385, 1290, 1120 – ^{1}H NMR ($CDCl_3$): 1.45 (s, 6H), 2.66 (m, 4H), 3.45 (s, 3H), 3.53 (s, 3H) 3.74 (s, 4H) – ^{13}C NMR ($CDCl_3$): $\delta = 26.9$, 41.0, 44.0, 47.6, 54.8, 67.1, 82.5, 100.5, 177.8 – Anal.: calc. for $C_{12}H_{20}N_2OS$: %C = 59.96, %H = 8.39, %N = 11.65; found: %C = 60.00, %H = 8.46, %N = 11.78.

2.2.13. N-Methyl-N-[4-methyl-1-(methylthio)-4-morpholin-4-ylpent-2-yn-1-ylidene] methanaminium iodide (5)

Prepared from **4** by treatment with methyl iodide according to [11]. Isolated by crystallization from Et_2O as a white solid – Fus. 111 °C (decomposition) C – IR (KBr): 2970, 2220, 1585 – ^{1}H NMR ($CDCl_3$): 1.57 (s, 6H), 2.65 (m, 4H), 2.93 (s, 3H), 3.73 (m, 4H), 3.75 (s, 3H), 3.99 (s, 3H) – Anal.: calc. for $C_{13}H_{23}N_2OS$: %C = 40.84, %H = 6.06, %N = 7.33; found: %C = 40.88, %H = 6.03, %N = 11.39.

2.3. Culturing cells for analysis by flow cytometry

DU145 cells (5 ml) at a density of 1×10^5/ml were seeded in 6 cm petri dishes and allowed to fix for 4 h. At the end of this period duplicate dishes were each treated with: 25 μl EtOH as controls, 25 μl of 2 mM DIMATE (final concentration 10 μM), 25 μl of 4 mM DIMATE (final concentration 20 μM). After 72 h incubation, the culture medium was collected and centrifuged. Adherent cells were harvested by treatment with 0.2% EDTA for 5 min at 37 °C, dishes were rinsed with 1 ml PBS and centrifuged. Pellets from the culture medium and from it corresponding dish were united after suspension in fresh medium supplemented with 7.5% FCS.

2.4. Measurement of redox potential

EtOH treated cells were suspended at 2.4×10^6/ml, 10 μM DIMATE-treated cells at 7.5×10^5/ml and 20 μM DIMATE-treated cells at 6.2×10^5/ml.

To each suspension was added a DMSO solution of 3-3-dihexyloxacarbocyanine iodide (DiOC6) from Molecular Probes, Oregon, pre-diluted to 1 μM in EtOH to a final concentration of 2.5 nM. After incubation for 15 min, cells were analyzed by a Beckman/Coulter EPICS (XL) flow cytometer.

2.5. Measurement of superoxide anion

A solution of hydroethidine (Molecular Probes, Oregon) pre-diluted to 1 mM in EtOH was added to controls and DIMATE-treated cells (as described above) to a final concentration of 1 μM. After incubation for 15 min, cells were analyzed by flow cytometry.

2.6. Measurement of the activity of ALDH1 and ALDH3

This was carried out on aliquots of 1 mg protein from cell lysates which had been centrifuged at 48,000g for 30 min. For the measurement of ALDH1 activity propanal was used as the substrate and for ALDH3 benzaldehyde was used. Full details of these methods have been described in Refs. [2,3].

3. Results

3.1. Pharmacomodulation of the N atom of the lead compound: 4-amino-4-methyl-pent-2-ynthioic acid, S-methyl ester (ampal thiolester: ATE)

This involved: changing the pK of the amine group, varying the steric hindrance on the N atom, modifying the lipophilic nature of the substituents on the N atom. The importance of the thiolester group was also assessed. All these new derivatives were screened for their growth-inhibitory effect on DU145 using DIMATE and MATE as reference compounds and only those with IC_{50}s (1–2 μM) substantially less than those of DIMATE and MATE (7–9 μM) were evaluated for selectivity using HPENC.

3.1.1. Effect of ATE derivatives on the growth of prostate normal and cancer cells

It is apparent (Table 2) that the introduction of a positive charge on the N atom as the quaternary ammonium compound **3** leads to an 80-fold decrease in inhibitory activity ($IC_{50} > 600$ μM). It seems difficult, to correlate the basicity of the compounds synthesized to their inhibitory activity. Indeed, **2g** and **2h** have effectively similar basicities, but **2h** with a piperidine ring instead of the pyrrolidine in **2g**, exhibited a three-fold decrease in activity. The same remark can be applied to **2i** (MATE) and **2j**, two other compounds with comparable pKs, yet **2j** showed a six-fold decrease when morpholine was changed to a thiamorpholine ring.

The lengthening of the alkane chains by two methylenes as in **2b**, or the replacement of the two dimethyl groups of DIMATE by two benzyl moieties as in **2f** did not lead to an improvement in inhibitory activity, all around 7–12 μM. It was only when one of the methyl groups of **2a** (DIMATE) was replaced by a benzyl as in **2d** a marked increase (5-fold) in inhibitory activity was observed, passing from IC_{50} 7 μM to 1.4 μM. The number of methylenes between the aromatic ring and the N atom in **2d** would also appear to be important because growth-inhibitory activity decreased when the ring was attached directly to the N atom as in **2c** (IC_{50} 12 μM) or when it was separated from the N atom by two methylenes as in **2e** (IC_{50} 6 μM).

When the N atom was part of a ring such as 2,3-dihydroindole or 1,3-dihydroisoindole or pyrrolidine, the corresponding derivatives **2k**, **2l** and **2g** retain inhibitory activity (IC_{50} 6–7 μM) similar to that of MATE. With a thiamorpholine

Table 2
IC_{50} values of ATE derivatives on prostate normal and cancer cell growth

Compound	**2a** DIMATE	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i** MATE	**2j**	**2k**	**2l**	**3** TRIMATE	**4**	**5**
DU145	7.2	12.6	12.5	1.4	6	7	6.7	22	9.3	54	7	6.2	>600	100	>100
HPENC	9.7			1.9					≫10				590		
MOP	3.0								10.0						
ME	3.0								10.0						

Values are means of three experiments, IC_{50} values are expressed in μM. HPENC, DU145, MOP and ME cells were seeded at a density of 1×10^5 cells in 1 ml culture medium per well of 24-well plates. Compounds were added at 4 h after seeding when cells had adhered. After 3 days in culture, the sheet of adhering DU145 was washed twice with PBS and harvested directly with 0.14M NaOH. Cell growth was measured by determining protein content. For MOP and ME growth was measured by cell-count using a Coulter counter. DIMATE and MATE values are the means of four determinations and for the other compounds, means of two determinations each done in duplicate.

and more curiously with a piperidine, the corresponding derivatives **2j** (IC_{50} 54 μM) and **2h** (IC_{50} 22 μM) were less inhibitory than MATE itself (IC_{50} 9 μM).

Taken together these results suggest that in addition to the residual charge on N, other factors, such as steric hindrance on the N atom or in the case of **2d** stabilization by a stacking interaction between the aromatic ring and an amino acid residue of the enzyme, play a role.

Changing the methyl thiolester group by other electrophilic groups was undertaken but the acetylenic dithioester function was excluded due to it's instability [11]. A thioamide **4** or it's more electrophilic iminium salt **5** led to a sharp decrease in inhibitory activity with IC_{50}s reaching 100 μM or more. It would therefore seem important to keep the thiolester group for inhibitory efficacy.

From this panel of 15 derivatives only **2d** showed a clear five-fold increase in inhibition over DIMATE or MATE. It was therefore chosen for investigations on selectivity using HPENC as controls. Unfortunately, the results (Table 2) showed that it's increased efficacy as an inhibitor of prostate cancer cell growth (IC_{50} 1.4 μM) was also accompanied by increased toxicity towards prostate epithelial normal cells (IC_{50} 1.9 μM). Further work on **2d** was therefore shelved and redirected to examine the selectivity of DIMATE and MATE.

3.2. Effect of DIMATE and MATE on the growth of normal and cancer cells

It is apparent (Table 2) that both DIMATE and MATE with IC_{50}s of 7and 9 μM, respectively, are good inhibitors of the growth of DU145 whereas on the normal counterparts HPENC, inhibition is a maximum of 10% with MATE at 10 μM but goes up to 52% with DIMATE at an equivalent concentration (Table 3). This result provided the first line of evidence that MATE might be a selective inhibitor of the growth of prostate cancer cells in culture. However, before investigating selectivity we first verified the effect of DIMATE and MATE on the hormone-sensitive cell line MOP and on it's hormone-resistant counterpart ME.

3.3. The effect of DIMATE and MATE on prostate cancer cell lines

As can be seen from the IC_{50}s (Table 2) the growth of all three cell lines DU145, MOP and ME is inhibited by DIMATE and MATE. However, the values for DIMATE are consistently lower than those of MATE, suggesting that DIMATE is more active than MATE. With growth-inhibition established on the hormone-sensitive and -resistant cancer cell lines it's reversibility was examined as a criterion of selectivity.

3.4. Reversibility of growth-inhibition

This was examined by replacing on the third day of culture, the medium containing inhibitors with fresh medium without inhibitors. After a further 5 days in culture, cells were examined under the microscope and growth assessed quantitatively by measuring the protein content of adhering cells. It is apparent (Fig. 1) that the growth of DU145 cells treated for 3 days with either DIMATE or MATE has been severely retarded compared to the growth of DU145 treated with vehicle alone (0.5% EtOH). Growth-inhibition was still as marked on day 8 with medium change at day 3 as was observed on day 8 without medium change. When the increase in cell number on day 8 with that on day 3 was compared it was found (Table 4) that no additional growth had taken place between days 3 and 8.

In contradistinction to the results obtained with DU145, the treatment of HPENC with DIMATE and MATE under conditions similar to those of DU145 showed that at 3 days MATE at 15 μM and DIMATE at 10 μM were growth-inhibitory and the cell sheets were less dense under the microscope (Fig. 2). Quantitative values for the inhibition by DIMATE and MATE were 60% and 37%, respectively (Table 5). However,

Table 3
Effect of DIMATE and MATE on prostate normal and cancer cell growth

Product	Concentration (μM)	% Inhibition of growth	
		DU 145	HPENC
DIMATE	0	0	0
	2	6	6
	5	34	23
	10	78	52
MATE	0	0	0
	2	0	3
	5	8	6
	10	54	11

See legend to Table 2. Values for HPENC are the means of three experiments each done in duplicate. Values for DU145 are the means of five experiments each done in duplicate.

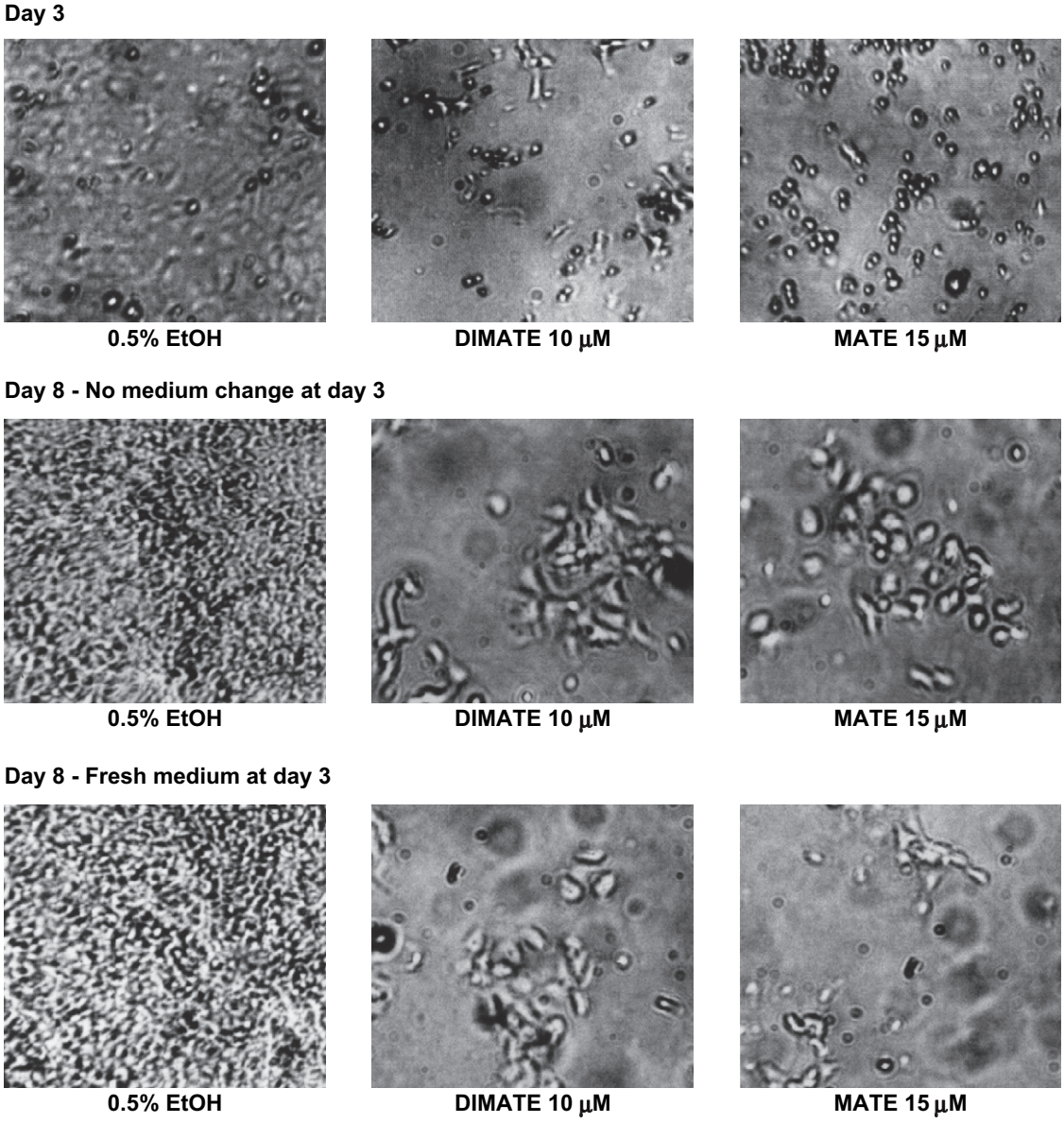


Fig. 1. Morphology by light microscopy at day 8 of DU145 treated with DIMATE and MATE with or without medium change at day 3. Magnification ×200.

the real difference with DU145 was seen with medium change at day 3. Indeed, HPENC restarted their proliferation in fresh medium without inhibitor (Fig. 2) and by day 8 cell number had increased about two-fold when pre-treated with MATE, three-fold when pre-treated with DIMATE and three-fold also in vehicle pre-treated cells (Table 5). These results show that DIMATE and MATE inhibit the growth of human prostate epithelial normal cells reversibly but that of human prostate epithelial cancer cells irreversibly. The mechanism of irreversible inhibition was therefore investigated.

3.5. Flow cytometric analyses for cell distribution, redox potential and superoxide anion

The results (Fig. 3) show quite clearly that in DU145 cells treated with DIMATE, changes in these three parameters take place. Apoptotic cells are present in H1 and H3, superoxide anions (hydroethidine labelling) are increased and redox potential ($DIOC_6$) is decreased. These changes are accentuated with 20 μM DIMATE and will be discussed further in the light of the perturbations which take place in mitochondria during early apoptosis.

3.6. The ALDH isoforms targeted by DIMATE and MATE

The results of the experiments on the action of these compounds on cells in culture showed that they were effective growth inhibitors. Nevertheless, their intracellular targets had to be different because DIMATE is a competitive irreversible inhibitor of yeast ALDH1 with an apparent K_i of 280 μM whereas MATE is a very weak inhibitor of yeast ALDH1 showing a maximum of 15% inhibition at 600 μM even when pre-incubated with the enzyme for 1 h [3]. To try to

Table 4
Effect of DIMATE and MATE on DU145 cell growth with and without medium change at day 3

Samples	% Growth-inhibition at day 3	Increase in cell number day 8/day 3	
		No medium change at day3	Fresh medium at day 3
Control	0	1.6	2.1
DIMATE 10 μM	67	2.5	2.7
MATE 10 μM	70	3	2.6
MATE 15 μM	88	1.9	1.6

Values are the means of two experiments each done in duplicate. DU145 cells were seeded in six wells and treated with compounds as described in the legend to Table 2. After 3 days in culture the medium was aspirated in two wells, the cell sheet washed with PBS and 1 ml of fresh medium added. Cells were incubated for a further 5 days and growth was measured on day 8. In two other wells cells were harvested after washing and protein content was determined. In the last two wells cells were left in the medium in which they were originally seeded and incubated for an additional 5 days. Cell growth was measured on day 8 by protein content of adherent cells. Control wells received 5 μl EtOH and were treated exactly as described for inhibitor-treated wells.

elucidate this obvious discrepancy, the ALDH1 and ALDH3 activities of DU145 cells treated for 3 days with the same molar concentrations of DIMATE or MATE were measured. The results (Table 6) show that the inhibition of ALDH3 is similar in both DIMATE-treated and MATE-treated cells whereas that of ALDH1 is less marked with MATE than that with DIMATE. It is clear that of the two isoforms it is the inhibition of ALDH3 enzyme activity that is better correlated with the inhibition of growth of DU145. These results are in accordance with many reports in the literature showing that ALDH3 is involved in controlling cell growth [18].

4. Discussion

The results obtained with this limited panel of α,β-acetylenic *N*-substituted aminothiol esters show that no clear-cut rationale emerged for ''designing'' growth inhibitors more effective and selective than DIMATE and MATE. Nevertheless,

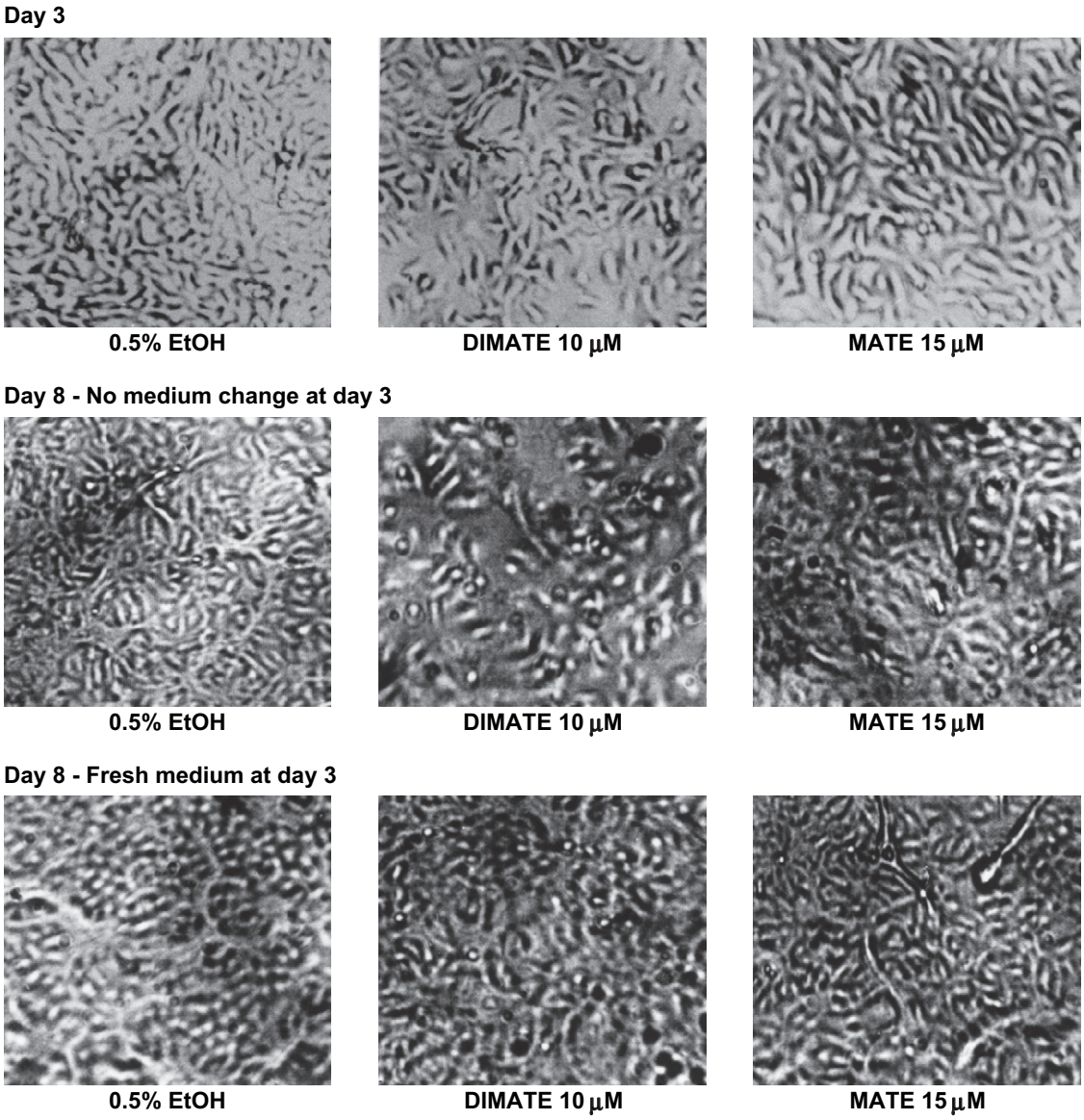


Fig. 2. Morphology by light microscopy at day 8 of HPENC treated with DIMATE and MATE with or without medium change at day 3. Magnification ×200.

Table 5
Effect of DIMATE and MATE on HPENC cell growth with and without medium change at day 3

Samples	% Growth-inhibition day 3	Increase in cell number day 8/day 3	
		No medium change at day3	Fresh medium at day 3
Control	0	0.7	2.2
DIMATE 10 μM	60	0.9	2.8
MATE 10 μM	0	0.4	1.6
MATE 15 μM	37	1.4	2.6

Values are the means of two experiments each done in duplicate. HPENC were subjected to the identical experimental conditions described in the legend to Table 4.

one moiety that contributes to growth-inhibitory activity is the thiolester. However, it does appear that it's contribution to growth inhibiton is not dependent on it's degree of electrophilicity since derivatives of MATE with other electrophiles such as the thioamide **4** or it's more electrophilic iminium salt **5** showed a sharp decline in inhibitory activity ($IC_{50} \geq 100$ μM). Our results with MATE (the thiolester) confirm earlier reports showing the importance of the ester functionality of the methyl and isopropyl esters of chlorpropamide for the inhibition of ALDH3 [19].

With regard to efficacy, the only criterion which improved growth-inhibition was hydrophobicity on N as in **2d**

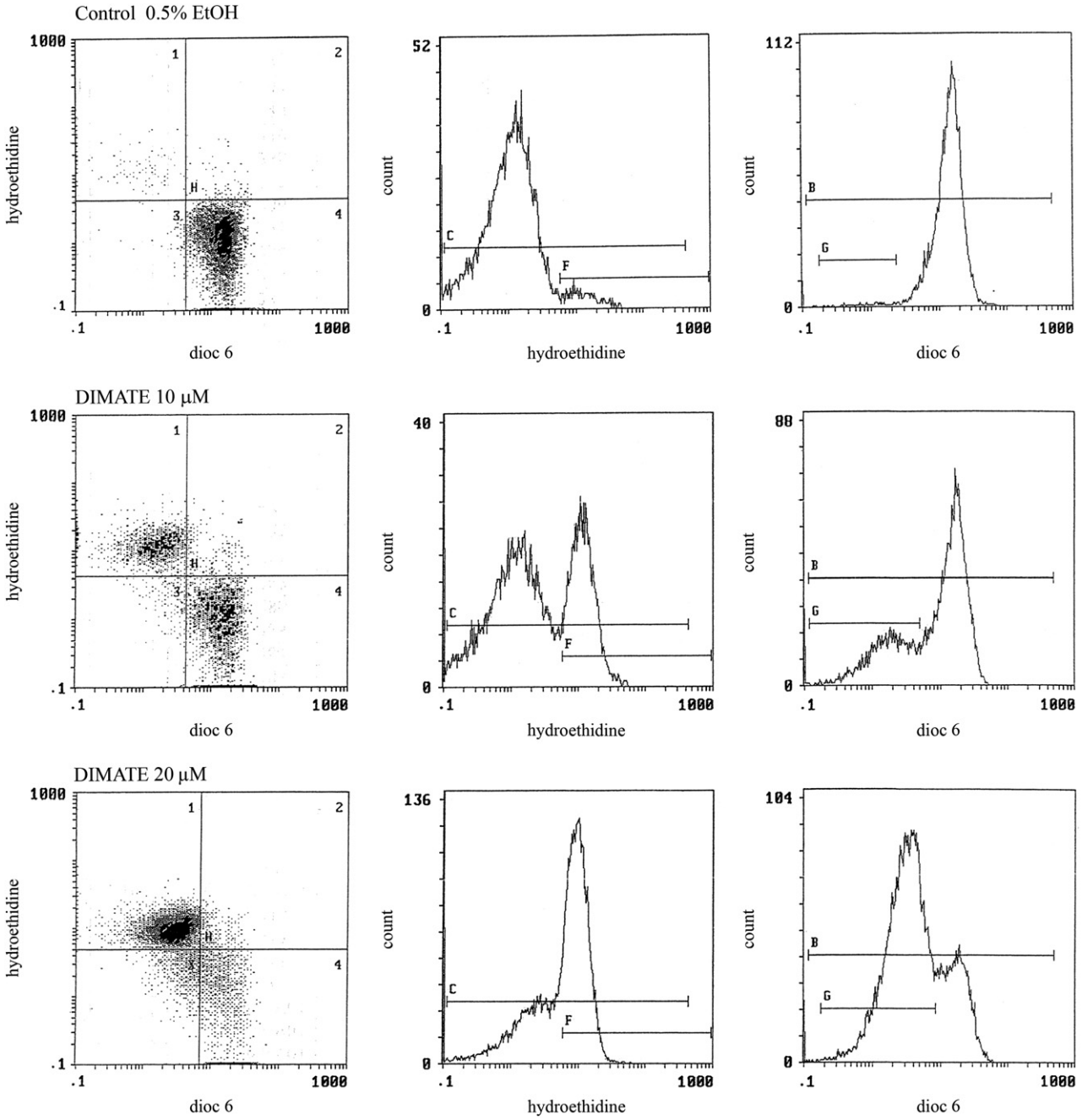


Fig. 3. Flow cytometric analysis for cell distribution, redox potential and superoxide anion content of DU145 treated with 10 and 20 μM DIMATE for 3 days.

Table 6
Effect on ALDH1 and ALDH3 of DU145 incubated with DIMATE and MATE for 3 days

Compound	Concentration μM	% Inhibition	
		ALDH1	ALDH3
DIMATE	2.5	15	40
	5	40	62
	7.5	78	100
MATE	2.5	3	40
	5	20	60
	7.5	32	78

Values are the means of two experiments each done in duplicate. DU145 and HPENC were seeded as described in the legend to Table 2 and treated with DIMATE or MATE at the concentrations indicated. After 3 days in culture, cells were harvested by trypsinisation, centrifuged, washed twice with PBS and the pellet lysed directly in 500 μl 60 mM sodium phosphate buffer of pH 7 containing 1% (v/v) TritonX-100, 1 mM EDTA for 30 min at 4 °C. Lysates were centrifuged at 48,000g for 30 min and the supernatants were used for measuring ALDH activity. Activity of ALDH1 was measured by adding 1 mg lysate protein to a reaction mixture containing 60 mM sodium phosphate buffer of pH 8.5, 1 mM EDTA, 100 mM KCl, 2.35 mM NAD. The reaction was started by the addition of 2 mM propanol and the final volume was 1 ml. ALDH activity was measured by the increase in OD_{340} of the NADH formed and inhibition as the % loss of activity of activity of DIMATE-treated vs that of non-inhibitor-treated cells. For measuring ALDH3 activity, 1 mg protein lysate was also used but with 5 mM benzaldehyde as substrate, 2.5 mM NADP as coenzyme in the presence of 1 mM pyrazole. Activity and percentage inhibition were determined as described for ALDH1.

(IC_{50} 1.4 μM) but this led to a concomitant decrease in selectivity as shown by it's IC_{50} (1.9 μM) on HPENC. This loss of selectivity for human prostate cancer cells could be due to the increased capacity of **2d** to diffuse across the hydrophobic cell membrane and/or it's increased affinity for the putative intracellular target enzyme ALDH. One argument in favour of the latter hypothesis was the finding that the concentration of **2d** which inhibited yeast ALDH1 activity by 50% was 73 μM, two-fold lower than that of DIMATE 165 μM (results not shown). The question of diffusion will only be answered when radiolabeled **2d** becomes available.

With regard to selectivity, MATE, from the IC_{50}s in Table 2, appeared to be less growth-inhibitory on HPENC than DIMATE. However, when an additional criterion of selectivity viz: the reversal of growth-inhibition was examined on HPENC, it was found that HPENC, which exhibited reduced growth after 3 days in the presence of both compounds (Fig. 2), proliferated anew when the medium containing inhibitor was changed and replaced by fresh medium without inhibitor (Fig. 2). Both compounds can therefore be classified as reversible growth inhibitors for human prostate epithelial normal cells, at least under the experimental conditions used here. The effect on reversibility of longer contact times with the inhibitors remains to be investigated.

In contradistinction to the observations made on HPENC, the cancer cell line DU145 did not re-proliferate when medium plus inhibitor was replaced by fresh medium minus inhibitor (Fig. 1). This strongly suggests that the inhibitors are irreversibly cytotoxic for cancer cells; a suggestion supported by the growth-inhibition also observed on the other two prostate cancer cell lines MOP and ME (Table 2).

When the mechanism of growth-inhibition was investigated, unequivocal evidence was obtained for inhibitor-induced apoptosis as shown by flow cytometric analysis of the changes in the distribution of DIMATE-treated DU145 cells (Fig. 3), in their redox potential and superoxide anion profiles (Fig. 3). The decrease in the mitochondrial redox potential ($DIOC_6$ labelling) and increase in superoxide anion (hydroethidine labelling) confirm that DIMATE (Fig. 3), which had previously been shown to also decrease mitochondrial glutathione [3], is acting on the execution phase of early apoptosis [20] before events typical of late apoptosis such as nuclear degradation and Annexin V positivity are manifested [20].

We can therefore conclude that DIMATE is apoptogenic on human prostate epithelial cancer cells as had previously been shown on mouse lymphoid cells BAF3 bcl2 [3]. Concerning the apoptotic activity of MATE, the above mentioned biochemical parameters of apoptosis were not examined, only DNA fragmentation was measured on ME and here too, there was evidence for an increase in low MW DNA on electrophoresis (results not shown).

As the results obtained so far on these two inhibitors showed that they were comparable as growth inhibitors but with some advantage for MATE in terms of selectivity ($IC_{50\ MATE} \gg 10$ μM on HPENC vs $IC_{50\ DIMATE}$ 9.7 μM on HPENC), their effects on their intracellular putative target enzymes (ALDH isoforms) were investigated. Additional reasons for this experiment were previous results showing that (a) DIMATE is a good competitive irreversible inhibitor of yeast ALDH1 whereas MATE is a very poor inhibitor of this enzyme showing a maximum of 15% inhibition even when pre-incubated at 600 μM with the enzyme for 1 h [3]; (b) the inhibition of ALDH1 activity in BAF3 bcl2 cells by DIMATE is accompanied by the increased fragmentation of DNA typical of apoptosis [3]. Hence, if ALDH1 in DU145 were as refractory as yeast ALDH1 to inhibition by MATE, the growth-inhibitory effect of MATE could not be attributed to it's inhibition of this isoform. The results shown in Table 4 permit this issue to be clarified. Indeed, in DU145 cells incubated with either inhibitor for 3 days, ALDH3 was the isoform that was inhibited to the same extent by both compounds. ALDH1 was two-fold less sensitive to inhibition by MATE than by DIMATE. This result confirms many other earlier reports in the literature showing that ALDH3 is involved in controlling cell growth [18,21], in protecting cells from the deleterious effects of xenobiotics [22,23] and of endogenous toxic aldehydes, e.g., 4-hydroxynonenal [24]. It is noteworthy that this enzyme is expressed constitutively in some tissues such as cornea, lung and stomach [18,25] whereas it is inducible in others such as liver [25]. Further, the existence of an apparently tumour-specific form of ALDH3 has been reported [26].

Of particular relevance to the work done here is the fact that the promoter region of the ALDH3 gene contains many different response elements among which are those for aromatic hydrocarbons and glucocorticoids [27]. These response elements control ALDH3 synthesis at the transcriptional level as shown by the differences in inducibility of the enzyme in adult life

when hormones are given during puberty [28]. Indeed, it has been reported that down-regulation occurred with progesterone but up-regulation with cortisone, cyproterone and tamoxifen [28]. These observations suggest that ALDH3 could be playing a pivotal role in hormone-dependent tumours such as prostate and breast cancers. Hence MATE, as an inhibitor of this enzyme could be used on the one hand for examining this role in hormone-sensitive cells in culture and on the other for investigating the effect of ALDH3 inhibition on the growth of xenografts of human prostate cancer cells *in vivo*.

Acknowledgments

Financial assistance for this work was provided in part by La Région Rhône Alpes and Galderma R&D, Sophia Antipolis, France.

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